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Role of Protein Kinase C in the Regulation of Cytosolic Ca^{2+} in A431 Cells: Separation of Growth Factor and Bradykinin Pathways

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Summary. Calcium signaling systems in nonexcitable cells involve activation of Ca^{2+} entry across the plasma membrane and release from intracellular stores as well as activation of Ca^{2+} pumps and inhibition of passive Ca^{2+} pathways to ensure exact regulation of free cytosolic Ca^{2+} concentration ($[Ca^{2+}]_i$). A431 cells loaded with fura-2 cells were used as a model system to examine regulation of Ca^{2+} entry and intracellular release. Epidermal growth factor (EGF) and transforming growth factor alpha (TGF-α) both stimulated Ca^{2+} entry and release while bradykinin appeared only to release Ca^{2+} from intracellular stores. The possible role of protein kinase C (PKC) in modulating the $[Ca^{2+}]_i$ response to these agonists was examined by four methods. Low concentrations of TPA (2×10^{-10} M) had no effect on Ca^{2+} release due to EGF, TGR-α or bradykinin but resulted in a rapid return of $[Ca^{2+}]_i$ to baseline levels for EGF or TGF-α. Addition of the PKC inhibitor staurosporine (1 and 10 nM) completely inhibited the action of TPA on EGF-induced $[Ca^{2+}]_i$ changes. An inhibitor of diglyceride kinase (R59022) mimicked the action of TPA. Down-regulation of PKC by overnight incubation with 0.1 or 1 μM TPA produced the converse effect, namely prolonged Ca^{2+} entry following stimulation with EGF or TGF-α. To show that one effect of TPA was on Ca^{2+} entry, fura-2 loaded cells were suspended in Mn^{2+} rather than Ca^{2+} buffers. Addition of EGF or TGF-α resulted in Ca^{2+} release and Mn^{2+} entry. TPA but not the inactive phorbol ester, 4-α-phorbol-12,13-didecanoate, inhibited the Mn^{2+} influx. Thus, PKC is able to regulate Ca^{2+} entry due to EGF or TGF-α in this cell type. A431 cells treated with higher concentrations of TPA (5×10^{-8} M) inhibited not only Ca^{2+} entry but also Ca^{2+} release due to EGF/TGF-α but had no effect on bradykinin-mediated Ca^{2+} release, suggesting differences in the regulation of the intracellular stores responsive to these two classes of agonists. Furthermore, sequential addition of EGF or TGF-α gave a single transient of $[Ca^{2+}]_i$, showing a common pool of Ca^{2+} for these agonists. In contrast, sequential addition of EGF (or TGF-α) and bradykinin resulted in two $[Ca^{2+}]_i$ transients equal in size to those obtained with a single agonist. Ionomycin alone was able to fully deplete intracellular Ca^{2+} stores, whereas ionomycin following either EGF (or TGF-α) or bradykinin gave an elevation of the $[Ca^{2+}]_i$ signal equal to that of the second agonist. These data indicate that there are separate pools of intracellular Ca^{2+} for EGF-mediated Ca^{2+} release which also respond differently to TPA.

Key Words protein kinase C · cytosolic Ca^{2+} · Ca^{2+} entry · A431 cells

Introduction

Activation of the epidermal growth factor receptor (EGFR)[1] triggers a cascade of events that are thought to be responsible for the initiation of DNA synthesis and cell proliferation. The EGFR mediates the transmembrane signaling of at least two other EGF-like molecules: transforming growth factor alpha (TGF-α) and vaccina virus growth factor [3]. The three growth factors show some amino acid sequence homology (approximately 22%) with conservation of all six cysteine residues. All three agonists show similar binding affinities to the EGFR in intact cells, induce autophosphorylation in membrane preparations at tyrosine and produce similar growth responses in target cells [3, 5, 11–13, 23]. A431 cells are used as a model for studying the biochemical events of EGF-induced signal transduction. In addition to the activation of tyrosine kinase, EGF has been shown to stimulate a rapid rise in 1,4,5-inositol triphosphate (IP_3) and free cytosolic Ca^{2+} ($[Ca^{2+}]_i$), and these events are thought to be important in transmembrane signaling [9, 16, 20–22, 26, 34, 37].

Regulation of $[Ca^{2+}]_i$ depends on changes in transmembrane Ca^{2+} channels, release of Ca^{2+} from intracellular stores and the activation of Ca^{2+} pumps

[1] *Abbreviations:* $[Ca^{2+}]_i$, free cytoplasmic Ca^{2+} concentration; DAG, 1,2-diacylglycerol; DMSO, dimethyl sulfoxide; EGTA, [ethylene-bis(oxyethylenenitrilo)]-tetraacetic acid; EGF, epidermal growth factor; EGFR, epidermal growth factor receptor; HBSS, hank balanced salt solution; HOC, hormone-operated Ca^{2+} channel; IP_3, inositol 1,4,5-triphosphate; IP_4, inositol 1,3,4,5-tetraphosphate; $[Mn^{2+}]_o$, extracellular Mn^{2+} concentration; PIP_2, phosphatidyl-inositol-4,5-biphosphate; PLC, phospholipase C; PKC, protein kinase C; TGF-α, transforming growth factor-alpha; TPA, 12-0-tetra-decanoyl-phorbol-13-acetate; VOC, voltage-operated Ca^{2+} channel.

for Ca^{2+} extrusion or receptors [2, 6]. In many cells, a key step in the action of Ca^{2+}-mobilizing agonists that release Ca^{2+} from intracellular stores is the binding of ligand to receptor and the transduction of the signal through a guanine nucleotide binding protein that activates a phosphoinositide-specific phospholipase C (PLC). Hydrolysis of phosphatidylinositol-4,5-biphosphate (PIP_2) generates IP_3, and 1,2-diacylglycerol (DAG) [1, 27]. IP_3, in turn, acts as a second messenger and binds to an intracellular receptor on the rough endoplasmic reticulum membrane releasing Ca^{2+} from intracellular stores [1, 27]. In the plasma membrane there are various Ca^{2+} entry modes, consisting of Ca^{2+} channels that are activated by hormones of various types (hormone-operated channels, HOC) as well as voltage-activated channels (VOC) [24]. In the same membrane there are Ca^{2+} export systems such as the cadmodulin-activated Ca^{2+} pump [6] and in some cells a Na^+/Ca^{2+} exchanger.

The second product of PIP_2 hydrolysis is DAG that is localized in the plasma membrane and activates protein kinase C (PKC; ref. [19]). A transient increase in DAG has been reported in A431 cells 5 min after EGF treatment [29]. Modulation of PKC by TPA has been shown to affect $[Ca^{2+}]_i$ [16, 21]. Thus, the $[Ca^{2+}]_i$ achieved in a given cell is the result of the activity of a variety of pathways in the two membrane systems. By changes of medium Ca^{2+}, by the use of channel blockers or by using other divalent cations such as Mn^{2+} that interact with Ca^{2+}-sensitive fluorescent dye probes, it is possible to determine the relative importance of Ca^{2+} release from intracellular stores and Ca^{2+} entry across the plasma membrane in a given signaling system.

Previous studies with A431 cells show that a major source of Ca^{2+} during the EGF-induced rise in $[Ca^{2+}]_i$ is from entry through the plasma membrane [16, 21, 26, 37]. A431 cells lack classical voltage-activated Ca^{2+} channels found in nerves and muscles [16] so that EGF-induced Ca^{2+} entry is coupled to EGFR by a receptor-operated mechanism. Initial studies of $[Ca^{2+}]_i$ which utilized the Ca^{2+}-sensitive fluorescent dye quin-2 showed only Ca^{2+} entry. This dye has limitations in that it buffers out the EGF-induced release of intracellular Ca^{2+} stores [16, 37]. Using fura-2 loaded cells, both components (the release of Ca^{2+} from intracellular stores and Ca^{2+} entry) that contribute to the rise in $[Ca^{2+}]_i$ were shown [21, 37]. Following loading of cells with fura-2, a fluorescence quench by Mn^{2+} can be used to study the divalent cation entry pathway through the plasma membrane [7, 14]. In the presence of extracellular Mn^{2+}, agonist-induced intracellular Ca^{2+} release causes an initial increase in fura-2 fluorescence that is followed by fluorescence quench as Mn^{2+} crosses the plasma membrane into the cell. This approach has been used to study Ca^{2+} entry in platelets where the PKC activator TPA preferentially inhibits release from intracellular stores before affecting Ca^{2+} entry [32]. In quin-2 loaded A431 cells a short 5–10 min treatment with TPA inhibits EGF-induced changes in $[Ca^{2+}]_i$, suggesting that activation of PKC inhibits Ca^{2+} entry [16]. Inositol phosphate production is also inhibited by 5-min treatment with TPA [33]. The same authors found treatment with phorbol ester for 24 hr resulted in an elevation of IP_3 and IP_4 production and elevation of $[Ca^{2+}]_i$ [21]. These results suggest that protein kinase C activity can play an important regulatory role in the control of $[Ca^{2+}]_i$ induced by activation of the EGFR, but the use of quin-2 prevented observations on the regulation of the intracellular Ca^{2+} store.

To further characterize modulation of intracellular Ca^{2+} by PKC in A431 cells, four experimental strategies that utilized Ca^{2+} or Mn^{2+} containing buffers were used: (*i*) Effects of general PKC activation by short (2–5 min) phorbol ester treatment on EGF/TGFα induced Ca^{2+} mobilization. (*ii*) Incubation of A431 cells with a diacyclglycerol kinase inhibitor, R59022 leading to an elevation of DAG and secondarily to an localized increase in PKC activity. (*iii*) Effects of PKC inhibition using staurosproine. (*iv*) Downregulation of PKC activity by long-term (24 hr) incubation of A431 cells with TPA [21] to determine Ca^{2+} signaling in the absence of this enzyme.

