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Epidermal Growth Factor-Stimulated DNA Synthesis Requires an Influx of Extracellular Calcium

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The dependency of normal cell proliferation on adequate extracellular Ca^{2+} levels was further investigated by determining the role of Ca^{2+} influx in epidermal growth factor (EGF)-induced rat liver epithelial (T51B) cell DNA synthesis. Fura-2-loaded T51B cells responded with an increase in $[Ca^{2+}]_i$ to EGF (5–50 ng/ml) that was blocked by low (25 μM) extracellular Ca^{2+} or by pretreatment with 50 μM La^{3+} to inhibit plasma membrane Ca^{2+} flux. Confluent T51B cells treated for 24 h with EGF (0.1–50 ng/ml) dose-dependently incorporated [^{3}H]-thymidine into cell nuclei. Low extracellular Ca^{2+} or addition of La^{3+} prevented the EGF-stimulated rise in labeled nuclei, indicating that a movement of Ca^{2+} into the cell was required for DNA synthesis. This was supported by our findings that bradykinin, which induced a rise in $[Ca^{2+}]_i$ by opening plasma membrane Ca^{2+} channels in T51B cells (but not A23187, thrombin or ATP, which raise $[Ca^{2+}]_i$ primary through mobilization of intracellular Ca^{2+} stores), potentiated DNA synthesis stimulated by submaximal doses of EGF. Potentiation of the action of EGF by the tumor promoter 12-0-tetradecanoyl-phorbol-13-acetate (TPA), indicates that activation of protein kinase C and an influx of Ca^{2+} share a common mechanism for initiating DNA synthesis.

Key words: EGF, DNA synthesis, Bradykinin, TPA, rat liver epithelial cells, calcium

Extracellular Ca^{2+} has been demonstrated to be required for cell proliferation in a wide variety of non-neoplastic cells of mesenchymal and epithelial origin both in vivo and in vitro [1–5]. Studies of rat liver epithelial (T51B) cell division have revealed two specific extracellular Ca^{2+}-dependent stages within the cell-division

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cycle [6–8]. The first is a brief 30 min period necessary for cells to transit the G_0 to G_1 boundary, and the second occurs during the 2 h immediately preceding the G_1 to G_S transition. In contrast, neoplastic cells require 10- to 50-fold less extracellular Ca^{2+} for proliferative activity and for these specific Ca^{2+}-dependent cell-cycle transitions than do their non-neoplastic counterparts [9,10].

The biochemical basis for the Ca^{2+} sensitivity in normal cells has been the focus of several investigations, which demonstrated that calmodulin, Ca^{2+}/calmodulin-dependent protein kinase, cyclic AMP, cyclic AMP-dependent protein kinase, and Ca^{2+} are involved in the progression of cells through the G_1 phase of their cell-cycle [for review, see 11]. These observations suggest that a surge of Ca^{2+} into the cytosolic compartment of cells is a necessary event for initiation of G_0 to G_1, and G_1 to S cell-cycle transitions. However, the requirement for a rise in free Ca^{2+}, or the source of Ca^{2+}, whether extracellular or intracellular, has not been established.

Ca^{2+} influx across the plasma membrane in response to mitogens binding to surface receptors occurs in a variety of cell types, yet the importance of membrane Ca^{2+} fluxes in cell proliferation is unknown [12]. This study examines the role of plasma membrane Ca^{2+} influx in T51B cell DNA synthesis induced by epidermal growth factor (EGF)—an agent shown to cause phosphotidylinositol turnover and a rise in intracellular free Ca^{2+} ($[Ca^{2+}]_i$) [13,23].

MATERIALS AND METHODS

Materials

EGF, bradykinin, thrombin, ATP, A23187, and $LaCl_3$ were purchased from Sigma (St. Louis, MO). TPA (12-0-tetradecanoyl-phorbol-13-acetate) was obtained from LC Services Corp (Woburn, MA). Fura-2am (acetoxymethyl ester) was obtained from Molecular Probes (Eugene, OR). [^{3}H] thymidine was acquired from ICN (Cambridge, MA).

