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Research Paper

EGF Activates Intracellular and Intercellular Calcium Signaling by Distinct Pathways in Tumor Cells

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ABSTRACT

Epidermal growth factor (EGF)-mediated Ca^{2+} signaling in multiple cell lines derived from human gliomas and in the A431 epidermoid carcinoma cell line was observed using fluorescence videomicroscopy. Bath application of EGF evoked an oscillatory increase in $[Ca^{2+}]_i$ in 4 different human glioma cell lines as well as the A431 cell line. This effect was blocked by the EGF receptor tyrosine kinase inhibitors gefitinib and erlotinib, as well as by the EGFR antibody cetuximab. In addition to this acute Ca^{2+} signaling response, transient exposure to EGF also potentiated subsequent Ca^{2+} signaling responses to other stimuli. Tumor cells transiently exposed to EGF (5 minutes), showed a sustained increase in propagation of intercellular Ca^{2+} waves, which have been previously shown to involve release of ATP and activation of purinergic receptors. Cells transiently exposed to EGF also showed a sustained potentiation of the Ca^{2+} signaling response to ATP. In contrast to the acute Ca^{2+} signaling response to EGF, this sustained potentiation of purinergic intercellular signaling was not blocked by gefitinib or erlotinib, while it was blocked by cetuximab. These results indicate that while the acute Ca^{2+} signaling response requires tyrosine kinase activation, the sustained potentiation of intercellular signaling occurs via a distinct pathway. Distinct intra- and intercellular Ca^{2+} signaling pathways may be mechanisms by which EGF modulates the growth and migration of tumor cells.

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INTRODUCTION

The epidermal growth factor receptor (EGFR) plays a critical role in the development and progression of a variety of cancers including glioblastoma multiforme.[1,2] It is involved in the regulation of cell growth through apoptosis, proliferation and differentiation, and also modulates the migratory behavior of cells.[3,4] EGFR may be involved in the pathogenesis of tumors via multiple mechanisms, including autocrine stimulation, overexpression and/or amplification of the EGFR gene, and deletions and mutations of the gene that produce a constitutively active receptor.[5,6] Glioblastoma multiforme is one of the common cancers linked with aberrations of the EGFR, with amplification seen in 40–60% of pathological samples and mutations seen in 60%.[7-13] Changes in the expression and/or function of EGFR have also been associated with a variety of cancers in a variety of other tissues including breast, lung, and prostate.[14-17]

EGFR has become an important molecular target for cancer therapy.[18-22] Several drugs have been developed that specifically block the ATP binding site of the tyrosine kinase. Gefitinib (Iressa, ZD1839) is an EGFR tyrosine kinase inhibitor that has recently been FDA approved for the treatment of nonsmall cell lung cancer, and is in clinical trials for other types of cancer.[23–26] Another drug, erlotinib (Tarceva, OSI-774) is currently in or has completed phase III trials investigating its efficacy in pancreatic and lung cancer among others.[27] A phase I trial is ongoing which is evaluating erlotinib for the treatment of glioblastoma (TARGET trial).

Another potential method of inhibiting EGFR is through monoclonal antibodies directed at the extracellular binding site of the epidermal growth factor (EGF). One such antibody is the human-mouse chimeric Mab C225 (cetuximab, Erbitux). It is currently in phase II and III clinical trials evaluating its usefulness in several cancer types.[28-30] C225 has also shown promise in vitro against glioblastoma that overexpresses EGFR by promoting apoptosis and decreasing proliferation.[31]

Although the EGFR has been extensively studied, the signaling mechanisms by which it exerts its effects are complex, and understanding of these mechanisms is incomplete. In this study, we characterize both acute and sustained changes in Ca^{2+} signaling in response to EGF. We show that EGF affects both intracellular Ca^{2+} signaling and intercellular communication. We also show that gefitinib and erlotinib as compared with cetuximab,

have specific and distinct effects on different components of the Ca^{2+} signaling response to EGF.

EXPERIMENTAL PROCEDURES

Cell Culture. Cell lines derived from four different human gliomas and A431 cells were grown in 35 mm plastic flasks, and passaged onto 18 mm diameter glass coverslips in 12 well plates. One glioma cell line in particular (GB-1) was used for most studies. Growth medium was comprised of DMEM-F12 supplemented with 5% fetal bovine serum, 5% horse serum, penicillin and streptomycin. Cells were grown at 37°C in a humidified incubator with 5% CO_2.