Similar studies were performed using the Ca^{2+}-mobilizing hormone, bradykinin [9, 10, 31], which, in contrast to EGF, produces a readily measurable elevation of IP_3 in our laboratory and elsewhere [9, 10].

In this paper, we show that activation of PKC by low concentrations of TPA or by the DAG kinase inhibitor R59022 suppresses EGF (or TGF-α) induced Ca^{2+} entry, whereas inhibition or downregulation of PKC either by staurosporine or 24-hr incubation with TPA, respectively, enhances Ca^{2+} entry. High concentrations of TPA inhibit Ca^{2+} release by EGF or TGF-α, but do not affect Ca^{2+} release due to bradykinin. Additional evidence for separation of the intracellular Ca^{2+} stores regulated by EGF or bradykinin was obtained by showing that these agonists were able to release intracellular Ca^{2+} independently in contrast to EGF and TGF-α.

Materials and Methods

Cells and Cell Culture

Human A431 (clone 8) epidermoid carcinoma cells were obtained from Dr. Gordon N. Gill, Department of Medicine, UCSD School

of Medicine, La Jolla, CA. Cells were plated in 75-mm flasks and grown in monolayer culture in RPMI 1640 containing 10% fetal calf serum, 10 mM HEPES buffer, penicillin (100 U/ml), streptomycin (100 U/ml), 2 mM L-glutamine and 40 μM 2-mercaptoethanol. Cells were used 2–3 days after plating when cultures were 80–90% confluent.

Reagents

Fura-2-AM was purchased from Molecular Probes (Junction City, OR) and was stored as a 1-mM stock solution in dimethyl sulfoxide at −70°C. EGF (culture grade) was purchased from Collaborative Research (Lexington, MA). Synthetic rat TGFα was purchased from Peninsula Laboratories, (Belmont, CA). 12-0-tetradecanoylphorbol-13-acetate (TPA) was purchased from LC Service (Woburn, MA) and 4-α phorbol 12,13-didecanoate, phorbol 12,13-dibutyrate, pertussis toxin, bradykinin, histone type III-S, phosphatidylserine and leupeptin were purchased from Sigma Chemical (St. Louis, MO). Staurosporine was purchased from Kamiya Biomedical (Thousand Oaks, CA). The diglyceride kinase inhibitor R59022 was purchased from Research Biochemical (Natick, MA). (γ-^{32}P) ATP was obtained from Amersham (Arlington Heights, IL).

Fura-2 Loading of Cells

Before trypsinization confluent A431 cells were washed twice with HBSS. They were detached using 1 × trypsin-EDTA for 5 min. Cells were immediately washed twice (600 × g centrifugation for 5 min) in a 20 mM HEPES buffer (pH 7.4) containing 120 mM NaCl, 6 mM KCl, 1 mM $MgSO_4$, 1 mg/ml glucose, 1 mg/ml pyruvate and 1.4 mM calcium (medium A). Approximately 5 × 10^6 cells per ml were suspended in medium A and incubated with 4 μM fura-2-AM for 20 min at 37°C. Fluorescence was recorded continuously with a Perkin-Elmer LS-5 spectrofluorometer. The excitation wavelength was set at 340 nm and emission wavelength set at 500 nm. The cell suspension was stirred, maintained at 37°C and equilibrated for approximately 5 min before addition of the various agents. $[Ca^{2+}]_i$ was calculated using the methods previously described [37] and is expressed as the mean ± SEM. It appears that trypsinization neither affected Ca^{2+} levels or the response of $[Ca^{2+}]_i$ to hormones.

Down Regulation of PKC Activity

A431 cells were allowed to reach 50 to 60% confluency (two days after plating) and then treated with TPA (0.1 or 1 μM) or vehicle (DMSO) for 24 hr. Cells were loaded with fura-2 (as described above) and fluorimetry analysis was done.

Protein Kinase C Assay

A431 cells grown in T150 cm^2 flask (five flasks per treatment group) were treated with TPA (1 pμM) or vehicle (DMSO) for 24 hr, washed three times with PBS and scraped off the flask with a rubber policeman. Identically treated cells were pooled and pelleted by centrifugation for 15 min at 200 × g. Cells were resuspended into 2 ml of ice-cold homogenization buffer which contained 250 mM sucrose, 20 mM Tris, pH 7.5, 50 mM 2-mercaptoethanol, 2 mM ethylenediamine tetraacetic acid (EDTA) and 10 mM EGTA. The cells were disrupted by 20 strokes with a Radnoti glass homogenizer. To remove nuclei and other cell debris the homogenate was centrifuged at 600 × g for 10 min. The 600 × g supernatant was then recentrifuged at 100,000 × g for 1 hr at 4°C.

Protein kinase C activity was determined by measuring the incorporation of ^{32}P from (γ-^{32}P) ATP into histone [30]. The assay was performed at 30°C in a total volume of 250 μl of incubation mixture which contained 20 mM Tris, pH 7.5, histone (200 μg), 5 mM $MgCl_2$, 0.2 mM leupeptin, phosphatidylserine (20 μg), TPA (54 ng), the addition of 500 μM Ca^{2+} and 10 μg of protein as source of enzyme. The reaction was started by the addition of 10 μM (γ-^{32}P) ATP (1 × 10^5 cpm per nmol). After incubation for 5 min, the reaction was terminated by the addition of 25% trichloracetic acid (TCA) (1 ml) and the acid precipitable material collected on Hoefer scientific cellulose nitrate filters (pore size 0.45 μm). After washing the filters three times with 10% TCA, the radioactivity was determined using a Beckman (LS 3801) scintillation counter. All protein determinations were made by the Lowry method using bovine serum albumin as the standard.

Inositol Methods

A431 cells were plated at 5 × 10^5 cells per well (6-well cluster dish) in RPMI 1640 containing 10% FBS two days prior to assay procedure to allow growth to confluency. The cells were pulsed overnight (~12 hr) with 4 μCi/ml ^{3}H-myo-inositol in RPMI 1640 containing 10% FBS. Two hr prior to the assay procedure the cells were placed in serum-free RPMI 1640 containing 4 μCi/ml ^{3}H-myo-inositol. After three washes with HBSS containing 5 mM myo-inositol (cold), 2 ml of medium A containing 10 mM LiCl were added to each well. After equilibration for 5 min at 37°C cells were stimulated with bradykinin (1 μM). The reaction was terminated by aspiration of the reaction buffer and addition of 10% TCA (1 ml). After 10 min the extracts containing inositol phosphates were collected and washed four times with water-saturated diethyl ether to remove TCA. Samples were neutralized by addition of 1 M Tris base (~10 μl). Sample volume was adjusted to 2 ml with double-distilled water (dd H_2O) and placed on an AGIX8 column (200–400 mesh, formate form; Bio-RAD) with a bed volume of 750 μl. After eluting with 4 ml of dd H_2O and 5 ml of 60 mM sodium formate/5 mM tetraborate, to remove inositol and glycerophosphoinositol, IP_1 was eluted by addition of 1.25 ml of 80 mM ammonium formate/100 mM formic acid five times. IP_2 was eluted by addition of 2.25/ml of 400 mM ammonium formate/100 mM formic acid five times. IP_3 was eluted by addition of 2 ml of 800 mM ammonium formate/100 mM formic acid five times. IP_4 was eluted by addition of 1.25 ml of 1.3 M ammonium formate/100 mM formic acid five times. Aquasol (15 ml) was added to each elution fraction and radioactivity determined by counting in a Beckman LS3801 scintillation counter.

Results

$[Ca^{2+}]_i$ Changes Due to EGF or TGF-α

The addition of EGF or TGF-α (30 ng/ml) induced a rapid biphasic rise in free cytosolic Ca^{2+} ($[Ca^{2+}]_i$) in fura-2 loaded cells (Fig. 1*A* and *B*). A maximal

response was obtained after 15–30 sec and slowly returned toward baseline over the next 20 min. Peak values ranged from 300–600 nM (425 ± 31, n = 10). A similar response was observed upon addition of EGF (30 ng/ml) to A431 cells (Fig. 1*B*). When A431 cells were first suspended in Ca^{2+}-free media and then treated with TGF-α there was a rapid fivefold increase in $[Ca^{2+}]_i$ (42 to 212 nM) that peaked within 20 sec but returned to baseline in 3 min as compared to the 20 min in Ca^{2+}-containing medium (Fig. 1*C*). An alternative means of showing intracellular Ca^{2+} release by an agonist is to block Ca^{2+} entry with La^{3+}. As shown in Fig. 1*D*, 10 μM La^{3+} was not able to prevent the TGF-α induced rise in $[Ca^{2+}]_i$, but, as in Ca^{2+}-free media, the activated cells reduced $[Ca^{2+}]_i$ to resting levels within about 3 min. Similar data were obtained with A431 cells grown on coverslips and loaded with fura-2. Therefore, the trypsinization procedure did not interfere with the EGF-induced changes in $[Ca^{2+}]_i$.

TGF-α and EGF-dependent rises in $[Ca^{2+}]_i$ were dose dependent with maximal responses at 30 ng/ml for each agonist (Fig. 2). The EC_{50} for Ca^{2+} mobilization was approximately 3.0 nM for both TGF-α and EGF.