Cell Culture

T51B cells were plated at a density of 7,000 cells/cm^2 on either 24 × 30-mm rectangular Thermanox coverslips (DNA synthesis) or 12-mm round glass coverslips (Ca^{2+} measurement) placed in multiwell sterile culture plates (Miles Lab., Naperville, IL) and grown to confluency (3×10^4 cells/cm^2) in Eagle's Basal Medium (BME; Gibco, Grand Island, NY) supplemented with 10% bovine calf serum (BCS; Colorado Serum Co., Denver, CO) and 25 μg/ml gentamicin (37°C; 95% air/5% CO_2, water saturated).

Measurement of Cytoplasmic Free Ca^{2+} Concentration

Monolayers of confluent T51B cells on glass coverslips (12 mm) were loaded with 5 μM fura-2am for 45 min in 90% BME:10% BCS, washed 5 min in HEPES buffer (25 mM HEPES, 125 mM NaCl, 6.0 mM KCl, 1.0 mM MgCl, and 0.55 mM glucose, pH 7.4), and placed in plastic cuvettes (1-cm light path) containing either low Ca^{2+} (0.025 mM) HEPES buffer or high Ca^{2+} (1.8mM) HEPES buffer Ca^{2+} (37°C). Changes in fluorescence were monitored at 340-nm excitation and 500-nm emission using a fluorescence spectrophotometer (Perkin Elmer). Calculations of $[Ca^{2+}]_i$ were made according to the formua $[Ca^{2+}]=K_d\,(F-F_{min})/(F_{max}-F)$; where

F_{min} is determined by the formula $F_{min}=(F_{Mn^{2+}}) + 0.243\ (F_{max}-F_{Mn^{2+}})$ as previously reported [14].

Measurement of DNA Synthesis

DNA synthesis was assessed according to the method of Boynton and Whitfield by determing the proportion of cell nuclei labeled with [^{3}H]thymidine (2.5 μCi/ml) following a 24 h incubation [15].

RESULTS

The ability of EGF to increase T51B whole cell cytosolic free Ca^{2+} was dependent on a high extracellular [Ca^{2+}] (Fig. 1). Maximum changes in intracellular Ca^{2+} levels were obtained with doses of EGF ranging between 25 and 50 ng/ml. Lowering the buffer [Ca^{2+}] from 1.8 mM to 0.025 mM abolished the rise in $[Ca^{2+}]_i$ induced by 50 ng/ml EGF (Fig. 1B). Plasma membrane Ca^{2+} channel blockage by addition of 50 μM La^{3+} prior to EGF eliminated the cell Ca^{2+} response (Fig. 1C). La^{3+} alone was unable to penetrate the plasma membrane, as evidenced by a lack of change in the fura-2 fluorescence signal upon treatment with La^{3+} alone (data not shown). Ca^{2+} mobilization from intracellular storage sites induced by EGF was not detected in T51B cells, although this response has been reported for other cell types [16,17].