Measurement of Calcium. $[Ca^{2+}]_i$ was measured using a fluorescence imaging system that has been described previously.[32] In brief, cells grown on glass coverslips were loaded with fluo-4 by incubation in 5 μM fluo-4-AM for 40–60 min. Coverslips were washed at least three times with HBSS prior to imaging. Coverslips were then placed on a Nikon inverted microscope and excited with a light emitting diode through a 488 nm bandpass filter, and fluorescence at 535 nm was recorded through a x 20 objective with a SIT camera. Images were digitized using an Axon Image Lightning board and Imaging WorkBench 4.0 software, and ΔF was calculated on a pixel-by-pixel basis as described previously.

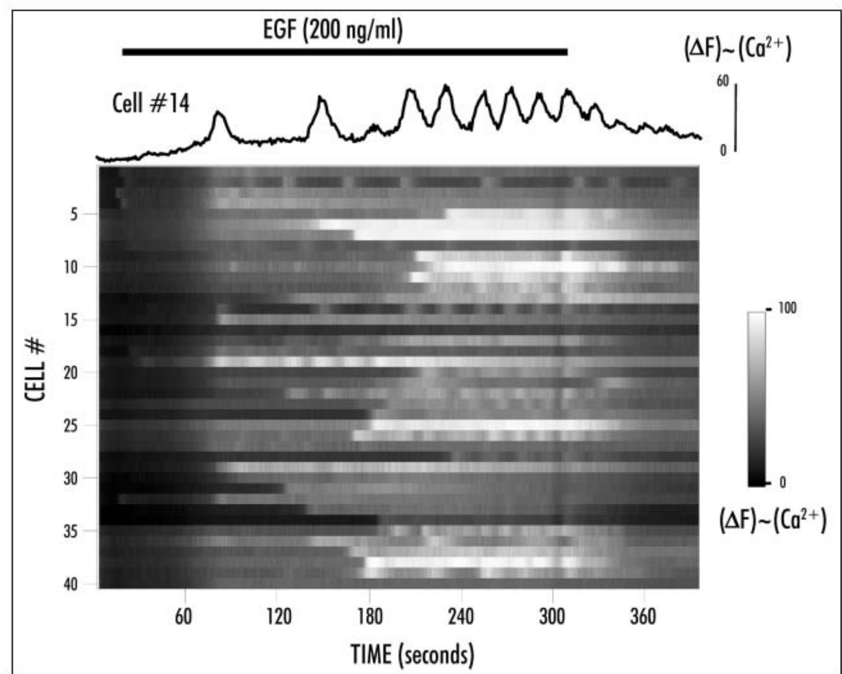

Figure 1. Ca^{2+} signaling response to EGF in the GB-1 human glioma cell line. Raster plot shows change in fluo-4 fluorescence (~$[Ca^{2+}]_i$) vs. time in 40 different cells in a microscopic field. Each lane represents an individual cell, with ΔF (~ $[Ca^{2+}]_i$) indicated by the grayscale bar. The top line trace shows a representative cell (#14). Bath application of EGF results in a delayed increase in $[Ca^{2+}]_i$ in the majority of cells in the field. Many cells show an oscillatory Ca^{2+} response that continues following washout of EGF.

Analysis of Fluorescence Recordings. Axon Imaging Workbench 4.0 software was used to analyze fluorescence recordings. Fourty to sixty cells were randomly selected from each microscopic field and analyzed using discreet regions of interest (ROI's) drawn above the individual cells. Outputs from the ROI's were visualized as line tracings (Δfluorescence vs. time). A response to a given stimulus was defined by an increase in fluorescence of at least 20% above baseline during the 120 seconds following the stimulus. Each cell trace was recorded either as a response or nonresponse. For the acute calcium response to EGF, additional information gathered included time of onset and frequency of oscillations. Three to twelve experiments were averaged to give a mean and a standard deviation. The student t-test was used when appropriate to determine significance between treatment verses baseline.

Intercellular Calcium Signaling. Intercellular Ca^{2+} waves were evoked by mechanical stimulation of a single cell. The membrane of one cell in the center of the image was briefly deformed using a glass micropipette with a micromanipulator. This is a reliable method of evoking intercellular calcium waves.[33] Any experiments where the cell was excessively deformed or showed leakage of dye indicating membrane rupture were discarded. Micropipette stimulations of single cells were done at baseline. Coverslips were washed and EGF was added for 5 minutes. Coverslips were again washed and $[Ca^{2+}]_i$ was allowed to return to baseline. At different time points following EGF exposure, micropipette stimulations were repeated on the same coverslip (different microscopic fields).