Effect of Modulation of Protein Kinase C by TPA, Staurosporine and R59022

Addition of 50 ng/ml of TPA to quin-2 loaded A431 cells has been shown to inhibit EGF-induced Ca^{2+} mobilization [16]. In the current experiments A431 cells were loaded with fura-2 to study both the release of Ca^{2+} from intracellular stores and uptake of Ca^{2+} through the plasma membrane. Addition of EGF (TGF-α *not shown*) to cells treated with 5 × 10^{-11} M, 1 × 10^{-10} M and 2 × 15^{-10} M TPA for 2 min still induced a rapid transient rise in $[Ca^{2+}]_i$. However, cytosolic Ca^{2+} returned to baseline more rapidly as the TPA concentration increased (Fig. 3*A*). Thus, the $T_{1/2}$ of the decline of the Ca^{2+} signal was reduced from 5 min to 75 sec at 2 × 10^{-10} M

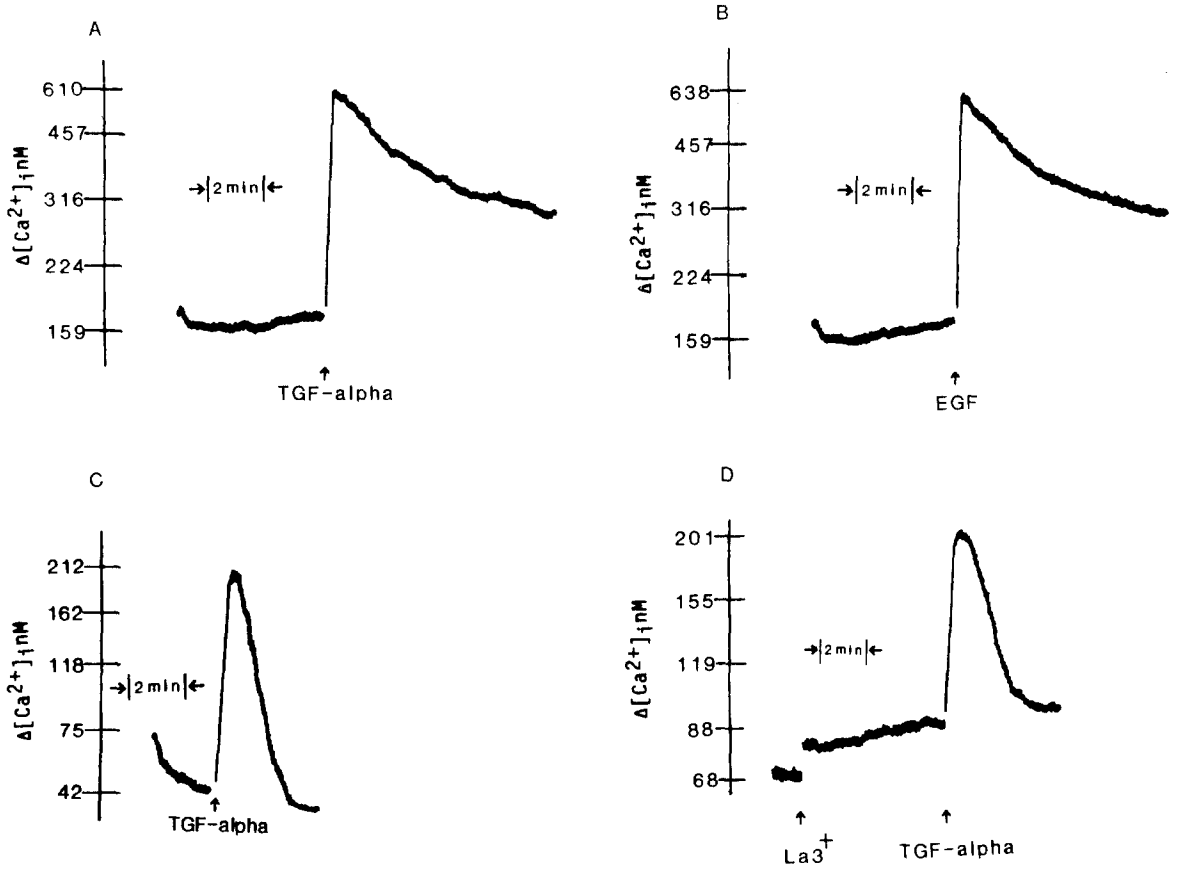

Fig. 1. TGF-α induced changes in $[Ca^{2+}]_i$. A431 cells (7 × 10^4 cells/ml) loaded with fura-2 AM (as described in Materials and Methods) were stimulated with TGF-α (30 ng/ml), and $[Ca^{2+}]_i$ was measured. (*A*) TGF-α response in medium A. (*B*) EGF (30 ng/ml) response in medium A. (*C*) TGF-α response in medium A without calcium containing 0.2 mM EGTA. (*D*) TGF-α response in medium A containing 10 μM La^{3+}

TPA. The finding that the peak value for $[Ca^{2+}]_i$ did not change at these levels of TPA suggests that Ca^{2+} entry does not contribute to the rapid rise of $[Ca^{2+}]_i$ in this cell line.

Addition of 1 or 10 nM staurosporine, a PKC inhibitor [18], before 2×10^{-10} TPA inhibited the faster return of $[Ca^{2+}]_i$ towards baseline (Fig. 3*B*). Thus, the effect of staurosporine on the TPA effect is consistent with an action of the chemicals on the same target, protein kinase C.

A431 cells treated with TPA for 5–10 min periods of time and/or higher concentrations of TPA (2×10^{-10} to 2×10^{-6} M) before addition of EGF/TGA-α showed inhibition of EGFR-induced Ca^{2+} release from intracellular stores as well as Ca^{2+} entry. The inactive phorbol ester 4-α-phorbol 12,13 didecanoate did not alter EGF/TGF-α induced changes at $[Ca^{2+}]_i$ in concentrations up to 1×10^{-7} M.

Treatment of A431 cells with the diglyceride kinase inhibitor R59022 was used as another approach to determine the role of PKC activation in Ca^{2+} signaling. R59022 is known to increase DAG and hence cellular PKC activity in platelets [4] and neutrophils [15]. Incubation of A431 cells for 5 min with 3–10 μM R59022 resulted in a dose-related effect on Ca^{2+}, namely noted as a more rapid return to baseline after TGF-α stimulation (Fig. 4). No effect on release from intracellular stores was observed in contrast to high TPA concentrations. Presumably TPA activates PKC in all cellular compartments, whereas increased DAG levels due to diglyceride kinase inhibition will occur almost exclusively in the plasma membrane. Thus, R59022 will affect Ca^{2+} entry, but not Ca^{2+} release from intracellular stores by activated plasma membrane bound PKC.

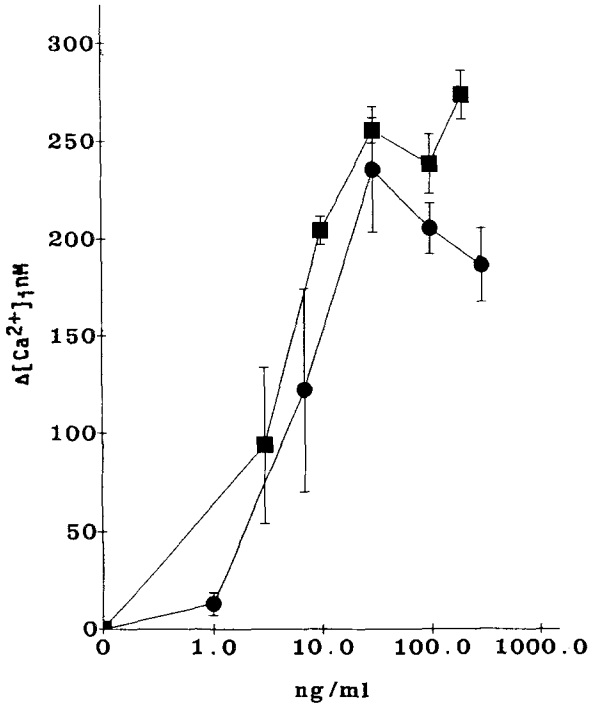

Fig. 2. Dose-response curve for TGF-α and EGF-induced increase in $[Ca^{2+}]_i$. Fura-2 loaded A431 cells ($7 + 10^4$ c/ml) were suspended in medium A, the indicated amount of TGF-α (■—■) or EGF (●—●) was added, and $[Ca^{2+}]_i$ was measured

Mn^{2+} as a Monitor of Ca^{2+} Entry

To distinguish between the effects of TPA treatment on Ca^{2+} entry, release from intracellular stores and activation of Ca^{2+} pumps fura-2 loaded cells were suspended in Ca^{2+}-free medium containing Mn^{2+}. Addition of EGF or TGF-α stimulated the release of

