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Role of Inositol (1,4,5)Trisphosphate in Epidermal Growth Factor-Induced Ca^{2+} Signaling in A431 Cells

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SUMMARY

The effects of epidermal growth factor on Ca^{2+} signaling in A431 cells were investigated. Epidermal growth factor induced a transient Ca^{2+} signal in the absence of external Ca^{2+} and a sustained response in the presence of extracellular Ca^{2+}, indicating an ability to mobilize intracellular Ca^{2+} as well as the ability to increase Ca^{2+} entry from the extracellular space. The Ca^{2+}-ATPase inhibitor thapsigargin also activated Ca^{2+} entry, and neither epidermal growth factor nor the guanine nucleotide-dependent protein-linked receptor agonist bradykinin activated additional Ca^{2+} entry over that due to thapsigargin. In nominally Ca^{2+}-free medium, the addition of bradykinin to A431 cells rapidly but transiently increased inositol 1,4,5-trisphosphate and, in parallel fashion, transiently increased cytosolic Ca^{2+}. Unexpectedly, under these experimental conditions, epidermal growth factor elicited a small but significant Ca^{2+} signal after the addition of bradykinin. Experiments were designed to determine whether the Ca^{2+} response to epidermal growth factor after bradykinin results from mobilization of Ca^{2+} by an inositol 1,4,5-trisphosphate-independent mechanism. Epidermal growth factor stimulated additional inositol 1,4,5-trisphosphate formation in bradykinin-treated cells. Furthermore, the Ca^{2+} signals elicited by both bradykinin and epidermal growth factor were blocked in cells microinjected with the inositol 1,4,5-trisphosphate receptor antagonist heparin, whereas the intracellular Ca^{2+}-ATPase inhibitor thapsigargin still mobilized Ca^{2+}. Finally, histamine, a less efficacious guanine nucleotide-dependent protein-linked receptor agonist, as well as photolyzed, microinjected, caged inositol 1,4,5-trisphosphate, also mobilized Ca^{2+} after bradykinin. The results of this study show (i) that epidermal growth factor activates intracellular Ca^{2+} release as well as Ca^{2+} entry, the latter most likely resulting from an indirect effect due to the depletion of intracellular Ca^{2+} pools, (ii) that the actions of epidermal growth factor on Ca^{2+} homeostasis can be fully accounted for by inositol 1,4,5-trisphosphate formation, and (iii) that the ability of A431 cells to produce Ca^{2+} signals when epidermal growth factor is applied after bradykinin can be explained by the rapid and complete desensitization of the bradykinin stimulated phospholipase C activity.

It has become widely accepted that a host of cell surface receptors elicit biochemical responses in their respective effector cells through phosphoinositide turnover and increases in cytosolic Ca^{2+} (1). Specifically, agonist activation of these receptors results in the phospholipase C-catalyzed hydrolysis of the minor plasma membrane lipid phosphatidylinositol 4,5-bisphosphate, which generates two biologically active molecules, (1,4,5)IP_3 and diacylglycerol. Diacylglycerol remains in the plasma membrane to activate protein kinase C, whereas the water-soluble (1,4,5)IP_3 diffuses to the cell interior, where it activates its receptor and releases intracellular Ca^{2+}. In many instances, depletion of this intracellular Ca^{2+} pool appears to be the signal for the sustained Ca^{2+} influx from the extracellular space that is observed in agonist-activated cells (2, 3).

Many of the neurotransmitter and peptide hormone receptors that stimulate phosphoinositide turnover appear to activate phospholipase C through an intermediary G protein(s). Recently, a second family of cell surface receptors, the growth factor receptors, also has been shown to activate phosphoinositide hydrolysis. Several growth factors, including EGF and platelet-derived growth factor, stimulate phosphoinositide turnover, and increase cytosolic Ca^{2+}, DNA and protein synthesis, and cell proliferation in several cell types (4–6). EGF- and platelet-derived growth factor-mediated activation of these cellular responses appears not to involve G protein intermediates (7) (but see Refs. 8 and 9). Rather, the actions of receptors of this class are believed to depend upon the intrinsic protein tyrosine kinase activity of the receptors (4, 5), which catalyzes the tyrosine phosphorylation of and thereby activates a specific isozyme of phospholipase C (10–14).

ABBREVIATIONS: (1,4,5)IP_3, D-*myo*-inositol 1,4,5-trisphosphate (all inositol polyphosphates are abbreviated according to the Chilton Convention); HEPES, 4-(2-hydroxyethyl)piperazineethanesulfonic acid; EGTA, [ethylenebis(oxyethylenenitrilo)]tetracetic acid; G protein, guanine nucleotide-dependent regulatory protein; caged IP_3, *myo*-inositol-1,4,5-trisphosphate 1-(2-nitrophenyl)ethyl ester; DMEM, Dulbecco's modified Eagle's medium; fura-2/AM, fura-2 acetoxymethyl ester; HPLC, high pressure liquid chromatography; EGF, epidermal growth factor.

The A431 human epidermoid carcinoma cell line, which overexpresses the EGF receptor (1–2 × 10^6 receptors/cell), has proved exceedingly useful in the elucidation of the mechanisms of action of EGF (15), and much of the information currently available on regulation of phospholipase C by EGF has been obtained with these cells. However, the nature of the Ca^{2+} signal due to EGF in these and other cell types is less well understood. For example, whereas the effect of (1,4,5)IP_3 on Ca^{2+} entry has been suggested to be indirect in several cell types, that is, a consequence of intracellular Ca^{2+} release, direct application of (1,4,5)IP_3 to isolated cell patches of A431 cells appears to mimic the actions of EGF on Ca^{2+} channel activity (16). Furthermore, whereas it is generally accepted that EGF elicits increases in cytosolic Ca^{2+} in A431 cells in the presence of extracellular Ca^{2+}, in the absence of extracellular Ca^{2+} EGF has been reported to stimulate a transient rise in cytosolic Ca^{2+} (17–21) or to fail to increase intracellular Ca^{2+} under these conditions (22, 23). A431 cells also possess bradykinin, histamine, and ATP receptors that purportedly activate phospholipase C through intermediary G proteins. Therefore, this cell line is valuable for comparing biochemical mechanisms for Ca^{2+} regulation between these two families of cell surface receptors, particularly in light of the disparate results reported for the actions of EGF. To this end, we have examined Ca^{2+} signaling in A431 cells in response to the G protein-linked bradykinin receptor and the EGF receptor, in cell populations and at the single-cell level. Unexpectedly, in the absence of extracellular Ca^{2+}, EGF was capable of mobilizing additional intracellular Ca^{2+} after incubation of the cells with the more efficacious (1,4,5)IP_3-generating agonist bradykinin. Rather than conclude that the mechanism of regulation of Ca^{2+} homeostasis by EGF is different from that by bradykinin, we present evidence that the bradykinin receptor desensitizes and, as it does, cytosolic Ca^{2+} replenishes an intracellular Ca^{2+} pool that is responsive to EGF. Furthermore, our results also suggest that the actions of EGF on Ca^{2+} homeostasis appear to be fully accounted for by an (1,4,5)IP_3-mediated release of intracellular Ca^{2+} and Ca^{2+} entry from the extracellular space.