Axon Imaging Workbench 4.0 software was also used to analyze the extent of intercellular Ca^{2+} waves. The maximum distance the wave traveled from the stimulated cell was determined with x and y coordinates. The radius and velocity of the waves were then calculated. At least six experiments per condition were recorded and an average radius and velocity were described. The student t-test was used to determine significant differences in Ca^{2+} wave propagation under different conditions.

Quantification of Total EGFR and Phosphorylated EGFR. Two phosphorylation sites of EGFR (1068 and 1173) that have been identified as critical to EGFR-mediated signaling were investigated.[9,34-36] Using an enzyme linked-immuno-sorbent assay (BioSource International, Inc.), total EGFR and phosphorylated EGFR were quantified under various conditions. Four flasks of GB-1 cells and A431 cells were grown to confluence in serum. To mimic the conditions observed during calcium imaging, cells were placed in HBSS without serum for 30–60 minutes and then incubated in either control HBSS, erlotinib or cetuximab. Three flasks were subsequently exposed to 200 ng/ml EGF, EGF + erlotinib, and EGF + cetuximab for 5–10 minutes and one flask served as a baseline. The cells were dissociated in a nonenzymatic cell dissociation solution and collected in cold PBS. Membrane proteins were extracted using 10^8 cells per milliliter of extraction buffer (Cellular and Organelle Membrane Solubilizing Reagent, Sigma) with 100 microliters of Protease Inhibitor Cocktail (Sigma). The cell pellet was lysed on ice with vortexing for 30 minutes. The lysate was centrifuged at 13,000 RPM's for 10 minutes and the supernatant was applied to the ELISA at a 1:10 dilution.

The ELISA quantified four EGFR samples: control HBSS, 200ng/ml EGF, EGF + erlotinib, and EGF + cetuximab. Three types of ELISA were used: total EGFR (ng/ml), pY1068 EGFR (units/ml), and pY1173 EGFR (units/ml). A ratio of the fluorescence of the phosphorylated EGFR over total EGFR was used to describe the

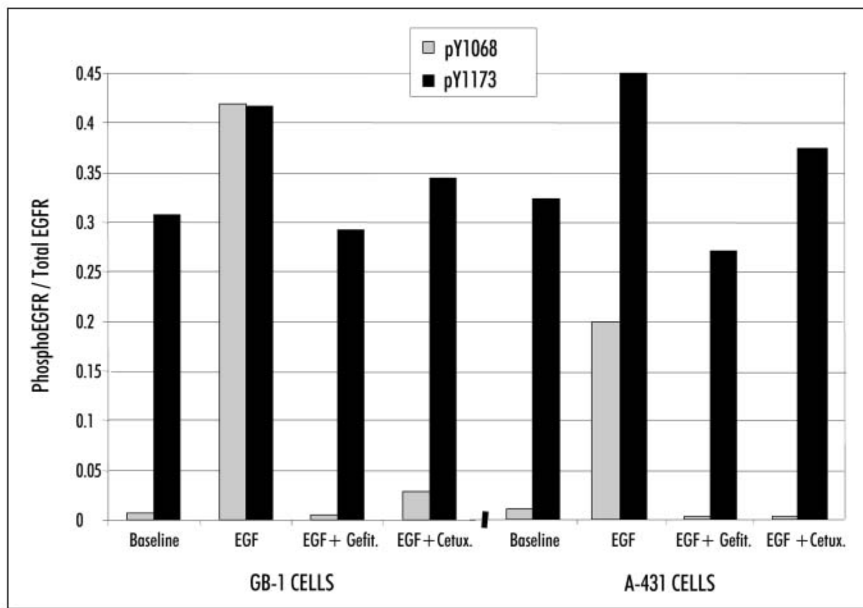

Figure 2. Effects of EGFR inhibitors on EGF phosphorylation. Total EGFR and two critical phosphorylation sites of EGFR (sites 1068 and 1173) were quantified by ELISA. A ratio of phosphorylated EGFR to total EGFR was used to describe the effect of EGF and EGFR inhibitors. Values represent averages of three different experiments. In both cell lines, EGF increased phosphorylation at both sites when compared to baseline. Erlotinib completely blocked phosphorylation at both sites. Cetuximab blocked the phosphorylation completely at pY1068 and significantly inhibited phosphorylation at pY1173.

efficacy of EGF in phosphorylating the two sites and the efficacy of the two clinical drugs in inhibiting this pathway. Coomassie dry protein assay plates (Pierce, Rockford, IL) were used to quantify the total protein in the membrane protein extraction.