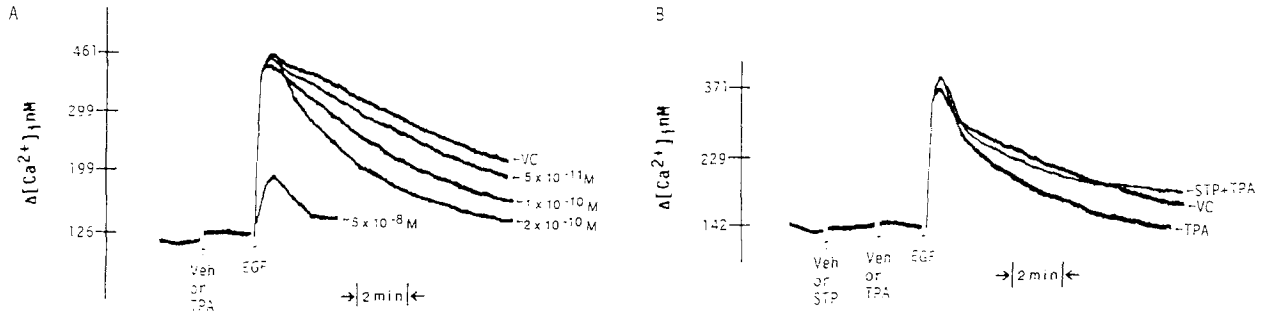

Fig. 3. Effect of the phorbol ester TPA on the EGF-receptor-mediated changes in $[Ca^{2+}]_i$. Fura-2 loaded A431 cells (7×10^4 c/ml) were suspended in medium A and incubated for 2 min with TPA (5×10^{-11} M, 1×10^{-10} M, 2×10^{-10} M, and 5×10^{-8} M), stimulated with EGF (30 ng/ml), and then $[Ca^{2+}]_i$ was measured. (*A*) EGF response in medium A after TPA pretreatment. (*B*) EGF response after 2-min treatment with staurosporine (*STP;* 1 nM) or vehicle (*Veh;* DMSO) and then treated with TPA (2×10^{-10} M) or vehicle (DMSO) for 2 min. *VC* = vehicle control

intracellular Ca^{2+} causing an initial increase in fura-2 fluorescence which was followed by fluorescence quenching as Mn^{2+} entered the cell through the plasma membrane. Figure 5*A* shows the effects of different concentrations of Mn^{2+} in the buffer medium on baseline $[Ca^{2+}]_i$. As $[Mn^{2+}]_o$ was increased, quenching of baseline $[Ca^{2+}]_i$ was observed suggesting a persistent divalent cation influx across unstimulated A431 plasma membranes. A concentration of 10 μM Mn^{2+} was chosen for subsequent EGF or TGF-α experiments to minimize quenching of baseline $[Ca^{2+}]_i$ and to provide good agonist-induced fluorescence quenching. Addition of 10 μM La^{3+} completely blocked Mn^{2+}-induced quenching (Fig. 5*B*). Nifedipine also blocked Mn^{2+} entry in a dose-related manner at 10 and 30 μM (Fig. 5*B*) but was not as effective as La^{3+}. No additional inhibition was observed at 100 or 300 μM nifedipine.

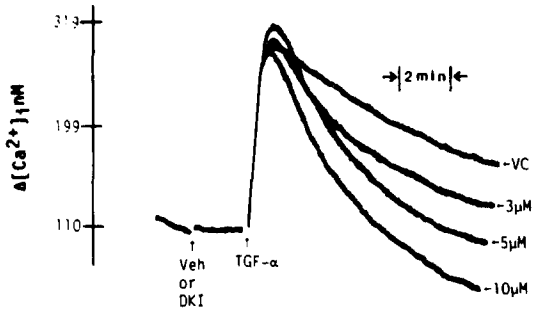

Fig. 4. Effect of R59022 on TGFα-induced $[Ca^{2+}]_i$. Fura-2 loaded A431 cells (7×10^4 c/ml) were suspended in medium A, incubated 2 min with R59022 or vehicle (ethanol/double-distilled water 50 : 50), stimulated with TGF-α (30 ng/ml), and then $[Ca^{2+}]_i$ was measured. Addition of R59022 to the cuvette resulted in a shift in baseline fluorescence that is concentration dependent. This baseline shift is caused by drug-induced autofluorescence and is not shown in the overlayed scans. *DKI* = diglyceride kinase inhibitor R59022; *Veh* = vehicle; *VC* = vehicle control

The effect of EGF (30 ng/ml) fura-2 fluorescence in A431 cells suspended in 10 μM Mn^{2+} containing buffers with and without TPA pretreatment is shown in Fig. 6. In vehicle-treated cells the addition of EGF resulted in a Ca^{2+} transient indicating the release of Ca^{2+} from intracellular stores followed by quenching of fura-2 fluorescence due to the influx of Mn^{2+}, which resulted in a signal that fell well below the baseline fura-2 signal. Pretreatment with TPA for 2 min inhibited the fluorescence quenching without affecting Ca^{2+} release from intracellular stores. A concentration of 2×10^{-10} M TPA is usually required to prevent Mn^{2+} entry. In some experiments, however, $2–5 \times 10^{-11}$ M TPA was sufficient for the selective effect of TPA on Ca^{2+} entry. PKC-mediated inhibition of Mn^{2+} entry is consistent with the conclusions derived from cells in Ca^{2+} media, namely that Ca^{2+} entry is inhibited by PKC activation in A431 cells. The use of Mn^{2+} shows that the

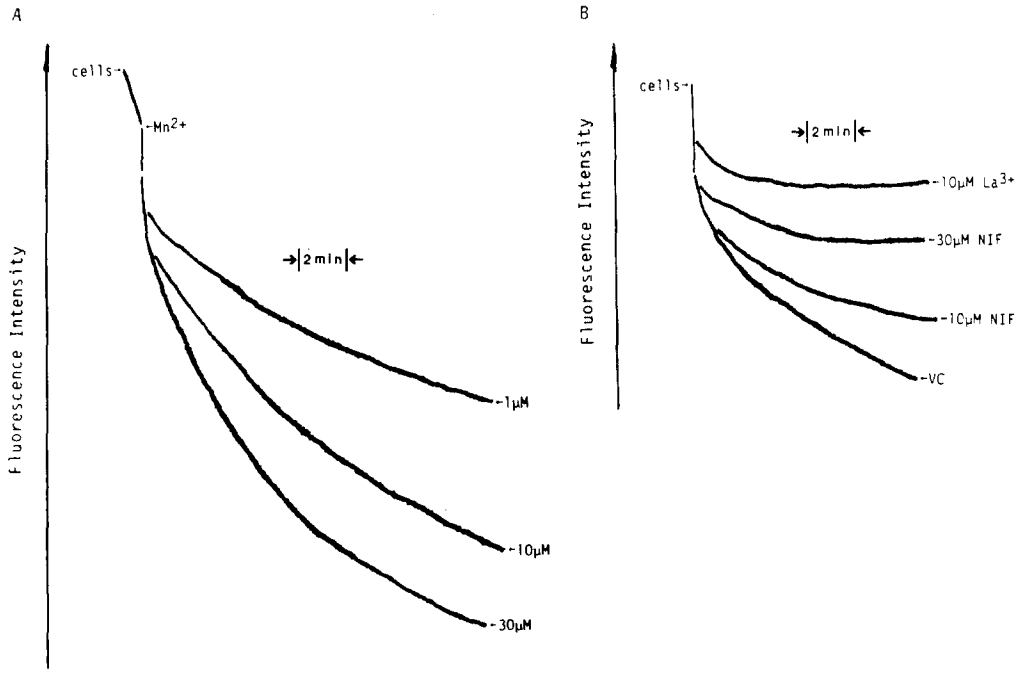

Fig. 5. Effect of lanthanium or and nifedipine on Mn^{2+} entry. Fura-2 loaded A431 cells (7×10^4 c/ml) were suspended in medium A without calcium and fluorescence quench was measured upon addition of Mn^{2+}. (*A*) Addition of Mn^{2+} (1, 10 or 30 μM). (*B*) Addition of vehicle (water), La^{3+} (10 μM) or nifedipine (*NIF;* 10 or 30 μM). *VC* = vehicle control

entry pathway is completely inhibited at 2×10^{-10} M TPA. Therefore, the decay of the Ca^{2+} signal found after TPA treatment in Ca^{2+} media is due to sequestration of Ca^{2+} into intracellular stores and export across the plasma membrane.

Effect of Downregulation of PKC

The results shown in Figs. 3 to 6 suggested that activation of PKC caused $[Ca^{2+}]_i$ to return more rapidly to baseline. If there were no PKC activity in the A431 cells the $[Ca^{2+}]_i$ would remain elevated for a prolonged period of time after TGF-α or EGF stimulation. It is difficult to measure PKC activation by TPA *ex vivo*, but it is possible to measure the level of PKC following downregulation.

A431 cells were treated with 0.1 or 1 μM TPA for 24 hr to downregulate PKC. PKC activity was 75 ± 5 (n = 3) pmol/mg protein/min in vehicle-treated cells *versus* 3 ± 2 (n = 3) in 24-hr TPA-treated cells. Rodriguez-Pena and Rozengurt [25] have also shown that long-term treatment with TPA results in loss of PKC activity in whole cells. These cells thus show downregulation of PKC. The baseline $[Ca^{2+}]_i$ was unchanged in these 24-hr treated cells as compared to cells treated with DMSO alone, 95 ± 7 nM (n = 9) and 96 ± 10 nM (n = 10). When TPA-pretreated cells were stimulated with 30 ng/ml of either EGF or TGF-α there was an enhanced rise in the $[Ca^{2+}]_i$ response that peaked at 358 ± 17 nm (n = 4) when compared to cells treated for 24 hr with DMSO only, 231 ± 20 nM (n = 4). Further, there was essentially a stable $[Ca^{2+}]_i$ response in the TPA-treated cells rather than the biphasic response seen in control cells (Fig. 7*A*, curves *a* and *c*). Addition of TPA prior to either growth factor had no effect on the $[Ca^{2+}]_i$ signal in the 24-hr TPA-pretreated cells (Fig. 7*A*, curve *b*), as might be expected from the measured marked decline of PKC, whereas the cells that had been exposed to only DMSO for 24 hr showed the usual more rapid reduction of $[Ca^{2+}]_i$ to baseline following TPA addition (Fig. 7*A*, curve *d*). These data show that reduction of PKC by 24-hr treatment of A431 cells by TPA results in both an increase of Ca^{2+} entry and release following EGFR activation.