Materials and Methods

[^{3}H]Inositol was purchased from American Radiolabeled Chemicals, Inc. (St. Louis, MO) and the [^{3}H]inositol phosphate standards were from DuPont-New England Nuclear (Boston, MA). Receptor-grade EGF was obtained from Collaborative Research Inc. (Bedford, MA) and bradykinin from Peninsula Laboratories (Belmont, CA). Grade I heparin (from porcine intestinal mucosa) and histamine were from Sigma (St. Louis, MO). Fura-2/AM and fluo-3 were purchased from Molecular Probes (Eugene, OR); the free acid form of fura-2 and caged IP_3 were purchased from Calbiochem (San Diego, CA). Thapsigargin was generously supplied by Dr. S. Brogger Christensen (Royal Danish School of Pharmacy, Copenhagen, Denmark). All other chemicals were of the highest purity available, as previously reported (24).

Cell culture. A431 human epidermoid carcinoma cells were generously provided by Dr. Graham Carpenter, Vanderbilt University. Cell stocks were grown in DMEM (high glucose) supplemented with 2.5 mM glutamine and 10% fetal bovine serum. The cells were detached from culture flasks with 0.5% (w/v) trypsin containing 5.3 mM EDTA (GIBCO, Grand Island, NY) and were switched to DMEM supplemented with 10% calf serum for experiments.

Determination of intracellular Ca^{2+} by fura-2 fluorescence. Because considerable leakage of the Ca^{2+} indicator fura-2 was observed when A431 cells were examined in suspension, A431 cells were grown on glass coverslips (40 × 40 mm; Bionique) for 2 days to near-confluency, in DMEM containing 10% calf serum, for $[Ca^{2+}]_i$ measurements. The coverslips were placed in a Teflon chamber (Bionique), and the cells were incubated with 1 μM fura-2/AM in serum-free DMEM at room temperature in the dark for 30 min. The cells were washed four times with phosphate-free HEPES-Ringer's buffer containing 1.8 mM $CaCl_2$, placed on the microscope stage, and incubated 20–30 min until the fura-2 fluorescence reached a stable baseline. The fluorescence was monitored with a photomultiplier-based spectrofluorimeter (Photon Technology International, Inc., Princeton, NJ) mounted around a Nikon Diaphot microscope equipped with a Nikon 40× (1.3 numerical aperture) Neofluor objective. The fluorescence light source was equipped with a light path chopper and dual excitation monochromators. The light path chopper permitted rapid interchange between two excitation wavelengths (340 and 360 nm), and the emission fluorescence of 510 nm (selected by a barrier filter in the microscope) (Omega Optical, Inc., Brattleboro, VT) was detected by a photomultiplier tube. Data were collected from fields of 8–25 cells. Calcium ratios for each coverslip were corrected for cell autofluorescence by incubation with 20 μM ionomycin plus 5 mM $MnCl_2$ for 15 min. Solutions bathing the cells were aspirated and replaced with fresh HEPES-buffered Ringer's containing agonists as appropriate (see figure legends for details). In some experiments, fluorescence ratios were converted to calcium concentrations, as described by Grynkiewicz *et al.* (25).

Microinjection of A431 cells. Fura-2 acid and heparin were injected into A431 cells, in preconfluent culture, by a 100-msec pressure injection with a glass pipette attached to a WPI PV830 Picopump (World Precision Instruments, New Haven, CT). The injection solution contained 10 mM fura-2 acid, with or without 200 mg/ml heparin, in phosphate buffer (27 mM K_2HPO_4, 8 mM NaH_2PO_4, 26 mM KH_2PO_4, pH 7.3). The fluorescence ratios were monitored for 15–30 min, and injections were considered successful if the ratios were similar to those of A431 cells loaded with fura-2/AM. For the microinjection experiments, in order to decrease the background fluorescence from adjacent noninjected cells, the aperture to the photomultiplier tube was decreased, to collect data from single cells. Cell autofluorescence was determined as previously described. In other experiments, A431 cells were injected with 40 mM fluo-3 plus 30 mM caged IP_3 (pipette concentrations) (26). Cell fluorescence was monitored at 510 nm in response to excitations at 490 nm. Caged (1,4,5)IP_3 was photolyzed by a 1-msec flash from a 35S strobe lamp (Chadwick-Helmuth, El Monte, CA), via a Dolan-Jenner UV-grade fiber optic light guide containing a 360-nm discriminating filter (Omega Optical, Inc). The fiber optic light guide was located directly above the cell chamber.

[^{3}H]Inositol phosphate accumulation. For measurement of inositol phosphate formation, cells were grown for 3 days on 100-mm tissue culture plates (Costar) in DMEM/10% calf serum containing [^{3}H]inositol (specific activity, 15 Ci/mmol; 12.5 μCi/ml). The radioactive medium was aspirated, and the cells were incubated at 37° for 30 min with fresh DMEM without [^{3}H]inositol. Immediately before the onset of the experiment, the cells were washed once with nominally Ca^{2+}-free HEPES-Ringer's buffer containing 0.2% bovine serum albumin. Cells were incubated in a 25° shaking water bath (to duplicate the temperature of the Ca^{2+} measurements) in fresh buffer with vehicle, bradykinin (100 nM final concentration), or EGF (30 nM final concentration) for the indicated times, and the reactions were terminated by aspiration of the medium and addition of 3 ml of ice-cold perchloric acid (3%, w/v), containing 1 mg/ml phytate, to each plate. The precipitated samples were incubated at 4° for 30 min, and the acidic supernatants (containing the [^{3}H]inositol phosphates) were removed from the monolayers and extracted with Freon-trioctylamine, as previously described (27). The aqueous phase of the extraction, containing the [^{3}H]inositol phosphates, was neutralized by the addition of ammonium hydroxide, taken to dryness in a vacuum concentrator (Savant), and resuspended in 500–700 μl of water. The samples were processed for HPLC analysis as previously described (24) and were applied to a Whatman Partisil 10 SAX column. Inositol phosphates were separated using the ammonium phosphate gradient (pH 3.8, 1 ml/min flow rate)

described by Dean and Moyer (28). Radioactivity was monitored on-line, and peaks of radioactivity (counts/min) were analyzed with a Radiomatic Flo-one detector (Radiomatic Instruments and Chemical Co., Inc., Tampa, FL). Inositol phosphates were identified on the basis of their retention times, as previously described (24). The integrated peaks of radioactivity are expressed as a percentage of the radioactivity in the phosphoinositides, permitting comparison between experiments (24).

