Epidermal Growth Factor Activates Store-operated Ca\textsuperscript{2+} Channels through an Inositol 1,4,5-Trisphosphate-independent Pathway in Human Glomerular Mesangial Cells*

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Wei-Ping Li‡§, Leonidas Tsiokas‡¶, Steven C. Sansom¶, and Rong Ma¶†
From the ¶Department of Cell Biology, University of Oklahoma Health Sciences Center, Oklahoma City, Oklahoma 73190, the ¶Department of Pharmacology, Anhui Medical University, Hefei, People’s Republic of China, and the ¶Department of Physiology and Biophysics, University of Nebraska Medical Center, Omaha, Nebraska 68198-4575

One of the fastest cellular responses following activation of epidermal growth factor receptor is an increase in intracellular Ca\textsuperscript{2+} concentration. This event is attributed to a transient Ca\textsuperscript{2+} release from internal stores and Ca\textsuperscript{2+} entry from extracellular compartment. Store-operated Ca\textsuperscript{2+} channels are defined the channels activated in response to store depletion. In the present study, we determined whether epidermal growth factor activated store-operated Ca\textsuperscript{2+} channels and further, whether depletion of internal Ca\textsuperscript{2+} stores was required for the epidermal growth factor-induced Ca\textsuperscript{2+} entry in human glomerular mesangial cells. We found that 100 nm epidermal growth factor activated a Ca\textsuperscript{2+}-permeable channel that had identical biophysical and pharmacological properties to channels activated by 1 μM thapsigargin in human glomerular mesangial cells or A431 cells. The epidermal growth factor-induced Ca\textsuperscript{2+} currents were completely abolished by a selective phospholipase C inhibitor, U73122. However, xestospongin C, a specific inositol 1,4,5-trisphosphate receptor inhibitor, did not affect the membrane currents elicited by epidermal growth factor despite a slight reduction in background currents. Following emptying of internal Ca\textsuperscript{2+} stores by thapsigargin, epidermal growth factor still potentiated the Ca\textsuperscript{2+} currents as determined by the whole-cell patch configuration. Furthermore, epidermal growth factor failed to trigger measurable Ca\textsuperscript{2+} release from endoplasmic reticulum. However, another physiological agent linked to phospholipase C and inositol 1,4,5-trisphosphate cascade, angiotensin II, produced a striking Ca\textsuperscript{2+} transient. These results indicate that epidermal growth factor activates store-operated Ca\textsuperscript{2+} channels through an inositol 1,4,5-trisphosphate-independent, but phospholipase C-dependent, pathway in human glomerular mesangial cells.

Cytosolic Ca\textsuperscript{2+} represents a convergent point of many signal transduction pathways and modulates a diverse array of cellular activities ranging from contraction to cell growth. Cells generate Ca\textsuperscript{2+} signals through both internal and external Ca\textsuperscript{2+} sources. In terms of external source, one mechanism for controlling Ca\textsuperscript{2+} entry involves the classical phosphoinositide pathway. Essentially, the binding of many hormones to G-protein-coupled receptors or tyrosine kinase-based receptors on the plasma membrane leads to activation of phospholipase C (PLC)\textsuperscript{β or γ} that hydrolyzes membrane lipids to produce inositol 1,4,5-trisphosphate (IP\textsubscript{3}), IP\textsubscript{3} binds to the IP\textsubscript{3} receptor located on the membrane of endoplasmic reticulum (ER) and subsequently stimulates release of Ca\textsuperscript{2+} from the lumen of ER to the cytoplasm. This release of Ca\textsuperscript{2+} is generally associated with an increase in Ca\textsuperscript{2+} entry across the plasma membrane, a process known as store-operated or capacitative Ca\textsuperscript{2+} entry, through a specific type of Ca\textsuperscript{2+} channel termed store-operated Ca\textsuperscript{2+} channel (SOC) (1, 2). In addition to the ubiquitous capacitative Ca\textsuperscript{2+} entry mechanism, non-capacitative Ca\textsuperscript{2+} entry involving the PLC-IP\textsubscript{3} cascade has also been described in a variety of cell types (3, 4). The molecular entity and the mechanisms of activation of the channels mediating Ca\textsuperscript{2+} entry in such instances still remain elusive.