This was demonstrated by the addition of Mn^{2+} to these cells. In Fig. 7*A* the scale was readjusted to allow superposition of the effect of Mn^{2+} under different conditions. The initial, time-independent effect of Mn^{2+} on fura-2 fluorescence of control and downregulated cells is due to interaction of the cation with fura-2 that has leaked from the cells. This effect was followed by a rapid fall of fluorescence showing rapid Mn^{2+} entry. This was faster and larger in downregulated cells (curves *a* and *b*) than cells treated with DMSO only (curves *c* and *d*) and was not affected by a second TPA treatment of the downregulated cells (curve *b*). In contrast, cells which had been incubated with only DMSO for 24 hr responded to the addition of TPA by blocking Mn^{2+} entry, as before (Fig. 7*A*, curve *d*).

The Mn^{2+} quench model was used as an additional means of showing that downregulation of PKC enhanced EGF or TGF-α mediated divalent cation (i.e., Mn^{2+}) entry through the plasma membrane. Downregulated and DMSO-treated cells were placed in buffers containing Mn^{2+} instead of Ca^{2+} and stimulated with EGF (TGF-α *not shown*). As shown in Fig. 7*B*, the EGF-induced Ca^{2+} transient was enhanced in downregulated cells (curve *b*) as also shown in Fig. 7*A* and more Mn^{2+} entered the A431 cells resulting in a greater rate and magnitude of fluorescence quenching.

PKC was not completely downregulated. An activity of 3 ± 2 pmol/mg protein/min was measured after 24-hr treatment with TPA. To determine if this small remaining activity could affect Ca^{2+} signaling a high concentration of TPA (1 μM) was added to TPA- and vehicle-treated cells. In 24-hr treated cells addition of more TPA- and vehicle-treated cells 2 min prior to EGF resulted in Ca^{2+} mobilization similar to the downregulated state, whereas it greatly inhibited vehicle-treated cells (Fig. 8). Thus, the residual PKC activity was unable to regulate Ca^{2+} entry.

An unambiguous demonstration of a relation-

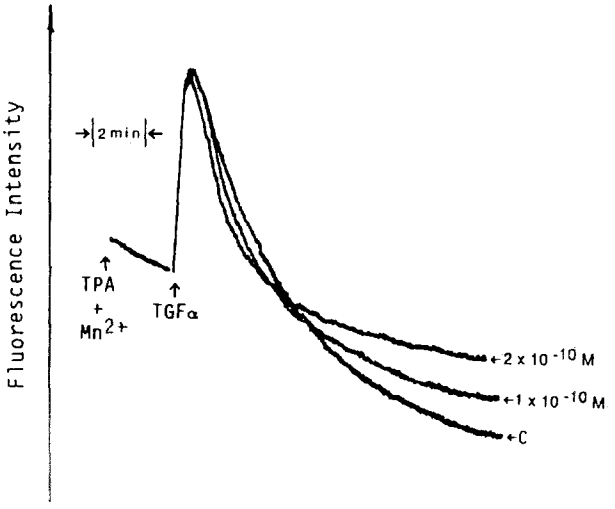

Fig. 6. Effect of TPA on release of Ca^{2+} and Mn^{2+} uptake. Fura-2 loaded A431 cells (7×10^4 c/ml) were suspended in medium A without Ca^{2+} containing Mn^{2+} (10 μM) and TPA (1×10^{-10} M or 2×10^{-10} M) or vehicle (DMSO) for 2 min and then stimulated with TGF-α (30 ng/ml)

ship between PKC-regulated Ca^{2+} influx and IP_3 accumulation was not possible with EGF. We previously reported that an EGF-induced rise in IP_3 was not observable in this A431 clone although Ca^{2+} mobilization is quite readily quantitated (Fig. 1; ref. [37]). The methods of Tilly [31] have recently been tried also without success. However, the inositol response following bradykinin was readily observed.

Bradykinin is an Ca^{2+}-mobilizing receptor agonist that induces PI turnover in A431 cells [9, 31]. In contrast to EGF the addition of bradykinin gave an easily measurable IP_3 signal and Ca^{2+} mobilization. We investigated the effects of TPA on bradykinin-induced IP_3 levels and Ca^{2+} mobilization in this A431 cell clone. Addition of bradykinin to A431 cells resulted in Ca^{2+} mobilization that appeared to be largely due to release from intracellular stores given the pattern of the change in $[Ca^{2+}]_i$ (Fig. 9*A* and *B*). Treatment of A431 cells with 10^{-10} M TPA for 2 min to activate PKC had little effect on $[Ca^{2+}]_i$ (Fig. 9*C*). Even higher TPA concentrations (10^{-10}–10^{-8} M) showed little inhibition of the $[Ca^{2+}]_i$ response over a wide bradykinin concentration range (10^{-10}–10^{-8} M; Fig. 10).

Addition of 1-μM bradykinin stimulated the release of inositol phosphates (Fig. 11*A*). IP_3 and IP_4 peaked at 30 sec and began to decline towards baseline at 1 min (Fig. 11*B*). There was no statistical differences between cells treated with 10^{-10} M TPA at 15 and 30 sec after bradykinin addition.

Twenty-four hr treatment of the cells with TPA did not modify the bradykinin-mediated release of Ca^{2+} from intracellular stores (*data not shown*).

Thus, modification of PKC activity had no measurable effect on the bradykinin-mediated Ca^{2+} release, in contrast to the pathways stimulated by the EGFR. It was therefore of interest to determine whether EGF, TGF-α and bradykinin shared the same intracellular Ca^{2+} pool. A separation of the intracellular stores could explain the marked difference in response to PKC modulation.

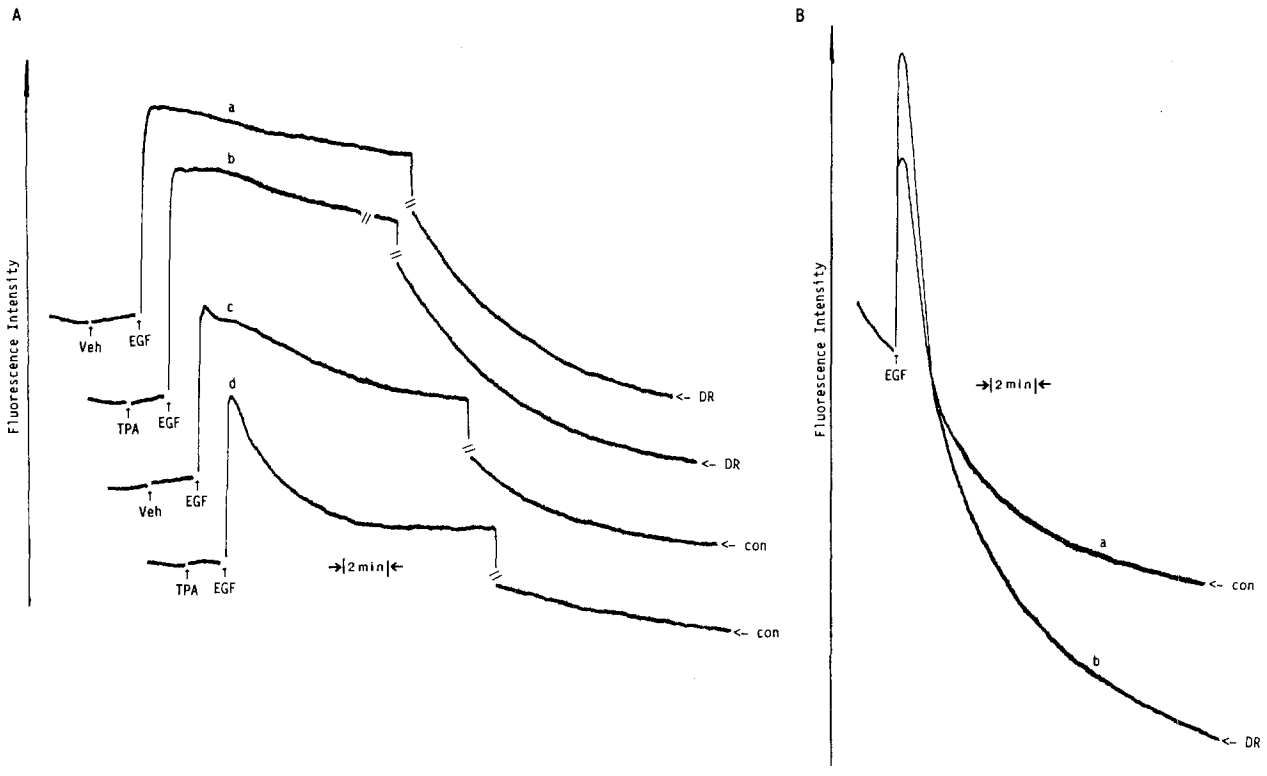

Fig. 7. Effect of PKC downregulation on the EGF-receptor response. A431 cells treated 24 hr with TPA (*DR;* 1 μM) or vehicle (*Veh;* DMSO) were loaded with fura-2 and suspended in Ca^{2+}-containing medium A (7×10^4 c/ml), and then $[Ca^{2+}]_i$ was measured. (*A*) Shows the effects of 2-min pretreatment with vehicle (DMSO) or TPA (2×10^{-10} M) and then stimulation with EGF (30 ng/ml). After 15 min, 10 μM Mn^{2+} was added to measure flux across plasma membrane. The top curve *a* shows downregulated cells with vehicle and then EGF; curve *b* shows downregulated cells with TPA added 2 min before EGF; curve *c* shows control cells with vehicle and then EGF; and curve *d* shows control cells treated with TPA 2 min prior to EGF addition. The initial drop after Mn^{2+} addition is not drawn to scale, and the curves are separated for clarity, although $[Ca^{2+}]_i$ started at the same value. (*B*) EGF (30 ng/ml) stimulation in medium A without Ca^{2+} but containing 10 μM Mn^{2+}. Curve *a* shows the effect of EGF addition to cells not preincubated for 24 hr with TPA and curve *b* shows the effect of TPA preincubation on the Ca^{2+} signal following EGF addition

Interactions of EGF, TGF-α, Bradykinin and Ionomycin

The response of A431 cells to EGF or TGF-α and bradykinin show characteristic differences in terms of the source of Ca^{2+} and the effect of modification of protein kinase C activity as discussed above. We therefore determined the effects on $[Ca^{2+}]_i$ of sequential addition of EGF, TGF-α and bradykinin, as well as ionomycin, in the presence of any of the agonists. If all the agonists share a common pool, then only a single release of Ca^{2+} would be expected. If there were separate pools, then a second release of Ca^{2+} would be found with the addition of a second agonist.