Drugs. Erlotinib (OSI-774, Tarceva) was generously provided by Genentech, South San Francisco, CA. Gefitinib (Iressa) was generously provided by Astra Zeneca, Wilmington, DE.

Cetuximab (C225, Erbitux) was generously provided by Bristol Myers Squib, New York, NY.

RESULTS

Acute Ca^{2+} Signaling Response to EGF. Bath application of EGF (200 ng/ml) evoked an increase in intracellular calcium concentration $[Ca^{2+}]_i$ in four different cell lines derived from different human gliomas. The responses of the different cell lines were qualitatively similar, but one particular line with the most consistent response was chosen for further study (GB-1). On average, 50.1% (range: 14.0–81.7, n = 180 cells in six experiments) of GB-1 cells responded to EGF with an increase in $[Ca^{2+}]_i$ (Figs. 1 and 2). Some of the responding cells (12.4% range: 2.0–38.3) showed repetitive Ca^{2+} oscillations in response to EGF. The average delay between application of EGF and the initial rise in $[Ca^{2+}]_i$ was 23.7 sec (range: 11–43). EGF-evoked Ca^{2+} signaling was also investigated in the A431 epidermoid carcinoma cell line, in which EGFR function has been extensively studied. An average of 67.8% (range: 45–93%, n = 90 cells in three experiments) of A431 cells responded to EGF with an increase in $[Ca^{2+}]_i$ (Fig. 2). Of these, 40.0% (range 31.6–50.0) showed repetitive Ca^{2+} oscillations. The average delay between application of EGF and the initial rise in $[Ca^{2+}]_i$ was 20.3 sec (range: 12–25). In both cell lines, a subset of cells showed a persistent increase in $[Ca^{2+}]_i$ or sustained Ca^{2+} oscillations lasting up to 10 minutes following washout of EGF.

Mechanism of Ca^{2+} Response to EGF. To investigate the Ca^{2+} signaling pathway involved in the response EGF, we examined the role of extracellular Ca^{2+}, intracellular Ca^{2+} stores, and phospholipase C. In the absence of extracellular Ca^{2+} or Mg^{2+}, the Ca^{2+} signaling response to EGF was similar to that observed in the presence of divalent cations (n = 60 cells, data not shown). Pretreatment with the endoplasmic reticulum pump inhibitor thapsigargin (100 nM) blocked the Ca^{2+} response to EGF in both cell lines (n = 60 cells, data not shown). The phospholipase C inhibitor U73122 (5 μM) also blocked the Ca^{2+} response to EGF (n = 120 cells, data not shown).

Effect of EGFR Inhibitors. We examined the effects of EGFR inhibitors on EGFR phosphorylation at two sites (pY1068, pY1173) using an ELISA (Fig. 2). A431 cells contained approximately 1000 fold more total EGFR/total protein than GB-1 cells (not shown). In both cell lines, EGF increased phosphorylation at both sites when compared to baseline. The EGFR-specific PTK inhibitor erlotinib (5 μM) completely blocked the EGF-induced phosphorylation at both sites, whereas the EGFR-specific antibody cetuximab (100 μM) inhibited but did not block EGF-induced phosphorylation at both sites (Fig. 2).