Results

EGF at 30 nM, a concentration previously demonstrated to be maximal for inositol phosphate formation and Ca^{2+} signaling (17, 29), evoked a biphasic Ca^{2+} signal in A431 cells, as measured by fura-2 fluorescence, that increased to a level approximately 5-fold greater than basal and then declined over a period of about 5 min to a sustained level that was slightly, but reproducibly, elevated above the basal level (Fig. 1A). As illustrated in Fig. 1, there was a considerable lag after the addition of EGF (which varied from 30 to 60 sec) before a detectable increase in cytosolic Ca^{2+} was observed. In the absence of extracellular Ca^{2+}, EGF only transiently elevated cytosolic Ca^{2+}, a result consistent with studies in A431 cells by several investigators (17–19) but in contrast to observations reported by two other laboratories (22, 23) (Fig. 1B). The sustained phase of the Ca^{2+} response could be restored by the addition of extracellular Ca^{2+} to the EGF-stimulated cells (Fig. 1B). To determine whether EGF stimulated Ca^{2+} release from the same intracellular store as bradykinin, bradykinin and EGF were sequentially added to A431 cells in the absence of extracellular Ca^{2+}. The addition of 100 nM bradykinin to naive A431 cells, in nominally Ca^{2+}-free solution, immediately and transiently increased cytosolic Ca^{2+} levels, again, a result attributed to Ca^{2+} release from intracellular stores (Fig. 2A). Surprisingly, 30 nM EGF caused additional release of Ca^{2+} from bradykinin-treated cells, although the magnitude of the EGF response, relative to that for bradykinin, varied among preparations. In contrast to EGF, a second application of bradykinin was unable to elicit a second Ca^{2+} response (data not shown). Generally, the magnitude of the EGF response was somewhat less in bradykinin-pretreated cells than in naive cells, a result consistent with the idea that bradykinin treatment at least partially depleted the EGF-responsive intracellular Ca^{2+} pool. Similar results were obtained if the order of the agonist application was reversed (Fig. 2B). EGF-treated cells also responded to bradykinin with a dramatic increase in cytosolic Ca^{2+}. The response to bradykinin was, in all cases, greater than the response to EGF, consistent with an observed greater increase in (1,4,5)IP_3. The

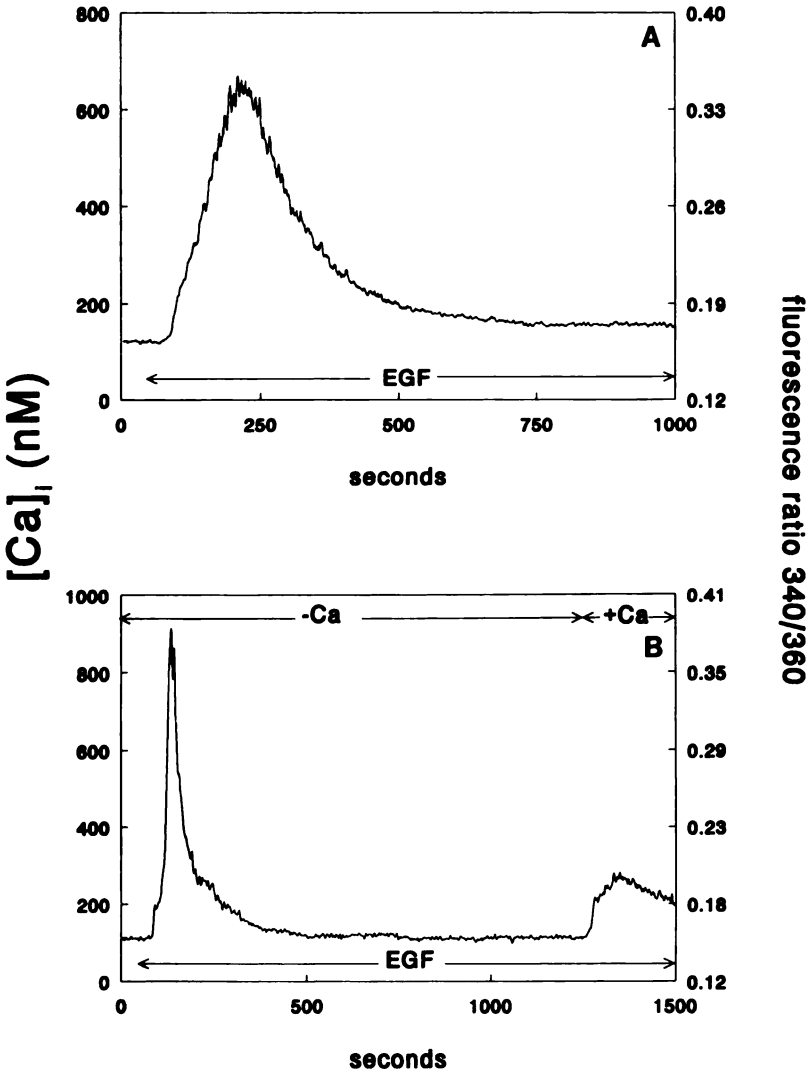

Fig. 1. Effect of EGF on cytosolic Ca^{2+} levels in A431 cells in the presence or absence of extracellular Ca^{2+}. A431 cells were grown on glass coverslips and injected with the Ca^{2+} indicator fura-2 (free acid), as described in Materials and Methods. A, A431 cells were incubated with 30 nM EGF in buffer containing 1.8 mM extracellular Ca^{2+}. The fluorescence data were corrected for cell autofluorescence and $[Ca^{2+}]_i$ was calculated as previously described (25). B, Cells were washed with nominally Ca^{2+}-free HEPES-buffered Ringer's solution immediately before the onset of the experiment. The cells were stimulated with 30 nM EGF from 50 to 1250 sec in the absence of extracellular Ca^{2+} and from 1250 to 1500 sec in buffer containing 1.8 mM $CaCl_2$. In a parallel trace, the addition of 1.8 mM Ca^{2+} to cells incubated in nominally Ca^{2+}-free buffer caused only a slight increase in $[Ca^{2+}]_i$ (from 128 to 152 nM; data not shown).