The addition of 30 ng/ml EGF to the cells in Ca^{2+}-free medium (to obviate Ca^{2+} entry) gave a transient Ca^{2+} signal that peaked and returned to baseline within 3 min. A subsequent dose of TGF-α gave no additional signal, and reversing the order of addition gave identical data (Fig. 12*A* and *B*). Simultaneous addition of maximally effective doses of EGF and TGF-α gave the same response as with either agonist alone. Thus, these two mediators appear to release Ca^{2+} from an identical intracellular Ca^{2+} store.

These data were in sharp contrast to the effects of combinations of EGF and bradykinin. When EGF was given and the Ca^{2+} signal allowed to return to baseline, the subsequent addition of bradykinin resulted in a second response of $[Ca^{2+}]_i$ (Fig. 12*A* and *B*). Reversing the order of addition gave similar results; namely, following the bradykinin response, EGF or TGF-α were still able to mobilize intracellular Ca^{2+} (Fig. 12*C*).

These data are simply interpreted if there are separate intracellular Ca^{2+} stores accessed by the EGF receptor and the bradykinin receptor. Ionomycin, in Ca^{2+}-free solutions, was used to provide further evidence for separate Ca^{2+} pools. Ionomycin added at 1 μM resulted in a transient increase of $[Ca^{2+}]_i$ that was about twice that of EGF, TGF-α or bradykinin alone (Fig. 13*A–D*). The addition of either mediator following ionomycin gave no change in $[Ca^{2+}]_i$, showing that this level of ionomycin had depleted the Ca^{2+} stores in A431 cells that responded to either class of agonist (Fig. 13*A*). Following the addition first of either EGF/TGF-α or

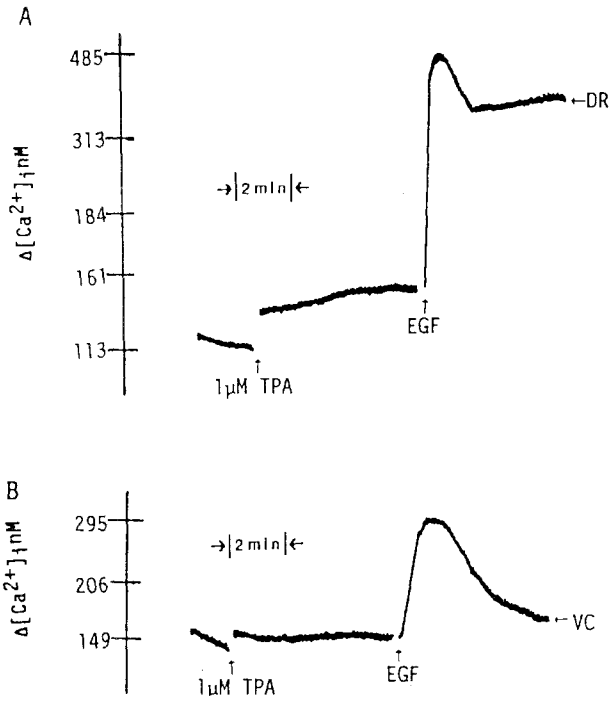

Fig. 8. Effects of downregulation of PKC. Fura-2 loaded cells (7×10^4 c/ml) were suspended in medium A and incubated 2 min with TPA (1 μM). (*A*) A431 cells treated 24 hr with 1 μM TPA to downregulate PKC activity. (*B*) Cells treated with DMSO for 24 hr

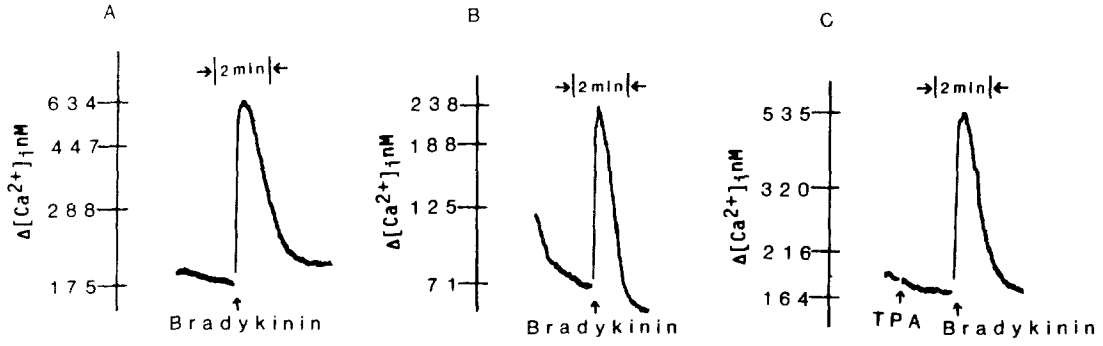

Fig. 9. Action of TPA on the bradykinin-induced release of $[Ca^{2+}]_i$. Fura-2 loaded A431 cells (7×10^4 c/ml) were suspended in medium A and stimulated with bradykinin (1 μM), and $[Ca^{2+}]_i$ was measured. (*A*) Bradykinin response in medium A. (*B*) Bradykinin response in Ca^{2+}-free medium A containing 0.2 mM EGTA. (*C*) Bradykinin response in medium A after a 2-min preincubation with TPA (2×10^{-9} M)

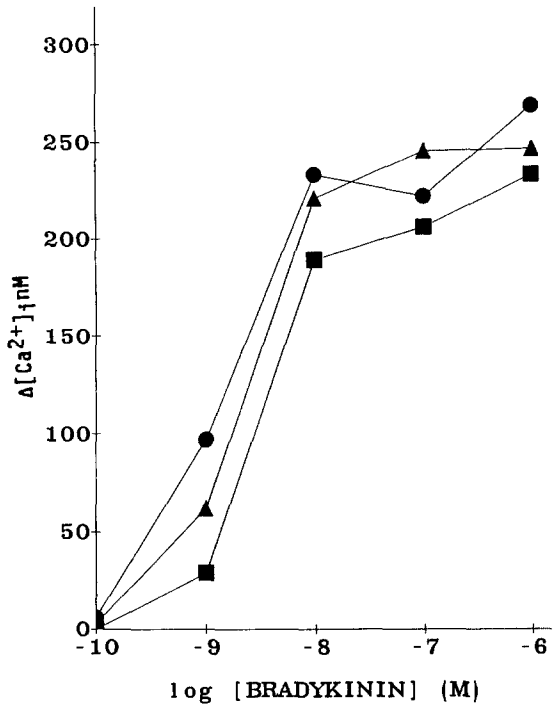

Fig. 10. Dose-response curve of action of TPA on bradykinin-induced increase of $[Ca^{2+}]_i$. Fura-2 loaded A431 cells (7×10^4 c/ml) were suspended in medium A, incubated 2 min with TPA or vehicle (DMSO), stimulated with the indicated concentration of bradykinin (10^{-10} to 10^{-6} M), and then $[Ca^{2+}]_i$ was measured. The vehicle control is represented by the (●—●), ▲—▲ represents cells pretreated with 10^{-10} M TPA, and the (■—■) represents cells pretreated with 10^{-6} M TPA. A 10^{-8} M TPA pretreatment was also used and was similar to 10^{-10} M (*data not shown*)

bradykinin with a normal transient change in $[Ca^{2+}]_i$, the addition of ionomycin produced a Ca^{2+} transient about half of that found with ionomycin alone. Subsequent addition of the appropriate agonist following ionomycin produced no further change in $[Ca^{2+}]_i$ (Fig. 13*C* and *D*). Ionomycin alone therefore released about twice as much Ca^{2+} as either class of agonist, and ionomycin after one of the agonists released as much Ca^{2+} as did an agonist. Thus, the action of ionomycin also allows the conclusion that there appears to be two pools of agonist-releasable intracellular Ca^{2+} of about equal magnitude, one released by the EGF receptor, the other by the bradykinin receptor.

Simultaneous addition of EGF and bradykinin, however, did not give an additive response (Fig. 13*B*). When this was followed by ionomycin, the ionophore gave an additional Ca^{2+} signal. This suggests that a simultaneous addition of the two classes of stimuli was not able to fully release Ca^{2+} from their intracellular target store. A possible explanation is that the agonists also induce mechanisms that turn off Ca^{2+} release (for example, Ca^{2+} inhibition of Ca^{2+} release), and, therefore, synergy may not be seen.