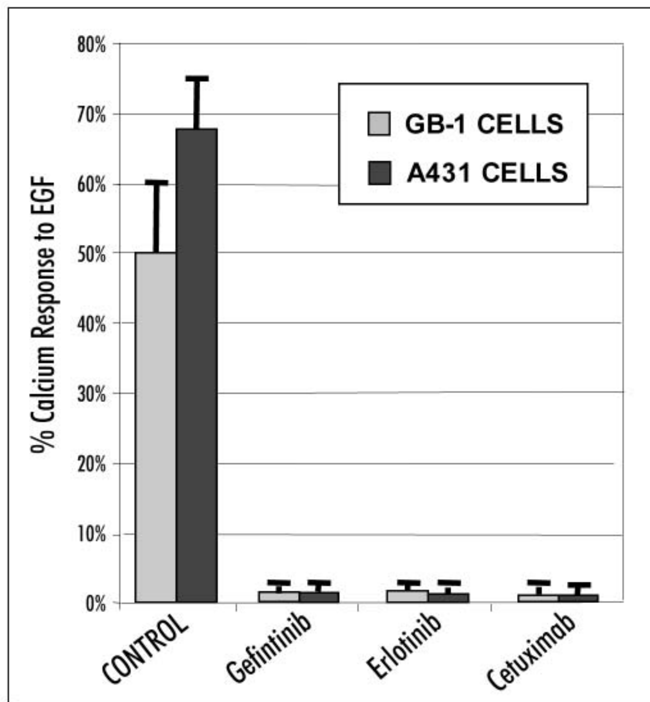

Figure 3. EGFR inhibitors block Ca^{2+} response to EGF. Bar graph shows the average percentage of GB-1 and A431 cells responding to bath application of EGF (200 ng/ml) with an increase in $[Ca^{2+}]_i$ (n = 180 cells in three experiments for each condition). Error bars represent standard deviation. The Ca^{2+} response to EGF was nearly abolished by gefitinib, erlotinib, and cetuximab (p<0.005 for each condition).

We also examined the effects of multiple inhibitors of EGFR on the Ca^{2+} response to EGF. The protein tyrosine kinase inhibitor tyrphostin 51 (10 μM n=120 cells in three experiments, data not shown) did not suppress the Ca^{2+} response to EGF. By contrast, the EGFR-specific PTK inhibitors erlotinib (5 μM) and gefitinib (5 μM) both abolished the acute Ca^{2+} signaling response to EGF. The EGFR-specific antibody cetuximab (100 μM) also completely abolished the Ca^{+2} response to EGF (Fig. 3).

EGF Mediates a Sustained Potentiation of Intercellular Ca^{2+} Signaling. Transient exposure to EGF resulted in a sustained increase in the propagation of intercellular Ca^{2+} waves. In cells treated with EGF for 5 minutes, the extent of propagation of intercellular Ca^{2+} waves evoked by mechanical stimulation of a single cell was markedly increased compared with untreated cells (Figs. 4 and 5). In GB-1 cells, transient EGF treatment resulted in a 156% increase in the average distance of Ca^{2+} wave propagation. In A431 cells, treatment with EGF resulted in a 77% increase in the average distance of Ca^{2+} wave propagation. The potentiation of intercellular Ca^{2+} waves persisted for up to 30 minutes following transient EGF exposure.

Since intercellular Ca^{2+} waves in glial cells involve release of ATP and activation of purinergic receptors.[32,37] we also investigated the effects of EGF on ATP-mediated Ca^{2+} signaling. We found that transient exposure to EGF also potentiated the Ca^{2+} signaling response to bath application of ATP (Fig. 6). An ATP concentration (0.5 μM) was chosen that evoked a Ca^{2+} response in only a small percentage of untreated cells. In cells treated with EGF for 5 minutes, the percentage of cells showing a Ca^{2+} response to ATP was markedly increased. This effect was observed up to 30 minutes after a 5 minute EGF exposure.

Although erlotinib and gefitinib both completely abolished the acute Ca^{2+} signaling response to EGF, they did not inhibit the potentiation of intercellular Ca^{2+} signaling in response to EGF. By contrast, cetuximab did inhibit the potentiation of Ca^{2+} waves by EGF (Fig. 5). Neither erlotinib nor gefitinib inhibited the potentiation of the response to ATP by EGF (Fig. 6). Cetuximab, however, abolished the sustained potentiation of the Ca^{2+} signaling response to ATP (Fig. 6). None of the compounds directly affected either intercellular Ca^{2+} waves or the response to ATP (not shown).