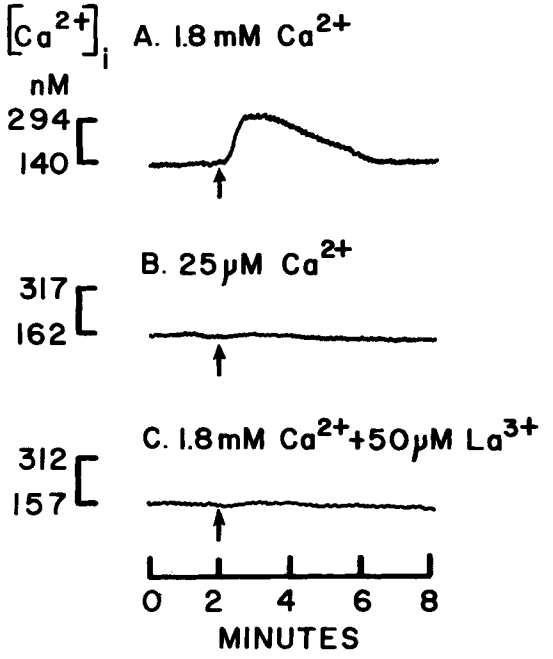


Fig. 1. Effect of low extracellular Ca^{2+}, or La^{3+} addition on the EGF-induced rise in free intracellular Ca^{2+}. T51B cells grown to a confluent monolayer on glass coverlsips and loaded with 5 μM fura-2 for 45 min (37°C) were placed in buffer that contained either **A)** 1.8 mM Ca^{2+}; **B)** 0.025 mM Ca^{2+}; or **C)** 1.8 mM Ca^{2+} and 50 μM La^{3+} (37°C). Two minutes later (arrow) 50 ng/ml EGF was added and the change in fluoresence recorded. Low extracellular Ca^{2+}, or La^{3+} addition, eliminated the Ca^{2+} response of EGF. The above tracings are single experiments which were repeated 20 times.

Addition of the surface binding agents thrombin, bradykinin, and ATP to fura-2-loaded cells in the presence of high extracellular Ca^{2+} (1.8 mM; Fig. 2A–C), or low extracellular Ca^{2+} (0.025 mM; Fig. 2D–F) resulted in the identification of the source of Ca^{2+} mobilized by these agents. Thrombin produced a rise in $[Ca^{2+}]_i$ through the liberation of intracellular Ca^{2+} stores alone, which was unaffected by the extracellular $[Ca^{2+}]$ (Fig. 2A,D). In contrast, the effect of bradykinin on $[Ca^{2+}]_i$ is completely dependent on a high level of extracellular Ca^{2+} (Fig. 2B,E). T51B cell response to ATP is biphasic, having a rapid, short-lasting spike of intracellularly released Ca^{2+}, followed with a prolonged tailing that is sustained from an influx of extracellular Ca^{2+} (Fig. 2C,F). In low Ca^{2+} buffer, the ATP-induced Ca^{2+} response pattern was condensed and lacked a broad tail indicating that the mobilized Ca^{2+} was derived exclusively from sites within the cell.

Fresh bovine calf serum (BCS) stimulated T51B cell DNA synthesis dose-dependently, causing a maximum proportion of labeled cells at a dose of 20% BCS/80% BME (Fig. 3A). EGF induced DNA synthesis in the absence of added fresh BCS (Fig. 3B) and caused a further twofold increase in the proportion of labeled nuclei when administered to cells in 1% BCS/90% BME medium (Fig. 3C). Low extracellular Ca^{2+} (0.025mM) or addition of La^{3+} (50 μM) inhibited the EGF-induced mitogenic response of T51B cells (Fig. 3D).

To investigate further the requirement of Ca^{2+} flux in mediating EGF mitogenic activity, various Ca^{2+} mobilizing agents and the tumor promoter TPA were examined alone or with a submaximal mitogenic dose of EGF to determine if Ca^{2+} fluxes could enhance the DNA synthetic response. Shown in Figure 4A,B bradykinin and TPA potentiated the effects of EGF 2- to 3-fold in media containing 1% BCS; with a similar, yet deminished effect on EGF in 100% BME media (Fig. 4C,D). Lowering the extracellular $[Ca^{2+}]$ completely abolished the potentiating action of these agents. The submaximal dose of EGF (0.1 ng/ml) used in these studies was unable to generate