Discussion

The EGFR-induced response of $[Ca^{2+}]_i$ in A431 cells loaded with fura-2 was biphasic. The initial transient was not affected by 10 μM La^{3+} and hence repre-

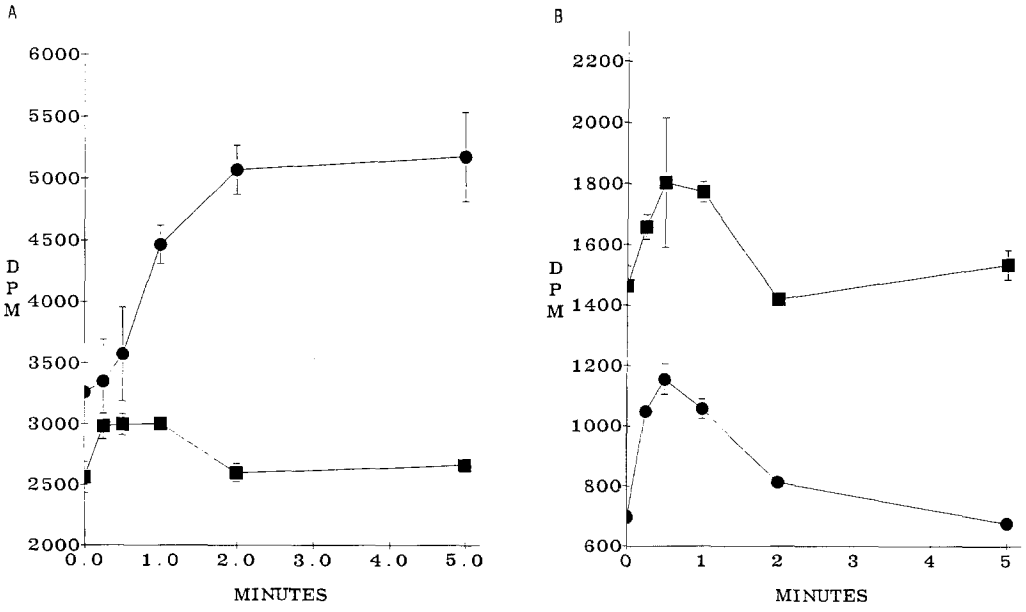

Fig. 11. Bradykinin-induced inositol phosphate production. A431 cells labeled with ^{3}H-myo-inositol (as described in Materials and Methods) were stimulated with 1 μM bradykinin and inositol phosphate production was measured. (*A*) IP_1 (●—●) and IP_2 (■—■) turnover. (*B*) IP_3 (●—●) and IP_4 (■—■) turnover

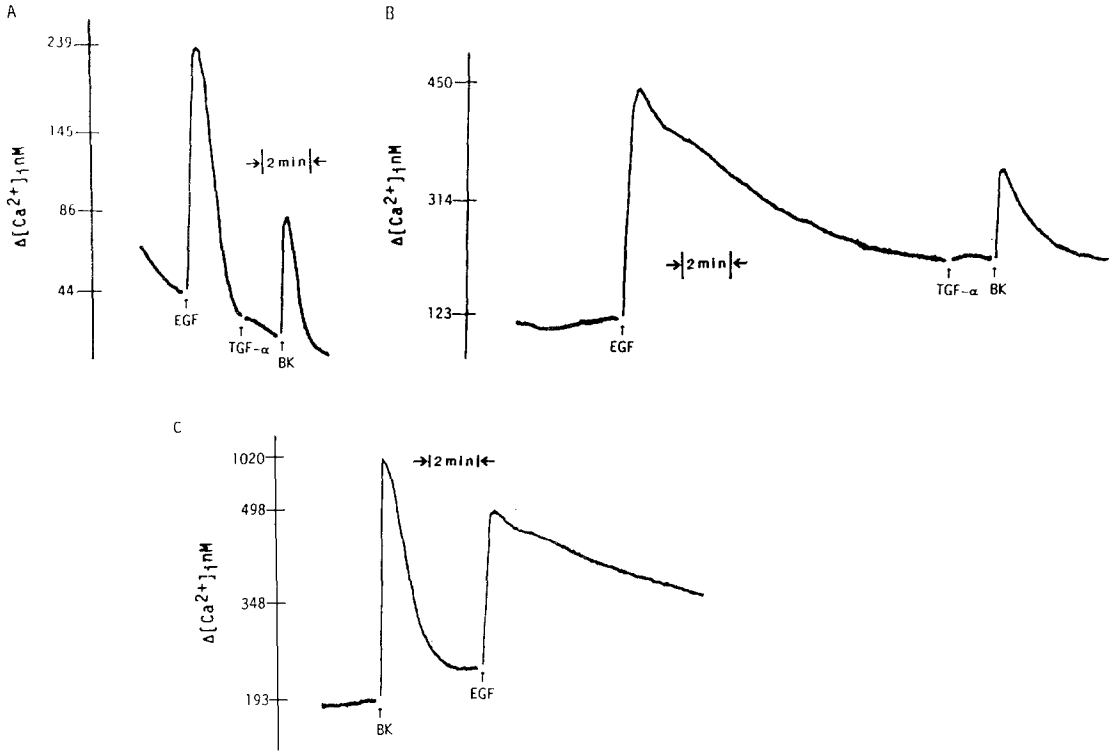

Fig. 12. Effect of sequential addition of EGF and bradykinin on $[Ca^{2+}]_i$. Fura-2 loaded A431 cells (7×10^4 c/ml) were suspended in medium A, stimulated sequentially with EGF, TGF-α and bradykinin (*BK*), and $[Ca^{2+}]_i$ was measured. (*A*) A431 Ca^{2+} response in medium A without Ca^{2+} containing 0.2 mM EGTA after sequential stimulation EGF (30 ng/ml), TGF-α (30 ng/ml) and bradykinin (1 μM). (*B*) Sequential addition of EGF (30 ng/ml), TGF-α (30 ng/ml) and bradykinin (1 μM) in medium A plus 1.4 mM Ca^{2+}. (*C*) Sequential addition of bradykinin (1 μM) and EGF (30 ng/ml) in medium A plus 1.4 mM Ca^{2+}

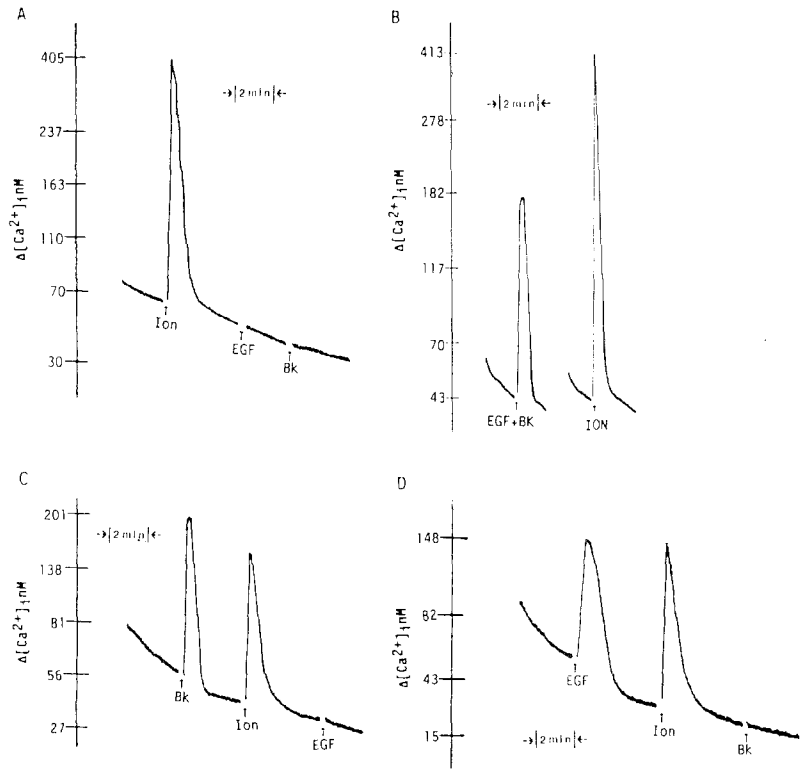

Fig. 13. Effect of sequential additions of ionomycin, EGF and bradykinin on Ca^{2+} release. Fura-2 loaded A431 cells (7×10^4 c/ml) were suspended in medium A without Ca^{2+} containing 0.2 mM EGTA. (*A*) Sequential addition of ionomycin (1 μM), EGF (30 ng/ml) and bradykinin (1 μM). (*B*) Ca^{2+} response after simultaneous addition of EGF (30 ng/ml) and bradykinin (1 μM) as compared to Ca^{2+} response after stimulation with ionomycin (1 mM). (*C*) Sequential addition of bradykinin (1 μM), ionomycin (1 μM) and EGF (30 ng/ml). (*D*) Sequential addition of EGF (30 ng/ml), ionomycin (1 μM) and bradykinin (1 μM)

sented the release of Ca^{2+} from intracellular stores. The second phase during which $[Ca^{2+}]_i$ is maintained above baseline was absent in Ca^{2+}-free medium and when La^{3+} was present, showing that this depended on Ca^{2+} entry via the hormone-operated channel [6, 21, 26]. No difference was found whether EGF or TGF-α was used to activate the receptor, showing a common site of action for these two peptides (Figs. 1 and 2).

Regulation of changes in $[Ca^{2+}]_i$ following the primary receptor-induced Ca^{2+} mobilization involves in part actions of PKC on the Ca^{2+} pathways affected by the receptor. This has been demonstrated by the effects of phorbol esters on changes in $[Ca^{2+}]_i$ with EGF and other types of receptors [1, 2, 6, 24, 27]. For example, in platelets [32] and human salivary cells [8], TPA was able to inhibit intracellular Ca^{2+} release, whereas in quin-2 loaded A431 cells, 10-min incubation with TPA prevented the rise in $[Ca^{2+}]_i$ due to Ca^{2+} entry [16]. It would seem that PKC activity is able to influence both plasma membrane and intracellular Ca^{2+} pathways. Here, we have shown that in one cell type, A431 cells, PKC activity modulates first, plasma membrane Ca^{2+} entry and at higher levels intracellular Ca^{2+} release. This action is selective for the EGF receptor and does not affect bradykinin action on cell Ca^{2+}.