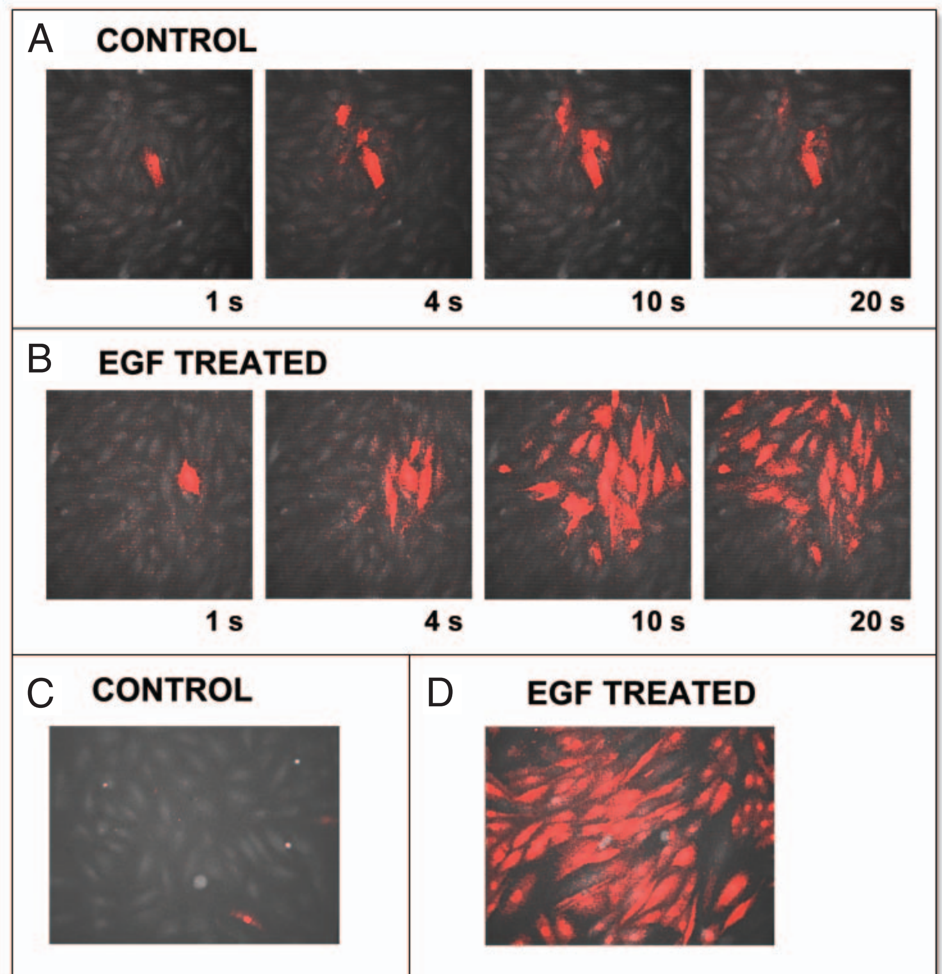

Figure 4. EGF potentiates intercellular Ca^{2+} waves and the response to ATP. (A) Image sequence shows an increase in $[Ca^{2+}]_i$ (indicated by red) in a field of GB-1 cells in response to mechanical stimulation of a single cell with a micropipette. The red color corresponds to a fluo-4 ΔF of 20–100. The response propagates from the stimulated cell to a few surrounding cells. (B) Sequence shows mechanically-evoked intercellular Ca^{2+} wave in a field of GB-1 cells 10 minutes after transient exposure to EGF (200 ng/ml) for 5 minutes. The extent of intercellular Ca^{2+} wave propagation is significantly increased compared with untreated controls. (C) Control response to a low concentration of ATP (500 nM) . The red color indicates the cells that show an increase in $[Ca^{2+}]i$ in response to ATP. Only a few cells show a response to ATP at this concentration. (D) Ca^{2+} response to ATP 10 minutes following transient exposure to EGF (200 ng/ml) for 5 minutes. After exposure to EGF, the majority of cells show a Ca^{2+} response to EGF. PTK inhibitors and EGFR inhibitors had different effects on the potentiation of Ca^{2+} signaling.

DISCUSSION

These studies show that EGF activates multiple distinct Ca^{2+} signaling pathways on different time scales in glioma cells and A431 cells. EGF-evoked Ca^{2+} signaling mediated by activation of PLCγ has been characterized in detail in other cell types.[38-41] The acute response to EGF in glioma cells is consistent with this mechanism. The absence of a requirement for extracellular Ca^{2+}, and the inhibition of the response by the PLC inhibitor U73122 and by depletion of intracellular Ca^{2+} stores with thapsigargin are consistent with PLC-dependent release of Ca^{2+} from intracellular stores. The delay in the response following exposure to EGF is also consistent with a process involving phosphorylation of PLC as opposed to a G-protein mediated response (such as the response to ATP) which occurs immediately upon exposure to the ligand. The response to EGF is also more sustained that that observed in response to activation of G-protein coupled receptors, and was observed to continue even after washout of the ligand. This extended duration of the response to EGF may be involved in its activation of a variety of cellular processes.

We also observed a distinct sustained response to EGF that includes increased intercellular Ca^{2+} signaling and an increased sensitivity of the Ca^{2+} response to activation of purinergic receptors with ATP. Intercellular Ca^{2+} waves in glial cells are mediated in part by release of ATP and activation of purinergic receptors on neighboring cells.[32,37] The increased sensitivity to ATP indicates that the sustained increase in intercellular Ca^{2+} waves in glioma cells in response to EGF involves a potentiation of purinergic extracellular signaling. The increased response to ATP could be explained either by a specific upregulation of purinergic receptors, or by a general increase in the signaling response generated by binding of ATP to its receptor.