Epidermal growth factor (EGF) mediates its effects on normal cell function via the activation of the epidermal growth factor receptor (EGFR), a typical tyrosine kinase receptor. Binding of EGF to its receptor results in the increased phosphorylation of a group of substrates, one of which is PLC-γ (5, 6). Activated PLC-γ then catalyzes the production of IP\textsubscript{3} and diacylglycerol. The earliest cellular responses to the activation of the EGFR includes a number of ionic changes, such as an increase in intracellular Ca\textsuperscript{2+} concentration ([Ca\textsuperscript{2+}])) (5, 7, 8). Some of these responses have been linked to the PLC cascade (5). The EGF-induced rise in [Ca\textsuperscript{2+}]\textsubscript{i} is derived from the activation of both release from ER (triggered by IP\textsubscript{3}) and Ca\textsuperscript{2+} influx across the plasma membrane (5, 7, 8). We previously found that EGF activated Ca\textsuperscript{2+}-permeable channels that possessed similar biophysical and pharmacological properties to those of SOC at single channel level in human glomerular mesangial cells (HMC) (9, 10). However, it remains uncertain whether the EGF-evoked Ca\textsuperscript{2+} influx is dependent on store depletion or other second messengers activated in response to EGF. In this study, we provide electrophysiological and pharmacological evidence that EGF activates SOC by a PLC-dependent but IP\textsubscript{3} receptor (IP\textsubscript{3}R) independent pathway in HMC.

EXPERIMENTAL PROCEDURES
Preparation of Cultures of Mesangial Cells—The procedures and methods for culturing HMC were described previously (11). Briefly,

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† To whom correspondence may be addressed: Dept. of Cell Biology, University of Oklahoma Health Sciences Center, 940 Stanton L. Young Blvd., Oklahoma City, OK 73190. E-mail: rong-ma@ouhsc.edu or leonidas-tsiokas@ouhsc.edu.
‡ The abbreviations used are: PLC, phospholipase C; IP\textsubscript{3}, inositol 1,4,5-trisphosphate; IP\textsubscript{3}R, IP\textsubscript{3} receptor; SOC, store-operated Ca\textsuperscript{2+} channel; EGF, epidermal growth factor; EGFR, EGFR receptor; HMC, human glomerular mesangial cells; TG, thapsigargin; Ang II, angiotensin II.
HMC were cultured in Dulbecco’s modified eagle’s medium (Sigma) supplemented with 10 mHEPES, 2.0 mglutamine, 0.66 mleucine, 1.0 mtsodium pyruvate, 0.1 mnonessential amino acids, 100 units/ml penicillin, 100 mglm streptomycin, and 20% fetal bovine se-
rum. The pH value of the medium was adjusted to the level between 7.2
and 7.4. Upon reaching confluence, cells were passed onto 22×22–1 mm
cover glasses (Fisher Scientific), which were served as the floor of the
perfusion chamber (Warner RC-20H, 23°C) used in either patch clamp
or fura-2 experiments. All cells were studied within 56 h after plated
and only subpassages less than eleven generations were used in the
present study.

Patch Clamp Procedures—The conventional whole-cell and cell-at-
tached voltage clamp configurations were performed in single HMC
cells at room temperature with a Warner PC-501A (for cell-attached)
amplifier (Warner Instrument Corp., Hamden, CT) and pClamp software
(Axon Instrument, Foster City, CA). Glass pipettes (plain, Fisher Scientific)
with resistances of 5–8 MΩ were prepared with a pipette puller and polisher
(PF-830 and MF-830, respectively, Narishige, Tokyo, Japan).

Single Channel Analysis—Single channel analysis was performed
using standard patch clamp techniques (11–13). Data were digitized for
single channel analysis using an analog-to-digital interface (Axon In-
struments) and recorded by a computer system. Low pass filter was set
at 500 Hz. The unitary current, defined as zero for the closed state (C),
was determined as the mean of the best-fit Gaussian distribution of the
amplitude histograms. Channels were considered to be in an open state
(O) when the total current (I) was >n–1/2|f| and <(n + 1/2)|f|, where
n is the maximum number of current levels observed. The open proba-
bility (Pₒ) was defined as the time spent in an open state divided by the
total time of the analyzed record. When multiple channels occupied a
patch, the channel activity was calculated as NPₒ = 2nPₒ, where Pₒ is
the probability of finding n channels open. Therefore, NPₒ could be
calculated without making assumptions about the total number of
channels in a patch or the open probability of individual single chan-
nels. The Axoscope acquisition program and pClamp program set 6.02
(Axon Instruments) were used to record and analyze currents.