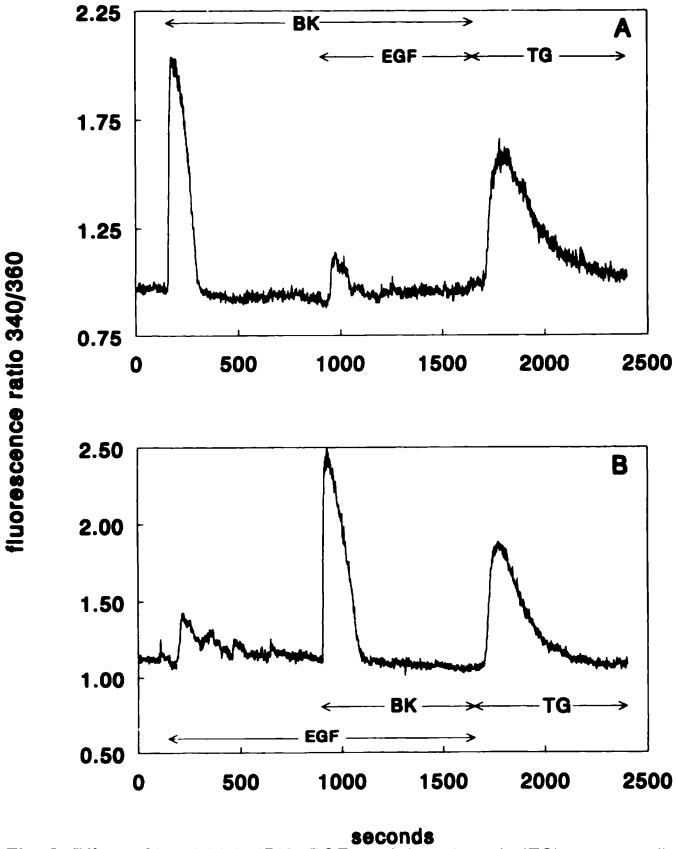

Fig. 2. Effect of bradykinin (*BK*), EGF, and thapsigargin (*TG*) on cytosolic Ca^{2+} levels in A431 cells in the absence of extracellular Ca^{2+}. A431 cells were grown on glass coverslips and loaded with the Ca^{2+} indicator fura-2/AM, as described in Materials and Methods. The cells were washed with nominally Ca^{2+}-free HEPES-buffered Ringer's solution immediately before the onset of the experiment. A, A431 cells were sequentially incubated with 100 nM bradykinin, 30 nM EGF plus 100 nM bradykinin, and 2 μM thapsigargin for the indicated times. B, Cells were sequentially incubated with 30 nM EGF, 100 nM bradykinin plus EGF, and 2 μM thapsigargin. The fluorescence data were corrected for cell autofluorescence, as described in Materials and Methods, and are expressed as the ratio of the fluorescence at an excitation of 340 nm to the fluorescence at 360 nm. The results are representative of results obtained on 4–17 coverslips in 4–13 independent experiments.

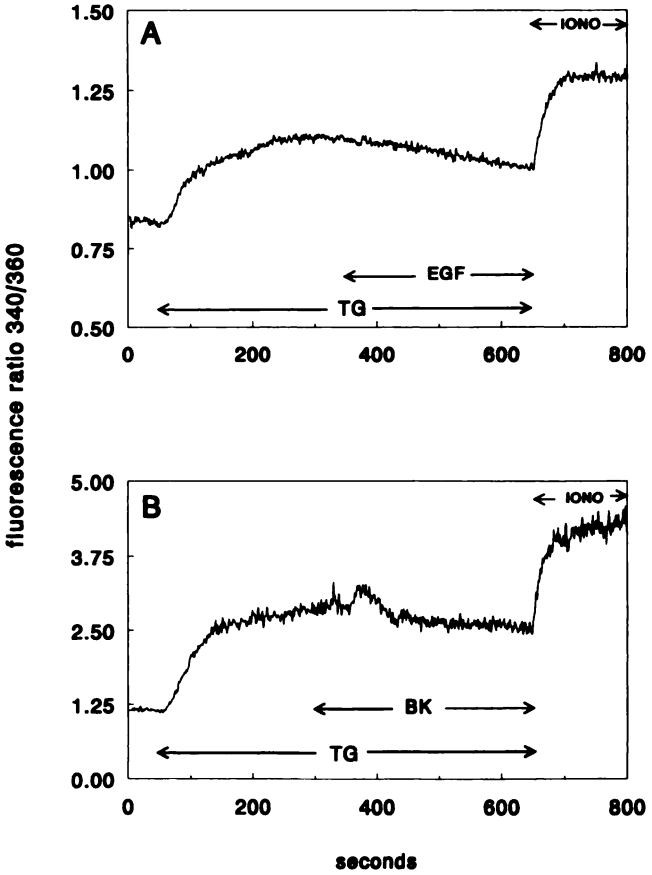

Fig. 3. Effect of bradykinin (*BK*) and EGF on thapsigargin (*TG*)-stimulated Ca^{2+} signaling in A431 cells. Fura-2/AM-loaded cells were sequentially stimulated with 2 μM thapsigargin from 50 to 650 sec and 30 nM EGF (A) or 100 nM bradykinin (B) from 350 to 650 sec, in HEPES-buffered Ringer's medium containing 0.3 mM $CaCl_2$. The response to 20 μM ionomycin (*Iono*) plus 3 mM $CaCl_2$ added to the cells at 650 sec demonstrates that the fura-2 was not saturated and would have detected agonist-stimulated increases in cytosolic Ca^{2+} had they occurred. The results are expressed as the ratio of fluorescence at 340 nm to that at 360 nm and are representative of results obtained in four independent experiments.

size of the bradykinin response was modestly diminished, if at all, by EGF pretreatment. Inclusion of 100 μM EGTA to the bathing solution to chelate any residual extracellular Ca^{2+} produced no qualitative or quantitative differences from the results illustrated in Fig. 2 (data not shown). Furthermore, agonist-treated cells displayed an additional transient Ca^{2+} signal in response to the microsomal Ca^{2+}-ATPase inhibitor thapsigargin (30, 31), in the absence of extracellular Ca^{2+} (Fig. 2). The substantial thapsigargin-sensitive Ca^{2+} signal, even in agonist-stimulated cells, suggests that the thapsigargin-sensitive Ca^{2+} pool is considerably larger than the agonist-sensitive Ca^{2+} pool(s). However, neither EGF nor bradykinin evoked a Ca^{2+} signal after thapsigargin treatment in the absence of extracellular Ca^{2+} (data not shown), indicating that the intracellular Ca^{2+} pools regulated by these agonists are entirely contained within the thapsigargin-sensitive pool. Consistent with results reported in several other cell types (32–35) (but see Ref. 36), thapsigargin stimulated sustained Ca^{2+} entry in the presence of extracellular Ca^{2+} (Fig. 3), an effect presumably secondary to the depletion of intracellular Ca^{2+} stores. However, the addition of either bradykinin or EGF to thapsigargin-stimulated cells failed to increase Ca^{2+} entry over that observed with thapsigargin alone.