The differential effects of EGFR inhibitors indicates that the acute Ca^{2+} signaling response involves a signaling pathway that is

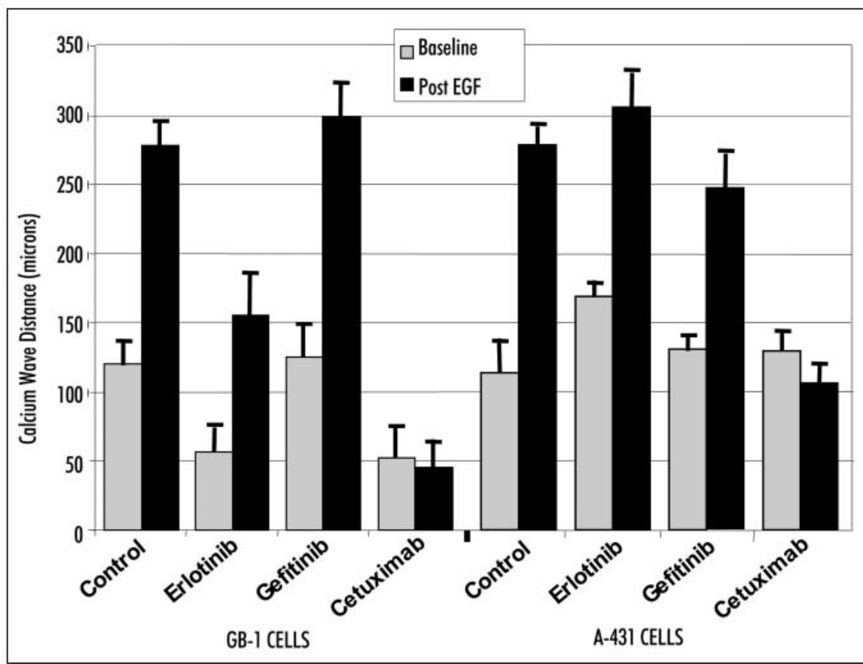

Figure 5. EGF potentiation of intercellular Ca^{2+} waves—differential effect of EGFR inhibitors. Bars represent the average distance of propagation of Ca^{2+} waves in human glioma (GB-1) cells and A431 cells in response to mechanical stimulation of a single cell. Transient exposure to EGF (200 ng/ml) for 5 minutes resulted in a sustained increase in the extent of propagation of intercellular Ca^{2+} waves (n = 10 experiments for cell type, p<0.005). This effect was blocked by cetuximab (n = 10, no significant difference before and after EGF), but not by erlotinib (n = 10, p<0.005) or gefitinib (n = 10, p<0.05). Error bars represent standard deviation.