Whole-cell Patch—When the whole-cell configuration was achieved,
cellular resistance was compensated before each recording period. From a holding potential of 0 mV, voltage steps were
applied from ~80 to 80 mV in 20 mV increments with 100 ms duration
at 5-s intervals. Current traces were filtered at 1 kHz and analyzed
off-line with pClamp and statistical (Sigmaplot/SigmaStat, Chicago, IL)
software.

Measurement of [Ca2+]i—[Ca2+]i was monitored in HMC using fura-2
and dual excitation wavelength fluorescence microscopy, as described
previously (14, 15). In brief, cells were loaded with fura-2 by incubation
for 60 min (23°C) in physiological saline solution containing 7 mura-2
AM, 0.09 g/dl Me2SO and 0.018 g/dl Fluoronic F-127 (Molecular Probes,
Eugene, OR). After loading with fura-2, HMC were placed in a perfusion
chamber by means of an open bath (O) when the total current (I) was
>23.3%.

Results

RESULTS

EGF Activated Store-operated Ca2+ Entry—In whole-cell
configuration, store-operated Ca2+ currents were induced
using a specific inhibitor of sarcoplasmic/endoplasmic reticulum
Ca2+ ATPase, thapsigargin (TG). Fig. 1A, panel a, shows
whole-cell currents before and after application of 1 μM TG
generated by step voltages (see “Experimental Procedures”).
Under basal condition (before TG), background currents were
observed at all tested potentials, suggesting a tonic activity
of the channels. Both inward and outward currents were greatly
enhanced by TG within 1–3 min, indicating a store-operated
property. Current-voltage relations plotted by current density
(normalized to cell capacitance) at 50 ms after establishing
whole-cell configuration against test potentials from six cells
exhibit a slight inward rectification from ~80 mV to ~40 mV,
and the currents were reversed around 20 mV for both before
and after TG (Fig. 1A, panel b), implying a slight selectivity
of this channel to Ca2+, TG significantly augmented inward
currents from ~80 mV to ~40 mV and outward currents at 80 mV
with ~222% increments at ~80 mV (Fig. 1A, panel c). To
further determine the store-operated nature of the channel,
the TG-induced responses were re-evaluated in the presence of
La3+, a widely used blocker for SOC (10, 16, 17). It is clearly
shown that 20 μM La3+ completely abolished the TG-evoked
increase in cell currents and even lowered the currents below
basal level (by ~23.3%, p < 0.05) (Fig. 1A, panel b).

Similar to TG, 100 nm EGF also increased whole-cell cur-
rents (Fig. 1B, panel a) and the responses reached significant
level from ~80 mV to ~20 mV and from 60 mV to 80 mV (Fig.
1B, panel b). The I-V curves reveal an almost identical I-V
relations and reversal potentials to those observed in TG-
treated cells (Fig. 1A, panel b, and B, panel b). At ~80 mV test
potential, EGF raised the current density from ~10.5 to 3.1
pA/pF to ~23.3 ± 6.3 pA/pF, representing 274.6 ± 40.6% of
background current (Fig. 1B, panel c). This EGF-induced
response was also abolished by 20 μM La3+ (Fig. 1B, panel c).