Although time courses for inositol phosphate formation have been reported for bradykinin (17, 18, 37) and EGF (17, 18, 29, 38) in A431 cells, in light of the findings in Fig. 2 it was particularly important to determine whether EGF stimulated (1,4,5)IP_3 formation in bradykinin-treated cells. The addition of bradykinin to [^{3}H]inositol-labeled A431 cells resulted in a large but transient increase in [^{3}H](1,4,5)IP_3 levels (Fig. 4A). [^{3}H](1,4,5)IP_3 was rapidly metabolized by dephosphorylation to [^{3}H](1,4)IP_2 and by its sequential phosphorylation to [^{3}H](1,3,4,5)IP_4 and dephosphorylation to [^{3}H](1,3,4)IP_3 (Fig. 4, B–D). In naive A431 cells, EGF stimulated small but significant increases (10–35%) in [^{3}H](1,4,5)IP_3 levels but much larger increases in [^{3}H]IP_4, [^{3}H](1,3,4)IP_3, and [^{3}H]IP_2 (Fig. 4). In some experiments, [^{3}H]inositol phosphates were separated by weak anion exchange chromatography, as previously described (27), and three IP_4 isomers, (1,3,4,6)IP_4, (1,3,4,5)IP_4, and (3,4,5,6)/(1,4,5,6)IP_4, were resolved, all of which increased upon incubation with EGF (data not shown). In bradykinin-treated cells, the increase in (1,4,5)IP_3 due to EGF was smaller than

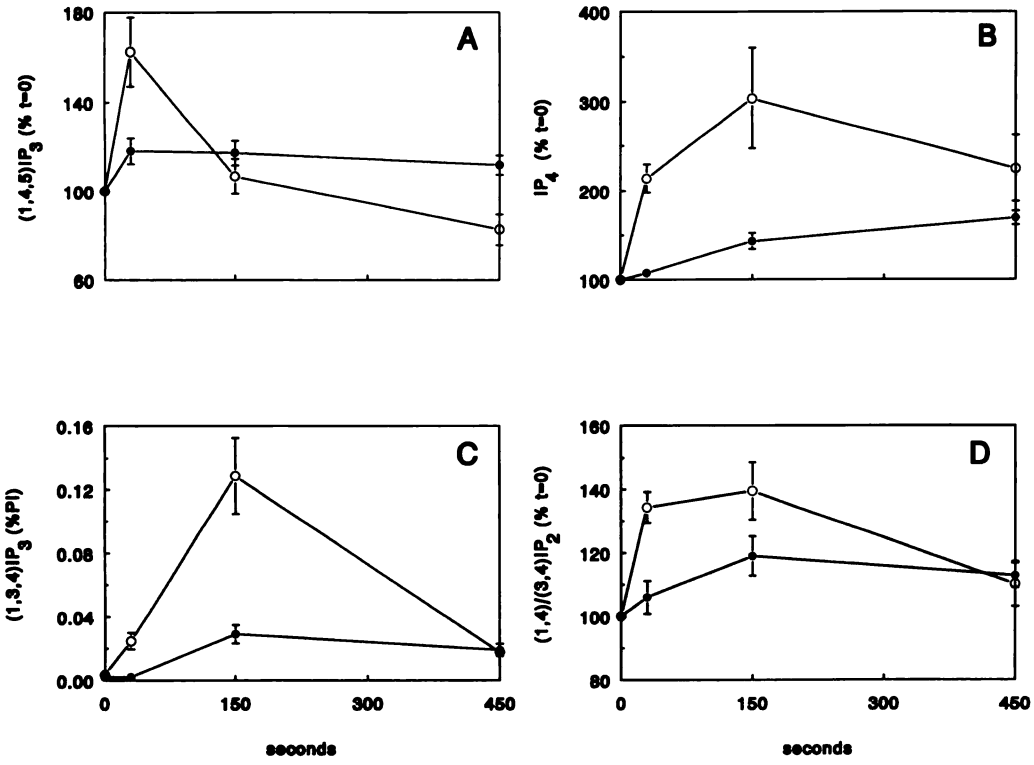

Fig. 4. Effect of bradykinin and EGF on [^{3}H](1,4,5)IP$_3$, (A), [^{3}H]IP$_4$ (B), [^{3}H](1,3,4)IP$_3$ (C), and [^{3}H]IP$_2$ (D) accumulation in A431 cells. [^{3}H] Inositol-labeled A431 cells were washed with nominally Ca^{2+}-free HEPES-Ringer's buffer and incubated with vehicle, 100 nM bradykinin, or 30 nM EGF for the indicated times, at room temperature. A431 cells were incubated for 750 sec with vehicle, at which time bradykinin (○) or EGF (●) was added to the cells (indicated as t = 0 on the figure). The reactions were terminated immediately before the addition of the agonists (t = 0) or at the indicated times after the second addition, by aspiration of the medium and addition of ice-cold perchloric acid containing 1 mg/ml phytate. The [^{3}H]inositol phosphates were separated on HPLC Partisil 10 SAX columns, and the integrated peaks of radioactivity were expressed as a percentage of the radioactivity in the phosphoinositides (%*PI*) and then relative to the basal value, except for (1,3,4)IP$_3$, for which the basal levels were not readily detectable. The data represent the mean ± standard error of results obtained in four experiments, each performed in duplicate.

that in naive cells and was not statistically significant (Fig. 5A). However, both [^{3}H]IP$_2$ and [^{3}H]IP$_4$ levels were modestly but significantly increased, and [^{3}H](1,3,4)IP$_3$ levels were increased dramatically (5-fold) in response to EGF (Fig. 5, B–D). Thus, the observation that the levels of the (1,4,5)IP$_3$ metabolites increased after the addition of EGF to bradykinin-treated cells indicates that EGF activates phospholipase C under these experimental conditions.