In contrast to quin-2 loaded A431 cells, high concentrations of TPA (5×10^8 M) inhibited most but never all of the EGF-induced rise in $[Ca^{2+}]_i$ in fura-2 loaded cells, since with fura-2 loaded cells both phases of Ca^{2+} mobilization can be visualized. The effects observed on intracellular Ca^{2+} release are consistent with the reported inhibition by TPA of PLC and IP_3 formation [1, 2, 6, 9, 27, 33, 34]. At lower concentrations of TPA (10^{-10} M), only the plasma membrane handling of Ca^{2+} was affected as evidenced by a reduced duration of the $[Ca^{2+}]_i$ plateau. The effects of low TPA concentrations were inhibited by staurosporine, confirming the involvement of PKC. Elevation of DAG by the diglyceride kinase inhibitor, R59022 [4, 15] mimicked the action of TPA on plasma membrane Ca^{2+} pathways (Fig. 4) by also reducing the duration of the change in $[Ca^{2+}]_i$. Again this finding is consistent with an involvement of PKC in regulation of the EGF receptor response of cell calcium. It is also likely that the PKC pathway that inhibits Ca^{2+} entry is rapidly activated since downregulation of PKC increases the $[Ca^{2+}]_i$ peak height.

The effect of PKC activation by low concentration of TPA or by R59022 can be explained by an effect on Ca^{2+} entry or plasma membrane Ca^{2+} pumps [6, 28]. Mn^{2+} was used to show that activation of PKC affected at least Ca^{2+} entry pathways (Figs. 6 and 7). The fluorescence quenching due to Mn^{2+} entry through hormone-operated Ca^{2+} channels was enhanced by stimulation of the EGF receptor and decreased by pretreatment of the cells with TPA, but not with an inactive phorbol ester. In contrast, similar measurements of Mn^{2+} entry in PAF-stimulated platelets [32] and carbachol-activated parotid acinar cells [8] showed no effect of TPA.

These agonists are coupled to PLC by G-proteins, in contrast to EGF which is tyrosine-kinase coupled [34, 35]. Activation of the EGFR tyrosine kinase by ligand is known to phosphorylate PLC and initiates inositol phosphate turnover [35, 36]. Experiments presented in the current study with fura-2 are consistent with TPA not affecting 1,4,5-IP_3 levels in the cell while inhibiting Ca^{2+} entry. The same amount of Ca^{2+} is released in the presence and absence of TPA although Mn^{2+} entry is inhibited (Fig. 6). Attempts were made to measure EGF-stimulated hydrolysis of PIP_2 to show it remained unaltered after TPA treatment. These experiments were unsuccessful in showing a statistically significant increase in EGF-inducible IP_3 in this A431 clone using the methods and conditions necessary to see effects on Ca^{2+} entry. We previously reported [37] that Ca^{2+} entry and release from intracellular stores were readily observed using fura-2 methods but no EGF-induced IP_3 signal was observed. Other investigators have reported differences in A431 clones and shown a 50 to 100% increase in IP_3 over the control [9, 22, 33]. Wahl and Carpenter [34] show that maximal production of IP_3 requires at least 75% receptor occupancy. This was achieved by incubation of A431 cells at 4°C for 3 hr. This method was tried so that simultaneous measurement of IP_3 and $[Ca^{2+}]_i$ could be done. However, it was not compatible with the experimental conditions necessary for using the Ca^{2+} dye binding techniques and for treating with TPA. Bradykinin was investigated as an alternative Ca^{2+}-mobializing receptor agonist that is much more effective at stimulating PI turnover with a 300–600% increase in IP_3 over control A431 cells [9, 10, 31], as also shown here. However, bradykinin induced little Ca^{2+} entry and TPA at concentrations as high as 10^{-6} M had little effect on Ca^{2+} mobilization.

The role of PKC in modulation of the $[Ca^{2+}]_i$ response was also examined in cells chronically depleted of PKC by 24-hr incubation in the presence of TPA. Here the initial rise in Ca^{2+} was greater and there was no return of $[Ca^{2+}]_i$ to baseline. The enhancement of the $[Ca^{2+}]_i$ transient might be due to the enhanced level of IP_3 or IP_4 following EGF stimulation in down regulated A431 cells [21, 33]. Alternatively, PKC may play a role more directly in regulation of the intracellular Ca^{2+} store response. Here, removal of PKC would allow greater Ca^{2+} release since elevation of PKC inhibited the initial rapid rise in $[Ca^{2+}]_i$. Elevation of PKC also inhibited Ca^{2+} or Mn^{2+} entry, hence removal of PKC would

allow prolonged elevation of $[Ca^{2+}]_i$, as was found. The effects of PKC alteration therefore suggest that both major Ca^{2+} pathways of EGF or TGF-α are continually subject to PKC regulation when activated, whereas bradykinin regulation is independent of PKC.

Distinctions between the EGF and bradykinin pathways were clearly demonstrated by the results presented here. EGF affects both plasma membrane entry of Ca^{2+} and release of Ca^{2+} from intracellular stores, whereas bradykinin affects only intracellular stores. In the case of bradykinin it seems that the action is mediated by an increase in IP_3 and IP_4. In contrast to some epithelial cells [17], this combination of second messengers does not affect Ca^{2+} entry in A431 cells, since there has been no evidence for a plasma membrane component of the $[Ca^{2+}]_i$ response of this clone of A431 cells. Presumably the G-protein coupled to the bradykinin receptor is not coupled to a Ca^{2+} channel. Changes of PKC activity were without measurable effect on the bradykinin response even at levels where PKC activation was affecting the intracellular release component of EGF-stimulated effects. This might suggest that the EGF-regulated intracellular Ca^{2+} is regulated differently from the bradykinin-regulated pool in this cell line, inasmuch as the bradykinin pool is apparently independent of levels of PKC.

Various explanations for separate pools can be suggested, such as separate anatomic locations or distinct second messenger pathways or second messenger compartmentation. Recent studies show that activation of A431 cells with EGF but not bradykinin results in tyrosine phosphorylation of PLC. Using an anti-phosphotyrosine antibody, immunoprecipitates of EGF-treated cells show enhanced PLC activity [35, 36]. The biochemical interaction of EGFR and the PLC enzyme may result in very localized release of inositol phosphates and coupled with rapid metabolism of IP_3 may explain the difficulty in measuring IP_3 under conditions where a Ca^{2+} signal is readily quantitated. However in IIC9 fibroblasts, EGF has been shown to increase diacylglycerol but without any increase in hydrolysis of polyphosphoinositides [6, 38]. Such a mechanism would account for activation of PKC, as implied here, and would require an alternative messenger for release of Ca^{2+} from intracellular stores.

It was also noted that the actions of EGF or TGF-α and bradykinin did not summate to equal the ionomycin-induced release of Ca^{2+} in these A431 cells. This may be evidence for crosstalk between the bradykinin and EGF intracellular Ca^{2+} stores in that both pools cannot be discharged simultaneously. One possibility is Ca^{2+} inhibition of Ca^{2+} release.

In summary, these results show that PKC regulates both Ca^{2+} entry and Ca^{2+} release in A431 cells when they are activated by the EGF receptor. However, the Ca^{2+} entry pathway is more sensitive to inhibition by PKC than the Ca^{2+} release pathway and indeed a component of Ca^{2+} release is not inhibitable by PKC activation. The difference in sensitivity to TPA may be accounted for by a plasma membrane location of the protein kinase C involved in regulation of plasma membrane Ca^{2+} entry and an endoplasmic reticulum location of the protein kinase C responsible for inhibition of release of Ca^{2+} from intracellular stores. The bradykinin-sensitive intracellular Ca^{2+} pool is not affected by changes in PKC and appears to be distinct from the EGF-releasable Ca^{2+} pool. These concepts are illustrated in Fig. 14. The bradykinin receptor (*BK*) is shown as coupled to G protein which in turn activates PLC. The IP_3 produced from PIP_2 releases Ca^{2+} from intracellular stores.The EGF/TGF-α receptor (*GF*) is a tyrosine kinase (*TK*) mediated system, activating PLC and a Ca^{2+} entry pathway also. However, from our data it is not clear that the Ca^{2+} release due to GF activation is mediated by IP_3, but the intracellular pool appears to be distinct. The DAG produced, however, activates a PKC isoform which when activated by receptor or TPA inhibits Ca^{2+} entry. At higher concentrations of TPA, most but not all of the Ca^{2+} can also be inhibited.

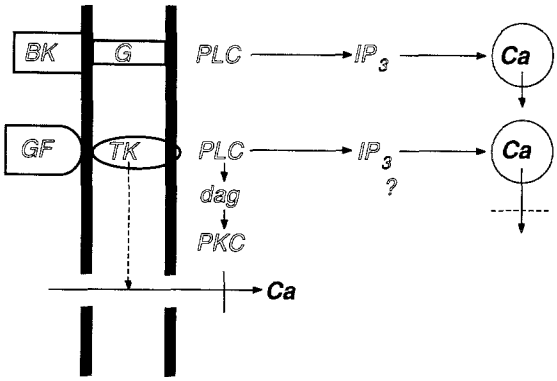

Fig. 14. Calcium signalling in A431 cells. The bradykinin receptor (*BK*) activates PLC by a G protein coupled system and the IP_3 produced releases Ca^{2+} from intracellular stores, but does not cause Ca^{2+} entry. The peptide hormone receptor (*GF*) is tyrosine kinase coupled, resulting in both Ca^{2+} entry and Ca^{2+} release. Coupling to PKC is considered to be via the formation of DAG. IP_3 may be the mediator for Ca^{2+} release, but this is not established by our data, and a different mediator may be responsible. Two separate intracellular Ca^{2+} stores are postulated for the two receptor pathways

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