The EGF-activated Ca2+ conductance was further examined
using fura-2 fluorescence measurement. In this series of exper-
iments, a Ca2+-readmission protocol was employed to assess
capacitative Ca2+ entry (10). In nominally Ca2+-free extracel-
lar solution, base-line intracellular Ca2+ concentrations
([Ca2+]) in all tested cells were in the range from 20 to 50 nm
(Fig. 2A, panel a). Replacing the bathing solution with 1 mM
Ca2+ solution only slightly elevated [Ca2+]. However, under
condition of preincubation with 100 nm EGF for 3–5 min,
admission of Ca2+ resulted in a striking increase in [Ca2+], (Fig.
2A, panels a and b, Control). The EGF-induced response was
partially but significantly attenuated by 5 μM diltiazem but
almost completely abolished by 2 μM La3+ (Fig. 2A, panels a
and b). The difference between basal [Ca2+], and peak [Ca2+],
in response to addition of Ca\(^{2+}\) (Δ[Ca\(^{2+}\)]) was well known to be derived from Ca\(^{2+}\) influx (4, 10, 18). Summary data for each group are presented in Fig. 2A, panel b, clearly showing that EGF significantly enhanced Ca\(^{2+}\) entry, and this enhancement was not significantly depressed by diltiazem and Cd\(^{2+}\) but abolished by La\(^{3+}\). Since both diltiazem and Cd\(^{2+}\) are selective blockers for voltage-gated Ca\(^{2+}\) channels in the range of concentrations used in the current study (11, 19), we infer that the EGF-evoked Ca\(^{2+}\) entry is unlikely to be predominantly mediated by voltage-gated Ca\(^{2+}\) channels, if any present in these cells. Thus, another type of channel, SOC, might play a major role in mediating the Ca\(^{2+}\) influx induced by EGF.

To further determine whether EGF is capable of activating SOC, whole-cell currents were measured in A431 cells, a human epidermoid cancer cell line. We chose this cell line because of two reasons: 1) A431 cells are rich in EGF receptors (20), and 2) whole-cell currents of SOC in this cell line are unique in that they show strong inward rectification and high Ba\(^{2+}\) conductance (21). We measured Ba\(^{2+}\) whole-cell currents in responses to EGF in six A431 cells and compared those with TG-induced currents. As shown in Fig. 2B, background currents were small and were inwardly rectified until they were reversed (at about 40 mV). These inward currents were significantly enhanced by TG or EGF without altering current-voltage relationships and reversal potentials. The EGF-sensitive currents are similar to TG-sensitive currents in terms of shape of current-voltage curves and reversal potentials (Fig. 2B, panel c), indicating that the EGF-activated currents were mediated through SOC.

**Fig. 1.** Whole-cell currents in response to application of TG (A) or EGF (B). Families of current responses to membrane voltage steps under basal conditions and 2 min after application of 1 μM TG and 100 nM EGF are shown in A, panel a, and B, panel a, respectively. Currents were normalized to cell capacitance. The dashed lines in current traces indicate 0 level of current. A, panel b, and B, panel b, show current-voltage (I-V) relations for TG treatment (n = 8) and EGF treatment (n = 7) groups, respectively. The currents at 50 ms after establishing whole-cell configuration at all tested potentials were measured for analysis. *, p < 0.05, comparison of currents before and after treatments at corresponding test potentials. A, panel c, and B, panel c, are summary data of percent changes in whole-cell currents induced by TG and EGF, respectively, and blocking effect of La\(^{3+}\) on the responses. The values of current at −80 mV from A, panel b, and B, panel b, were used to plot the bar graphs, respectively. The currents before treatments are normalized to 100%. *, p < 0.05, compared with other two groups.

EGF-induced Ca\(^{2+}\) Entry Was Dependent on PLC but Independent on IP\(_3\) Receptor—Next, we aimed to determine whether the EGF-induced response was mediated by PLC activation and IP\(_3\) generation using a membrane-permeable PLC inhibitor, U73122. A 3–5 min preincubation of cells with 10 μM U73122 has been demonstrated to fully and irreversibly prevent PLC activation upon agonist stimulation in smooth muscle cells (22). Likewise, a 3 min pretreatment of HMC with 10 μM U73122 was sufficient to depress the EGF-activated Ca\(^{2+}\) current in cell-attached mode. As shown in Fig. 3A, administration of 100 nM EGF to the bath raised NP\(_0\) of the Ca\(^{2+}\) channels from 1.06 ± 0.08 to 1.55 ± 0.11 (p < 0.05), and this activation was not observed in the presence of 10 μM U73122. However, U73122 itself did not affect the base-line activity of the channels. Taken together, these data suggested that PLC activation was an essential step in the EGF-mediated activation of SOCs.