Heparin is a competitive antagonist for the (1,4,5)IP$_3$ receptor that regulates intracellular Ca^{2+} stores (39–41). In order to

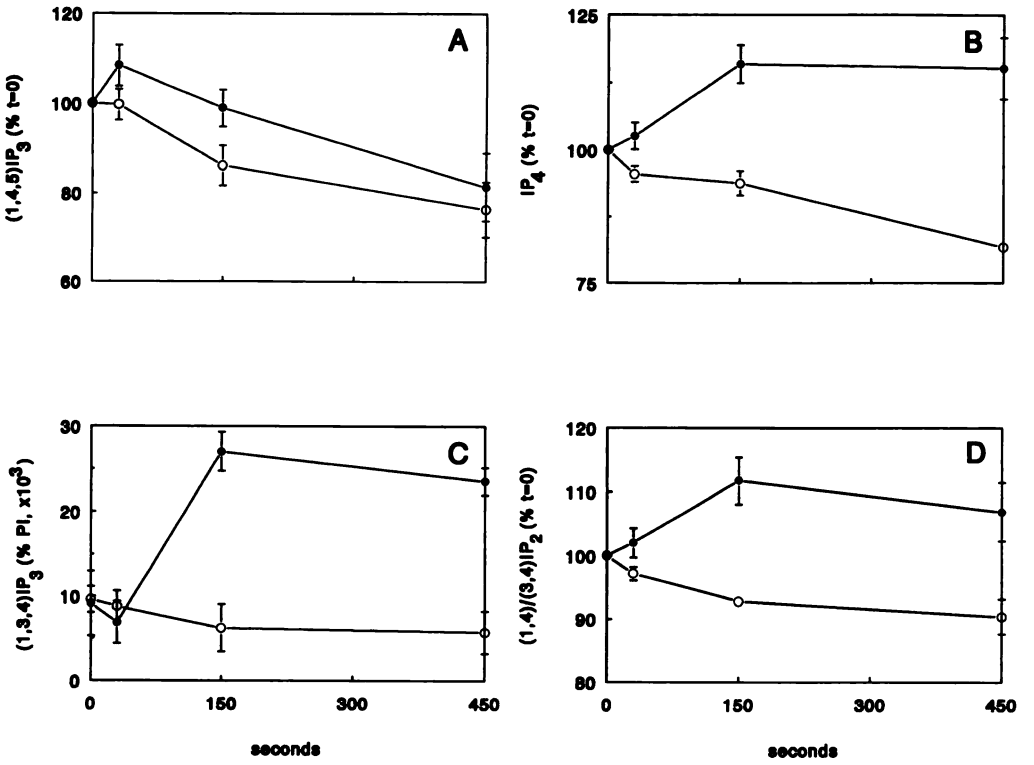

Fig. 5. Effect of EGF on [^{3}H] (1,4,5)IP$_3$, (A), [^{3}H]IP$_4$ (B), [^{3}H] (1,3,4)IP$_3$ (C), and [^{3}H]IP$_2$ (D) accumulation in bradykinin-stimulated A431 cells. [^{3}H]Inositol-labeled A431 cells were washed with nominally Ca^{2+}-free HEPES-Ringer's buffer and incubated with 100 nM bradykinin for 750 sec. Then, at t = 0, either vehicle (○) or 30 nM EGF (●) was added to the bradykinin-treated cells for the indicated times. The [^{3}H]inositol phosphates were separated on HPLC Partisil 10 SAX columns, and the integrated peaks of radioactivity were expressed as a percentage of the radioactivity in the phosphoinositides (%*PI*) and then relative to the basal value, except for (1,3,4)IP$_3$, for which the basal levels were not readily detectable. The data represent the mean ± standard error of results obtained in four experiments, each performed in duplicate.

assess further whether (1,4,5)IP$_3$ formation was responsible for EGF-mediated Ca^{2+} signaling in A431 cells, the capacity of EGF to mobilize Ca^{2+} was examined in the presence of heparin. A431 cells were microinjected with heparin and fura-2 acid, and Ca^{2+} signaling was examined. Again, as was observed with the fura-2/AM-loaded cells (Fig. 2), in the absence of extracellular Ca^{2+}, bradykinin and EGF sequentially (and in either order) elicited transient Ca^{2+} signals in single A431 cells injected with fura-2 acid alone (Fig. 6A; data not shown). The ability of EGF to generate a Ca^{2+} signal in single cells also indicates that EGF and bradykinin mobilize Ca^{2+} from the same cell and that the observations on cell populations are not due to different cells that selectively respond to each agonist. The bradykinin concentration was decreased 10-fold in these experiments in order to obtain Ca^{2+} signals similar in magnitude to those elicited by EGF. Under these experimental conditions, heparin consistently blocked both the EGF- and bradykinin-stimulated Ca^{2+} signals. Heparin also blocked or, in some cases, at the very least greatly attenuated the EGF- and bradykinin-mediated Ca^{2+} signals observed in the presence of extracellular Ca^{2+} (Fig. 6B). Similar results were observed if the order of agonist application was reversed in the presence of extracellular Ca^{2+} (data not shown). In contrast to the results observed with receptor agonists, heparin did not block the IP$_3$-independent thapsigargin-mediated Ca^{2+} signaling in A431 cells (data not shown).

Because the EGF receptor requires its intrinsic tyrosine kinase activity to activate phospholipase C, it is conceivable that this kinase activity might permit additional activation of phospholipase(s) C over the bradykinin receptor/G protein-activated process and account for the capacity of EGF to regulate Ca^{2+} after bradykinin. In order to determine whether this second stimulation of phosphoinositide turnover and Ca^{2+} signaling was specific for tyrosine kinase-activating receptors or could occur through other pathways as well, the capacity of a second G protein-linked receptor, the histamine H$_1$ receptor, to mobilize Ca^{2+} in A431 cells was examined. In these cells, in agreement with the findings of Hepler *et al.* (17), histamine was found to be a less efficacious agonist than bradykinin for activation of phosphoinositide hydrolysis (data not shown). In the absence of extracellular Ca^{2+}, 1 mM histamine elicited a smaller Ca^{2+} signal in A431 cells, compared with bradykinin (Fig. 7A). This result notwithstanding, histamine added 750 sec after bradykinin also generated a second Ca^{2+} signal in A431 cells (Fig. 7B).

Finally, intracellular Ca^{2+} could be mobilized by receptor-independent formation of (1,4,5)IP$_3$ in bradykinin-treated cells. Specifically, photolysis of microinjected caged IP$_3$ to release (1,4,5)IP$_3$ also generated a second Ca^{2+} signal in bradykinin-stimulated A431 cells in the absence of extracellular Ca^{2+} (Fig. 8).