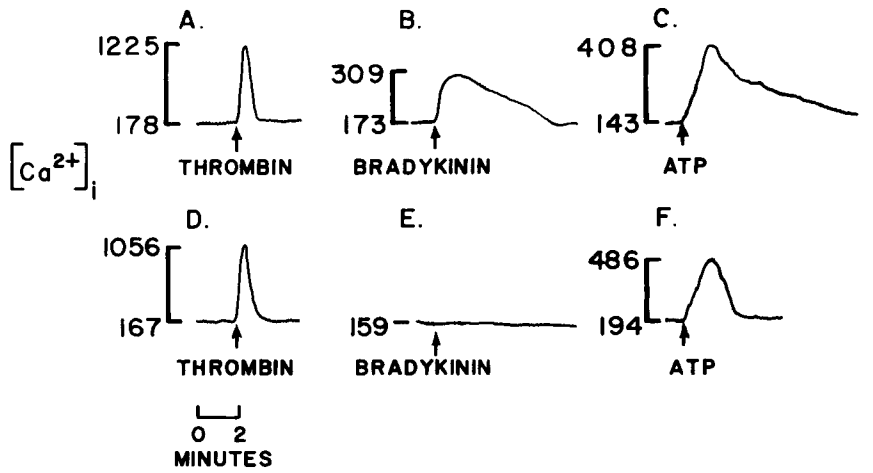
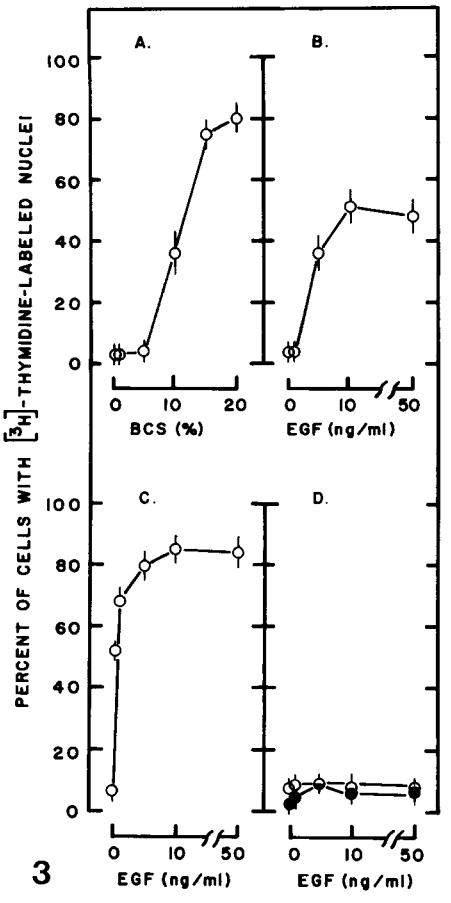


Fig. 2. Effect of thrombin, bradykinin, and ATP on T51B cell intracellular Ca^{2+} levels. Fura-2-loaded cells were placed in buffer containing either 1.8 mM Ca^{2+} (**A–C**) or 0.025 mM Ca^{2+} (**D–F**) and exposed to thrombin (0.05 U/ml), bradykinin (50 μM), and ATP (100 μM). The action of bradykinin and the prolonged elevated Ca^{2+} component of the ATP response were blocked by low extracellular Ca^{2+} levels, while the response to thrombin remained unaffected. These are representative tracings from experiments performed 20 times.



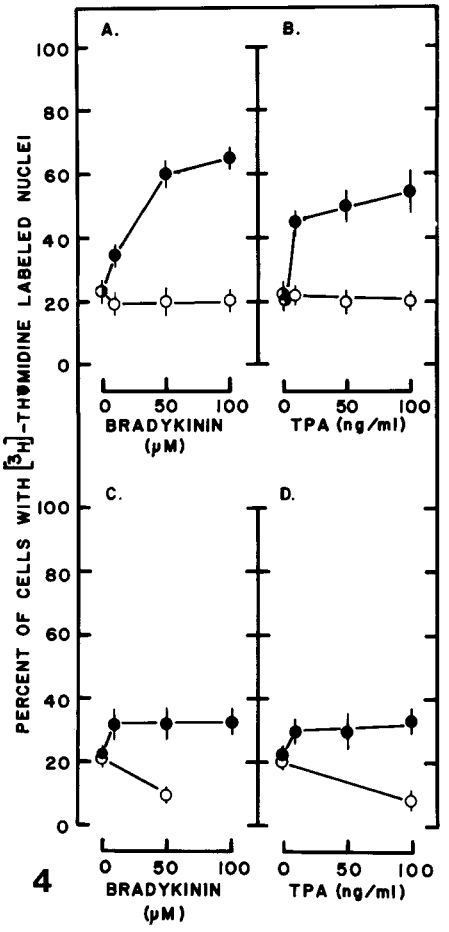


Fig. 3. Influence of low extracellular Ca^{2+}, or La^{3+} addition on EGF-stimulated DNA synthesis. Confluent and proliferatively quiescent T51B cells were treated at the start of the experiment as follows: **A:** Fresh bovine calf serum in BME (1.8 mM Ca^{2+}). **B:** EGF in fresh BME (1.8 mM Ca^{2+}). **C:** EGF in fresh 1% BCS/99% BME (1.8 mM Ca^{2+}) **D:** EGF in fresh 1% BCS/99% BME (0.025 mM Ca^{2+}) (open circles); or 1% BCS/99% BME (1.8 mM Ca^{2+} and 50 μM La^{3+}) (closed circles). Cells were incubated 24 h with [^{3}H] thymidine and fixed; and labeled nuclei were visualized by autoradiography. Effects of EGF were inhibited by lowering the media Ca^{2+} content, and by La^{3+} blockage of plasma membrane Ca^{2+} channels. Values are mean ± SEM; n = 4.