Because PLC activation is upstream of IP\(_3\) production and intracellular store depletion, and EGF-activated channels had similar properties to SOCs, we reasoned that IP\(_3\) generation might be an essential step in the EGF-activated Ca\(^{2+}\) conductance. If this was the case, blocking IP\(_{3}\)R function should partially or completely attenuate the Ca\(^{2+}\) entry induced by EGF. Xestospongion C is a potent membrane-permeable IP\(_{3}\)R inhibi-
tor (23) and has been widely used to investigate the role of IP₃R in a variety of intracellular signaling pathways (3, 24). HMC were preincubated with 10 μM xestospongin C for 30 min, and whole-cell currents generated from step voltages were measured before and after application of EGF. Basal currents were greatly reduced under treatment with xestospongin C (Fig. 3, B and D), suggesting that endogenous IP₃R activity in the resting state may regulate the activity of Ca²⁺-permeant channels. Interestingly, in the presence of xestospongin C, EGF still increased membrane conductance at all tested potentials (Fig. 3C), and the responses reached significant levels from −80 mV to −40 mV and from 40 mV to 80 mV (Fig. 3D). Specifically, at −80 mV, the EGF-induced current was 282.6 ± 39.9% of background levels (Fig. 3D, inset), comparable with the EGF-evoked enhancement in the absence of xestospongin C (Fig. 1C). These data indicate that IP₃R function is not required for the EGF-induced activation of Ca²⁺-conducting channels.

To assure that PLC and IP₃R were efficiently depressed by U73122 and xestospongin C, respectively, angiotensin II (Ang II)-induced Ca²⁺ release from ER was examined in the presence or absence of the two inhibitors. Ang II has been demonstrated to mobilize intracellular Ca²⁺ through PLC and IP₃R pathway in glomerular mesangial cells (11, 25). As shown in Fig. 3E, Ang II triggered a clear rise of cytosolic Ca²⁺, which, apparently, was originated from internal Ca²⁺ stores because the cell was bathed in nominally Ca²⁺-free solution. The Ang II-stimulated Ca²⁺ release was nearly abolished by pretreating HMC with 10 μM U73122 (3 min) or 10 μM xestospongin C (30 min) (Fig. 3, E and F). Interestingly, the basal intracellular Ca²⁺ concentration ([Ca²⁺]ᵢ) was significantly lowered by U73122 or xestospongin C treatment (Fig. 3F), suggesting that the tonic activity of endogenous PLC and IP₃R might play a role in Ca²⁺ homeostasis under resting state.

Effect of Depletion of Internal Ca²⁺ Stores on EGF-activated Membrane Currents—To further test whether the cascade of IP₃-IP₃R-depletion of ER contributes to the EGF-induced responses, the effect of EGF on membrane currents were re-evaluated after depleting intracellular Ca²⁺ stores by TG in cell-attached and whole-cell patch modes. Single channel recordings (Fig. 4A) revealed that even though the channel number (three in this particular experiment) did not increase after TG, the opening time of the channels was greatly prolonged. This prolongation was further potentiated by addition of EGF. Summary data show that the NP₀ of the channels significantly increased after administration of TG and was further significantly raised by EGF (Fig. 4B).

In whole-cell patch configuration, inward current at −80 mV was significantly enhanced by TG from −6.4 ± 1.9 pA/pF to −14.3 ± 3.1 pA/pF. Under this circumstance, EGF still resulted in an additional and significant increase in inward current (−27.6 ± 6.4 pA/pF). Again, the TG- and EGF-activated currents were abolished by 20 μM La³⁺ (−5.6 ± 1.8 pA/pF) (Fig. 4C).