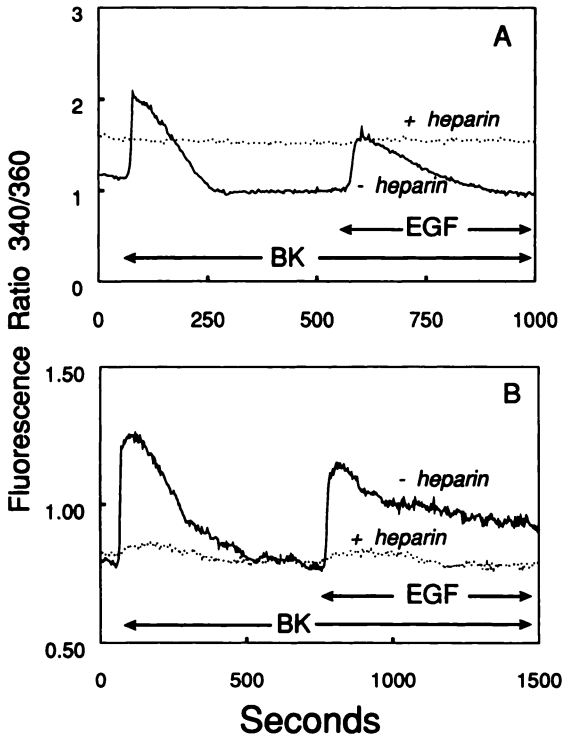

Fig. 6. Effect of heparin on the ability of bradykinin (*BK*) and EGF to elevate cytosolic Ca^{2+} in fura-2-injected A431 cells. A431 cells were injected by pipettes containing fura-2 acid (10 mM), with or without 200 mg/ml heparin, as described in Materials and Methods. Cells were challenged with 10 nM bradykinin or 10 nM bradykinin plus 30 nM EGF, in the absence (A) or presence (B) of extracellular Ca^{2+}, for the intervals indicated. The fluorescence data were corrected for cell autofluorescence and are expressed as the ratio of the fluorescence at excitation wavelengths of 340 and 360 nm, and are representative of results obtained in three to five independent experiments.

Discussion

Although a number of laboratories have demonstrated that EGF stimulates phosphoinositide turnover (17–19, 29, 38, 42), the role of inositol phosphates in EGF-induced Ca^{2+} signaling has not been extensively examined. This study was designed to investigate this Ca^{2+} signaling pathway, especially in light of discrepancies in the literature regarding the relationship be-

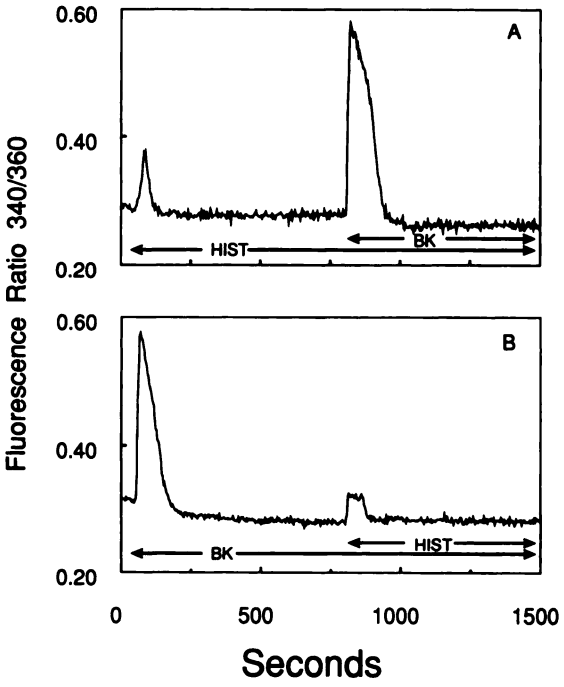

Fig. 7. Effect of sequential addition of bradykinin (*BK*) and histamine (*HIST*) on cytosolic Ca^{2+} levels in A431 cells in the absence of extracellular Ca^{2+}. Cells were sequentially incubated with 1 mM histamine followed by 100 nM bradykinin plus histamine (A) or vice versa (B), for the indicated intervals. Similar results were observed in two additional experiments.

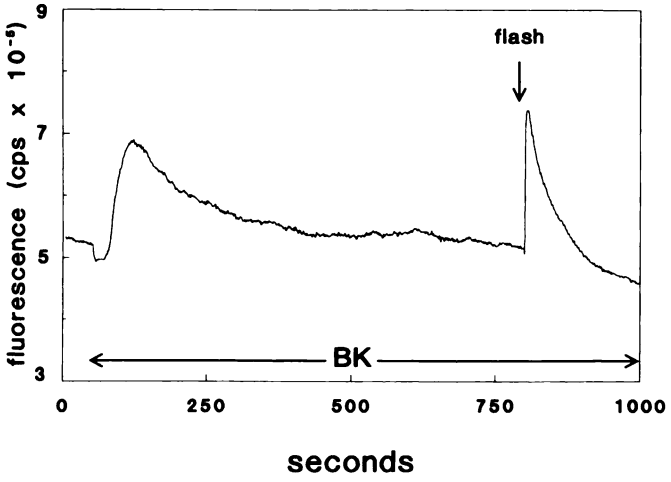

Fig. 8. Effect of photolysis of caged (1,4,5)IP_3 on cytosolic Ca^{2+} levels in bradykinin (*BK*)-treated A431 cells. A431 cells were microinjected with 40 mM fluo-3 plus 30 mM caged (1,4,5)IP_3, as described in Materials and Methods, and the fluorescence was monitored from single cells. The cells were washed with Ca^{2+}-free HEPES-buffered Ringer's solution, containing 100 μM EGTA, immediately before the onset of the experiment. Cells were challenged with 10 nM bradykinin from 50 to 1000 sec and exposed to a single 1-msec flash at 800 sec. The fluorescence data are expressed as the counts/sec at 510 nm. Similar results were observed in two other experiments.

tween EGF-stimulated phosphoinositide turnover and Ca^{2+} signaling in A431 cells.