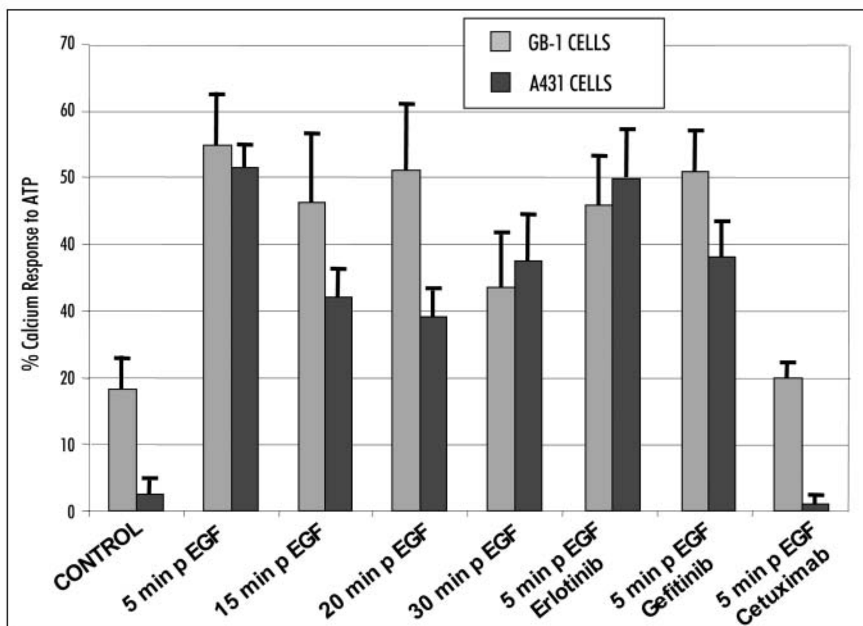

Figure 6. EGF potentiation of response to ATP—differential effect of EGFR inhibitors. Bars represent the percentage of GB-1 cells and A431 cells responding to 500 nM ATP with an increase in $[Ca^{2+}]_i$. Transient exposure to EGF (200 ng/ml for 5 minutes) resulted in a sustained increase in the percentage of cells responding to ATP. This increase was blocked by cetuximab but not by erlotinib or gefitinib in both cell types. (n = 6 experiments for each condition, 60 cells per experiment). All values were significantly different from control (p<0.005), except for C225-treated cells, which were not significantly different from control.

distinct from the sustained Ca^{2+} signaling response. Erlotinib blocked phosphorylation of EGFR and completely blocked the acute Ca^{2+} response to EGF. However, neither erlotinib nor gefitinib blocked the sustained increase in intercellular Ca^{2+} signaling or potentiation of the response to ATP observed following transient EGF exposure. By contrast, the EGFR specific antibody cetuximab blocked both the acute and sustained responses to EGF. These results indicate that binding of EGF to its receptor activates a sustained increase in purinergic intercellular Ca^{2+} signaling that does not require either phosphorylation of two primary EGFR phosphorylation sites or acute PLCγ-mediated Ca^{2+} signaling.

A dissociation of autophosphorylation of EGFR and signaling through AKT and ERK has been observed in response to low concentrations of EGFR inhibitors.[42] It is therefore possible that this pathway was involved in the sustained potentiation of the purinergic intercellular signaling. However, the concentration of the erlotinib and gefitinib used in these studies (5 μM) were relatively high, and have been shown to inhibit AKT and ERK signaling in A431 cells (data not shown) as well as other preparations.[42,43]

The mechanism of the sustained response to EGF is not clear at present. One question that arises is whether the potentiation of the Ca^{2+} signaling response to ATP indicates a specific upregulation of purinergic receptor signaling vs. a generalized increase in G protein coupled receptor mediated signaling. Our preliminary studies indicate that Ca^{2+} signaling evoked by bradykinin in GB-1 cells is not potentiated by EGF treatment, indicating that the sustained response to EGF may be specific to purinergic receptors.

Activation of G protein-coupled receptors has been shown to potentiate EGFR-mediated signaling[38,44,45] through multiple mechanisms. Conversely, activation of EGFR has been reported to potentiate G-protein coupled receptor mediated signaling in astrocytes via the MAP kinase pathway.[46] Acute application of EGF has also been observed to increase the duration and amplitude of injury associated Ca^{2+} waves in epithelial cells.[47]

The functional consequences of the acute and sustained responses to EGF are not certain. Oscillatory Ca^{2+} signaling has been associated with both cell migration and cell proliferation.[46,48–51] PLC gamma signaling has been reported to be specifically involved in tumor cell migration and invasion as opposed to proliferation.[52,53] However, depletion of intracellular Ca^{2+} also blocks the proliferative response to EGF.[49] Different spatial and temporal patterns of Ca^{2+}

signaling may be important in determining the specificity the functional cellular response.

Purinergic receptor mediated signaling modulates both proliferation and migration in glial cells and other cells.[54–57] Knockout of CD39 (Nucleoside triphosphate diphosphohydrolase-1 or NTPDase1), the major ectonucleotidase of endothelial cells, has been reported to inhibit both cellular migration and angiogenesis,[57] indicating an important role for purinergic receptor signaling in these processes. EGFR's sustained effects on purinergic signaling could therefore be involved in its effects on migration, as well as on angiogenesis.

Agents targeting EGFR are a focus of intense interest as therapies for cancer. Ca^{2+} signaling responses to EGF represent an efficient physiological assay for screening of ligands that affect EGFR mediated signaling. The results described here also identify a sustained effect of EGF on Ca^{2+} signaling that has not been previously characterized. The insensitivity of this sustained effect to erlotinib and gefitinib indicates that it does not involve the same tyrosine kinase mediated pathway as has been described for the acute Ca^{2+} signaling response to EGF. An increased understanding of this sustained intercellular signaling in response to EGF may lead to a new understanding of the role of EGF in tumor cell proliferation and migration, and may identify new therapeutic approaches to gliomas and other tumors.

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