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**Fig. 2.** A, fura-2 fluorescence measurements of [Ca²⁺], demonstrating the EGF-induced capacitive Ca²⁺ entry. Representative tracings (panel a) and summary data (panel b) of [Ca²⁺], in response to readmission of Ca²⁺ (1 mM) containing solution to the bath in the presence or absence of 100 nM EGF with or without various Ca²⁺ channel blockers. The initial bathing solution is nominally Ca²⁺-free. The arrow indicates when 1 mM Ca²⁺ solution was added. *, p < 0.05, compared with control and La³⁺ groups. Dil, diltiazem. B, whole-cell I-V relations before and after treatment of 1 mM TG (panel a) or 100 nM EGF (panel b) in A431 cells. Panel c shows TG or EGF sensitive currents (the difference in current before and after application of TG or EGF) at all tested potentials. The currents at 50 ms after establishing whole-cell configuration at all tested potentials were measured for analysis. *, p < 0.05, comparison of currents before and after treatments at corresponding test potentials. The data were averaged from six cells in each group.
Focal activation of Store-operated Ca\(^{2+}\) Channels by EGF.

In sum, the single channel and whole-cell current measurements exhibited that depletion of internal Ca\(^{2+}\) stores did not impact the EGF-induced ionic conductance, strongly suggesting that the EGF-evoked conductance is independent of store depletion.

Effect of EGF on Ca\(^{2+}\) Release Transient—To further test the store depletion dependence of EGF-induced Ca\(^{2+}\) entry, we examined whether EGF could efficiently empty the internal Ca\(^{2+}\) pools in HMC. To this end, cytosolic Ca\(^{2+}\) concentration in response to EGF was assessed using fura-2 fluorescence measurement. As a positive control, TG was employed and a rapid Ca\(^{2+}\) transient was observed immediately after applying TG (Fig. 5A), consistent with our previous report (10). However, this transient was not observed in the cell challenged with 100 nM EGF. The rise in Ca\(^{2+}\), was sluggish and low (Fig. 5B). Ang II, another physiological agent, also led to a striking Ca\(^{2+}\) spike, which has been known to originate from IP\(_3\)-triggered Ca\(^{2+}\) release from ER (Fig. 5C) (11), implying that the PLC-IP\(_3\) pathway is functional. The differences of peak [Ca\(^{2+}\)]\(_i\) from basal level (Δ[Ca\(^{2+}\)]\(_i\)) in each group are summarized in Fig. 5D, showing a significant difference in Ca\(^{2+}\) release between TG or angiotensin II treatment and EGF treatment. These data show that while the PLC-IP\(_3\) pathway was fully functional in these cells, EGF failed to activate a measurable Ca\(^{2+}\) release. It has been documented that EGF-stimulated Ca\(^{2+}\) release from ER varies with cell types (4, 8, 26, 27). Apparently, in HMC, EGF-induced mobilization of intracellular Ca\(^{2+}\) represented a minor fraction of the total stored Ca\(^{2+}\). These results were consistent with the results obtained from xestospongin C experiment showing that store depletion was unlikely to account for activation of SOC channels in response to EGF stimulation of HMC.

This notion was further verified in the cell-attached patch clamp experiments. As described previously (Fig. 3A), application of 100 nM EGF substantially activated SOC (NP\(_0\): 0.9 ± 0.1 and 1.35 ± 0.09, before and after EGF, respectively, p < 0.01) (Fig. 6). Subsequent addition of 1 μM TG (in the presence of EGF) significantly enhanced the channel activity (NP\(_0\): 1.71 ± 0.16, p < 0.01, compared with EGF alone) (Fig. 6), indicating that the size of internal Ca\(^{2+}\) pools did not change as a result of EGF stimulation.

**DISCUSSION**

In the present study, we provide evidence that EGF activates SOCs via a PLC dependent but IP\(_3\)R independent mechanism in HMC. Pharmacological and electrophysiological data showed that first, the biophysical properties of EGF-activated channels were similar to SOCs. Second, pharmacological inhibitor of IP\(_3\)R did not affect EGF-induced currents. Third, EGF still significantly potentiated SOC currents when internal stores were predepleted. The implication of our study is that SOCs can be activated by second messenger systems independ-
ently of store depletion in response to EGF stimulation in HMC.