Fig. 4. Influence of bradykinin and TPA on EGF-induced DNA synthesis. At the start of the experiment confluent and quescent T51B cells were treated as follows. **A:** Bradykinin + 0.1 ng/ml EGF in fresh 1% BCS/99% BME. **B:** TPA + 0.1 ng/ml EGF in fresh 1% BCS/99% BME. **C:** Bradykinin + 3 ng/ml EGF in 100% BME. **D:** TPA + 3 ng/ml EGF in 100% BME. The above treatments contained either 1.8 mM Ca^{2+} (closed circles) or 0.025 mM Ca^{2+} (open circles). Cells were incubated 24 h in the presence of [^{3}H] thymidine, and the fraction of labeled nuclei determine by autoradiography. In the presence of serum, the proportion of label nuclei induced by EGF was increased 2–3-fold by both bradykinin and TPA. The effect was reduced in the absence of serum. Values are mean ± SEM; n = 3.

a Ca^{2+} influx and had no effect on the Ca^{2+} influx induced by bradykinin (Table I). T51B cell $[Ca^{2+}]_i$ was unaffected by TPA treatment (Table I).

The potentiating action of bradykinin and TPA was not an additive effect since neither agent is mitogenic for T51B cells (Table II). Agents that raise the $[Ca^{2+}]_i$ through complete or partial depletion of intracellular Ca^{2+} stores (thrombin, ATP, A23187) were ineffective as mitogens or in potentiating EGF-induced DNA synthesis (Table II). In addition, combinations of bradykinin and thrombin (Ca^{2+} influx and intracellular Ca^{2+} release), bradykinin and TPA (Ca^{2+} influx and protein kinase C activation), and TPA and A23187 (Ca^{2+} influx, intracellular Ca^{2+} release, and protein kinase C activation) were all incapable of stimulating DNA synthesis (Table II).

DISCUSSION

The results presented here demonstrate that EGF-induced DNA synthesis requires an influx of extracellular Ca^{2+}. Stimulation of T51B cells with EGF causes an initial rapid and transient influx of Ca^{2+}, and culminates in DNA replication and cell division several hours later. Both early and late events in cell proliferation were inhibited by placing cells in low Ca^{2+} media, or by treatment with La^{3+} to displace

TABLE I. Effect of EGF, Bradykinin, and TPA on $[Ca^{2+}]_i$

Treatment	Change in $[Ca^{2+}]_i$ (nM)
50 ng/ml EGF	169
5 ng/ml EGF	87
0.1 ng/ml EGF	0
50 μM Bradykinin	217
0.1 ng/ml EGF + 50 μM Bradykinin	224
100 ng/ml TPA	0
0.1 ng/ml EGF + 100 ng/ml TPA	0

Fura-2-loaded cells grown on glass coverslips were placed in 1.8 mM Ca^{2+} buffer and measured by spectrofluorometry.

TABLE II. Influence of Ca^{2+}-Mobilizing Agents on EGF-Induced DNA Synthesis

	Ca^{2+} flux[a]		% [^{3}H] thymidine-labeled nuclei	
	Extra	Intra	−0.1 ng/ml EGF	+0.1 ng/ml EGF
Control (EGF)	+	−	3.6 ± 1.4[b]	25.1 ± 3.8
Bradykinin (50 μM)	+	−	4.3 ± 0.8	63.8 ± 1.1
Thrombin (0.05 U/ml)	−	+	3.1 ± 1.3	24.4 ± 2.7
ATP (100 μM)	+	+	4.2 ± 1.5	9.3 ± 0.6
A23187 (1 μM)	+	+	1.8 ± 0.9	3.2 ± 1.1
TPA (100 ng/ml)	−	−	3.4 ± 1.2	56.1 ± 3.6
Bradykinin (50 μM) + Thrombin (0.05) U/ml)	+	+	3.6 ± 1.7	—
Bradykinin (50 μM) + TPA (100 ng/ml)	+	−	5.8 ± 0.9	—
TPA (100 ng/ml) + A23187 (1 μM)	+	+	2.2 ± 1.5	—