Our finding that EGF can generate a Ca^{2+} signal in the absence of extracellular Ca^{2+} confirms previous reports (17–19) but contradicts the finding of at least two other groups (22, 23). However, one group observed EGF-stimulated Ca^{2+} release in the absence of extracellular Ca^{2+} with the Ca^{2+} indicator indo-1 but not with quin2, a Ca^{2+} indicator with greater buffering capacity (23) (but see Ref. 18). Alternatively, subclones of A431 cells may have developed that could contribute to the heterogeneity of the results reported. Again, although most laboratories agree that EGF stimulates phosphoinositide hydrolysis (17–19, 29, 38, 42), the role of inositol phosphates in EGF-mediated Ca^{2+} signaling has not been confirmed. One patch-clamp study by Chapron *et al.* (16) reported that (1,4,5)IP_3 mimicked the actions of EGF on Ca^{2+} channel activity of isolated A431 cell patches. However, the results presented in the current study argue against a direct effect of EGF on plasma membrane Ca^{2+} channels. That is, thapsigargin was found to be an efficient activator of Ca^{2+} entry in A431 cells (Fig. 2); this is generally taken to mean that it is the status of the intracellular Ca^{2+} pool that regulates Ca^{2+} entry, rather than a direct effect of receptors or inositol polyphosphates (33). Furthermore, EGF did not increase Ca^{2+} entry when added to thapsigargin-activated cells; this may indicate that additional mechanisms for activation of Ca^{2+} entry are not available to EGF (Fig. 3). One possible explanation for the findings of Chapron *et al.* (16) is that excised patches may retain portions of the cellular infrastructure, including (1,4,5)IP_3-sensitive organelles that, upon application of (1,4,5)IP_3, discharge their Ca^{2+}, leading to Ca^{2+} entry.

In the absence of extracellular calcium, the addition of EGF to cells previously stimulated with bradykinin results in the transient elevation of cytosolic Ca^{2+}. Similar findings were reported by Wheeler *et al.* (20), who interpreted this as evidence for separate Ca^{2+} pools regulated by the different agonists. This observation also contrasts with most previous reports on multiple phospholipase C-linked receptors, wherein it has been generally found that the discharge of internal Ca^{2+} by an agonist acting on one receptor type precludes any additional release of Ca^{2+} upon addition of an agonist for a second receptor type (43–45). Therefore, experiments were undertaken to elucidate the mechanism(s) by which EGF regulates Ca^{2+} homeostasis.

In this study as well as in other studies (17, 18), it is clear that bradykinin is much more efficacious at stimulating (1,4,5)IP_3 formation than is EGF. Although we could measure EGF-mediated increases in (1,4,5)IP_3 levels in naive cells, it was difficult to detect EGF-stimulated increases in bradykinin-treated cells; however, the (1,4,5)IP_3 metabolites, (1,3,4)IP_3, IP_4, and IP_2, consistently increased with EGF treatment. These small changes in (1,4,5)IP_3 levels in A431 cells after incubation with EGF are consistent with the increases in (1,4,5)IP_3 levels (0–50%) reported by a number of other laboratories (17–19). Both the modest changes in (1,4,5)IP_3 levels and the delayed Ca^{2+} signal observed in response to EGF are likely due to a slow association of EGF with its receptor. Wahl and co-workers (29, 38) overcame this obstacle by a 2-hr incubation of [^{3}H] inositol-labeled A431 cells with EGF at 5° and assay of [^{3}H] inositol phosphate accumulation at 37°. Under these experimental conditions, EGF stimulated (1,4,5)IP_3 levels 2–8-fold. Unfortunately, the experimental protocols used in the current study preclude such a procedure.

Again, in light of these modest changes in inositol phosphates, the capacity of EGF to mobilize intracellular Ca^{2+} stores after bradykinin stimulation was unexpected. Several possible mechanisms may explain this observation. First, EGF may regulate cytosolic Ca^{2+} by a non-IP_3-mediated mechanism. This is unlikely, given that the competitive IP_3 receptor antagonist heparin (39–41) blocked the actions of EGF both in naive cells and in cells previously incubated with bradykinin. A second explanation for the Ca^{2+} data might be that EGF-stimulated IP_3 formation may release Ca^{2+} from a pool distinct from the IP_3-sensitive pool mobilized by bradykinin, as originally suggested by Wheeler *et al.* (20). Whereas stimulation of A431 cells with EGF results in considerably less (1,4,5)IP_3 formation than that observed in response to bradykinin, perhaps the tyrosine kinase activity of the EGF receptor, acting on as yet unidentified substrates, together with IP_3 formation reveal cryptic Ca^{2+} pools not released by IP_3 alone. However, the ability of EGF to induce Ca^{2+} release after bradykinin does not appear to be related to its mechanism of action in activating phospholipase C, because histamine, a much less efficacious G protein-linked receptor agonist with no reported tyrosine kinase activity, as well as (1,4,5)IP_3 photolytically released from its caged precursor, also mobilize Ca^{2+} after bradykinin. Therefore we conclude that, at least in A431 cells, the existence of a distinct, growth factor-sensitive Ca^{2+} pool appears unlikely.

A contributing factor for these experimental observations may be desensitization of the bradykinin receptor. Cunningham *et al.* (46) have reported in A431 cells that the desensitization of the bradykinin response is homologous, in the sense that the capacity of EGF to stimulate (1,4,5)IP_3 formation in bradykinin-treated cells was not diminished. The results illustrated in Figs. 4 and 5 are consistent with this finding. As the bradykinin receptor desensitizes, the levels of (1,4,5)IP_3 fall and some of the cytosolic Ca^{2+} is taken back up by the IP_3-sensitive intracellular stores for release by the addition of a second receptor

agonist. Furthermore, there is evidence in the literature for reuptake of mobilized Ca^{2+} into (1,4,5)IP_3-sensitive stores in intact cells. For example, in rabbit parietal cells, at least some of the intracellular Ca^{2+} released in response to the muscarinic receptor agonist methacholine can be resequestered by intracellular organelles and subsequently released by a second addition of agonist. Specifically, addition of the muscarinic receptor antagonist atropine to cholinergically stimulated parietal cells, at a time when cytosolic Ca^{2+} was maximally elevated, promoted uptake of the Ca^{2+} back into IP_3-sensitive intracellular stores (47).

In summary, the findings in this study indicate that activation of the EGF receptor induces Ca^{2+} signals in A431 cells by mechanisms dependent on the formation of (1,4,5)IP_3, which are not experimentally distinguishable from those involved in the actions of the more extensively studied G protein-coupled receptors. Specifically, these include an initial release of intracellular Ca^{2+} stores, followed by activation of Ca^{2+} entry across the plasma membrane. The Ca^{2+} entry activated by EGF may result from an indirect mechanism dependent on the depletion of intracellular Ca^{2+} stores. When the actions of EGF were compared with those of bradykinin, marked differences were noted in the degree of activation of this pathway, the time course of inositol phosphate and $[Ca^{2+}]_i$ changes, and the extent and time course of receptor desensitization. It is possible that these differences are significant in the timing of activation of a multitude of signaling processes that constitute the physiological mitogenic signal generated through this ubiquitous growth factor receptor.

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