An extensive body of evidence suggests that the earliest cellular responses to the activation of the EGF receptor include a number of ionic changes, such as a transient plasma membrane hyperpolarization and increase in $[\text{Ca}^{2+}]_i$ (5, 7, 8, 28). The $\text{Ca}^{2+}$ influx across plasma membrane constitutes a large component of the total increase in $[\text{Ca}^{2+}]_i$ (4, 7, 8). No convincing clues as to the nature of $\text{Ca}^{2+}$ channel mediating the $\text{Ca}^{2+}$ influx and the mechanism responsible for the initiation of $\text{Ca}^{2+}$ influx have been provided thus far. In the present study, we show that the EGF-activated $\text{Ca}^{2+}$ conductance has identical properties to those of SOCs. Our observation was based on four lines of evidence. First, EGF- or TG-induced currents showed similar ion selectivity, as determined by the shape of $I$-$V$ curves and reversal potentials. Second, both currents were sensitive to $\text{La}^{3+}$, a relatively selective SOC blocker. Third, the EGF-induced $\text{Ca}^{2+}$ entry estimated by ratiometric fluorescence measurement was not blocked by selective voltage-gated $\text{Ca}^{2+}$ channel blocker diltiazem or $\text{Cd}^{2+}$, but by $2 \mu\text{M La}^{3+}$, which have been described to be features of capacitative $\text{Ca}^{2+}$ entry (10). Fourth, EGF activated a $\text{Ba}^{2+}$-selective current in A431 cells, which is characteristic of SOC currents in this type of cells (21). Our results are in agreement with earlier reports from Moolenaar’s group (29) that EGF-induced $\text{Ca}^{2+}$ signal in A431 cells was not affected by nifedipine, a potent antagonist of voltage-gated $\text{Ca}^{2+}$ channel, indicating that EGF-induced currents are most unlikely to be carried by voltage-activated $\text{Ca}^{2+}$ channels. In the same type of cells, Peppelenbosch’s group (7) demonstrated that EGF activated a selective $\text{Ca}^{2+}$ channel the single channel conductance (1–3 picosiemens) of which is in the range of that for SOC described in HMC (10). Taken together, these results lead us to propose that the EGF-activated $\text{Ca}^{2+}$ conductance is most likely mediated by SOCs.

The finding that EGF activated SOCs was not surprising, as it was expected that EGF may have indirectly activated these channels through the production of $\text{IP}_3$ and subsequent depletion of the internal $\text{Ca}^{2+}$ stores. However, this idea was challenged by the results showing that blocking $\text{IP}_3$ receptor function with xestospongin C and predepleting internal $\text{Ca}^{2+}$ stores with TG did not impact on EGF-induced responses. The possibility that the xestospongin C was used at a concentration too low to block $\text{IP}_3$ receptor could be excluded, because it was used at a concentration of $10 \mu\text{M}$, which represents 30-fold higher concentration of its $\text{IC}_{50}$ (358 nM) (23). In addition, preincubation of HMC with $10 \mu\text{M xestospongin C}$ for 30 min greatly reduced basal membrane currents (Fig. 3B) and intracellular $\text{Ca}^{2+}$ concentration (Fig. 3, E and F), suggesting that, at this concentra-

![FIG. 4](http://www.jbc.org/)

**Fig. 4.** The effects of depletion of internal $\text{Ca}^{2+}$ stores with TG and additional application of EGF on $\text{Ca}^{2+}$ channel activity in the cell-attached (A and B) and whole-cell patch configuration (C). A, representative tracings of SOC in basal condition (upper), application of TG (middle), and TG combined with EGF (bottom) at a holding potential ($-V_p$) of $-80 \text{mV}$. Arrows indicate closed state. Dashed lines indicate the different current levels. Inward currents are downward. B, bar graph summarizing SOC activity ($NP_O$) under basal condition and different treatments. *, $p < 0.05$, compared with baseline. #, $p < 0.05$, compared with base-line and TG groups. C, summary data from whole cell recordings show that EGF potentiates $\text{Ca}^{2+}$ entry after the internal stores have been depleted with TG and the blocking effect of $\text{La}^{3+}$ on the response. *, $p < 0.05$, compared with baseline. #, $p < 0.05$, compared with TG and $\text{La}^{3+}$ groups.
tion, it may have inhibited tonic activity of IP3R. Furthermore, xenostospong C almost completely abolished Ang II-stimulated ER Ca2+ release through IP3R (Fig. 3, E and F) (11, 25).