[a]Source of Ca^{2+}: Extra, extracellular Ca^{2+}; Intra, intracellular Ca^{2+}.
[b]Values are mean ± SEM.

cell-surface Ca^{2+} and block transmembrane Ca^{2+} fluxes [18]. Previous reports of DNA synthesis induced by serum [19] and insulinlike growth factor II [20] have shown a similar requirement for a flux of Ca^{2+}, which was sensitive to both La^{3+} and the depletion of extracellular Ca^{2+}. This evidence suggests that cellular reactions leading to DNA synthesis are initiated by a surge of Ca^{2+} (down its concentration gradient) across the plasma membrane, which temporarily raises intracellular free Ca^{2+} levels. This concept is further supported by the observation that bradykinin, an agent that induces a rise in $[Ca^{2+}]_i$ only through the opening of plasma membrane Ca^{2+} channels and has no mitogenic activity with T51B cells, was able to potentiate the effects of EGF on DNA synthesis. Moreover, dose-dependency of EGF stimulation of T51B cells may be related to the extent of Ca^{2+} influx produced at each dose, rather than the number of EGF receptors occupied on the cell plasma membrane surface.

Intracellular Ca^{2+}-mobilizing agents, thrombin and ATP, are known to cause phosphotidylinositol turnover and formation of inositol trisphosphate ($Ins(1,4,5)P_3$) [21,22]. Since these agents are not mitogenic with T51B cells and showed no potentiation of EGF-induced DNA synthesis, a rise in $[Ca^{2+}]_i$ supplied by emptying internal Ca^{2+} stores appears to be insufficient for initiating cellular processes involved in cell proliferation. The Ca^{2+} ionophore A23187, and ATP were inhibitory toward the action of EGF, indicating that high $[Ca^{2+}]_i$ may activate opposing regulatory mechanisms. In support of this hypothesis is the observation that EGF induces $Ins(1,4,5)P_3$ formation and a high transient increase in $[Ca^{2+}]_i$ in A431 cells, but inhibits DNA synthesis [23].

TPA alone was not mitogenic but in combination with EGF enhanced the fraction of T51B cells that had initiated DNA synthesis to a similar degree as bradykinin, despite the absence of a detectable increase in $[Ca^{2+}]_i$. One possible explanation for this discrepancy between bradykinin and TPA is that the sensitivity of the spectrofluorometric method is incapable of detecting a Ca^{2+}flux induced by TPA. Video-imaging microscopy has recently been utilized to demonstrate a $[Ca^{2+}]_i$ increase in response to TPA in Balb/c 3T3 cells [24]. However, activation of protein kinase C in the absence of Ca^{2+} surge could also explain the potentiating effects of TPA [25]. Both EGF binding to surface receptors and TPA activation of protein kinase C in A431 cells were demonstrated to cause phosphorylation of the same threonine residue in EGF receptors, suggesting that EGF indirectly influences protein kinase C activation [26]. In another study, TPA initiated DNA synthesis within an hour in Ca^{2+}-deprived T51B cells arrested in G_1, demonstrating the ability of active protein kinase C to participate in the processes which are necessary to elicit a DNA synthetic response [27].

Recent reports of the influence of TPA and EGF on arachidonic acid release and prostaglandin production in porcine thyroid cells indicate a possible common pathway for these agents in mediating cell proliferative activity [28,29]. Prostaglandins are known to be mitogenic for certain cell types [30]. In addition, bradykinin stimulates arachidonic acid release and prostaglandin synthesis in a variety of tissues [31], which could be the basis for its potentiating action. Further work to establish the mechanism of action of protein kinase C and Ca^{2+} influx in potentiating the proliferative action of EGF is clearly indicated.

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