Non-store-operated Ca2+ entry has been described in a variety of cell types (3, 4, 22, 30). Several second messengers might be involved in such instances, for example arachidonic acid and IP3. However, the receptor-activated, store depletion-independent channels were not characterized in those experiments, and thus, whether those channels are the same channels as the ones observed in this study is unknown. Recently, Vazquez and Putney’s group (3) demonstrated that stimulation of DT40 cells with anti-IgM, the B cell receptor agonist, resulted in a non-capacitative cation entry assessed by Ba2+ influx, presumably via the nonreceptor tyrosine kinase-PLC-IP3 cascade. This store depletion independent Ca2+ conducted entry mechanism was attributed to IP3 binding to IP3 receptor on plasma membrane, but not on ER membrane (3). However, this might not be the case in HMC based on several lines of evidence. First, xenostospong C did not attenuate the EGF-induced Ca2+ influx (Fig. 3, B–D). Second, EGF was not able to release Ca2+ from ER as TG or angiotensin II did (Fig. 5), indicating only a minor production of IP3 in EGF-stimulated HMC. Third, EGF treatment did not affect the size of internal Ca2+ pools as additional administration of TG following EGF evoked a further and significant activation of SOC (Fig. 6). In accordance, we recently found that TRPC3 was not detected in HMC examined by Western blotting and immunofluorescence staining (data not shown). TRPC3, one member in the TRP superfamily, has been considered to have physical interaction with IP3 receptor on ER membrane (31–35), supporting a conformational coupling mechanism of SOC activation (1, 36, 37). Indeed, EGF-stimulated production of IP3 varies with cell types. In A431 cells, the Ca2+ release from ER constitutes only a minor component of the total EGF-induced increase in [Ca2+], (7). However, a rapid and transient Ca2+ release from ER could be clearly observed in a human salivary cell line stimulated with EGF (4). Interestingly, the EGF-elicited depletion of the intracellular Ca2+ stores in this cell line was unable to activate store-operated Ca2+ entry (4). Hence, it appears that depleting internal Ca2+ stores is not required for EGF-induced Ca2+ entry. Our study has further expanded this notion in that SOC could be activated by mechanism(s) independent of store depletion.

How should we interpret the paradoxical results that EGF activates SOC but store depletion is not required? It is noteworthy to mention that PLC has been recently proposed to play a critical role in SOC activation (18). The action of PLC in this pathway is not due to production of IP3 and diacylglycerol (18). Indeed, EGF-stimulated entry was augmented by PLC inhibitor (U73122) in this study. In a detailed study conducted by Patterson et al. (17), PLC-γ was proposed to be a predominant component to mediate capacitative Ca2+ entry. Overexpression of PLC-γ1 augmented capacitative Ca2+ entry induced by either direct depletion of internal stores with TG or activation of a G-protein-coupled receptor with UTP or ATP. This action of PLC-γ is independent of production of IP3, because its catalytic function was not required. If PLC-γ was knocked out using a novel RNA interference technique, while the receptor-activated Ca2+ entry was eliminated, the Ca2+ entry induced by store emptying in response to TG or ionomycin was still conserved. Hence, this group proposed that SOC is activated not only by lowering of ER luminal Ca2+, but by a receptor-mediated activation of PLC-γ as well (17). Our results fit this assumption given that EGF-EGFR links to PLC pathway. In support, we found that the EGF-evoked single channel currents of SOC were almost completely abolished by specific PLC inhibitor (U73122) in this study.

Whether PLC-γ interacts directly with SOC proteins is de-
batable. The members of the TRP family of ion channels have been implicated as candidates for SOC (38–42). TRPM7, one member in this superfamily, has been defined as a channel interacting with PLC (including both β and γ isoforms) (43, 44). PLC-γ has also been demonstrated to interact with TRPC3 and TRPC4, another two members of TRP family. We recently found that TRPC4 is present in HMC as determined by immunofluorescence staining (data not shown). New studies are underway to directly address the possibility that TRPC4 is an essential component of EGF-induced conductance in HMC.

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Epidermal Growth Factor Activates Store-operated Ca^{2+} Channels through an Inositol 1,4,5-Trisphosphate-independent Pathway in Human Glomerular Mesangial Cells

Wei-Ping Li, Leonidas Tsiokas, Steven C. Sansom and Rong Ma

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