Epidermal Growth Factor Stimulates the Rapid Accumulation of Inositol (1,4,5)-Trisphosphate and a Rise in Cytosolic Calcium Mobilized from Intracellular Stores in A431 Cells*

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Exposure of A431 human epidermoid carcinoma cells to epidermal growth factor (EGF), bradykinin, and histamine resulted in time- and concentration-dependent accumulation of the inositol phosphates (InsP) insitol monophosphate, inositol bisphosphate, and inositol trisphosphate (InsP3). Maximal concentrations of EGF (316 ng/ml; ~50 nM), bradykinin (1 μM), and histamine (1 mM) resulted in 3-, 6-, and 3-fold increases, respectively, in the amounts of inositol phosphates formed over a 10-min period. The Kₐₜ values for stimulation were approximately 10 nM, 3 nM, and 10 μM for EGF, bradykinin, and histamine, respectively. EGF and bradykinin stimulated the rapid accumulation of the two isomers of InsP₃, Ins(1,3,4)P₃, and Ins(1,4,5)P₃ as determined by high performance liquid chromatography analysis; maximal accumulation of Ins(1,4,5)P₃ occurred within 15 s. EGF and bradykinin also stimulated a rapid (maximal levels attained within 30 s after addition of hormone) and a sustained 4- and 6-fold rise, respectively, in cytosolic free Ca²⁺ levels as measured by Fura-2 fluorescence. EGF and bradykinin also produced a rapid, although transient, 3- and 5-fold increase, respectively, in cytosolic free Ca²⁺ after chelation of extracellular Ca²⁺ with 3 mM EGTA. These data are consistent with the idea that EGF elevates intracellular Ca²⁺ levels in A431 cells, at least in part, as a result of the rapid formation of Ins(1,4,5)P₃ and the consequential release of Ca²⁺ from intracellular stores.

The EGF receptor is a single-chained, 170,000-dalton transmembrane glycoprotein that possesses intrinsic tyrosine-specific protein kinase activity (1–5). Activation of the EGF receptor initiates a complex series of events that includes the phosphorylation of several protein substrates (6, 7) including autophosphorylation of the receptor (2), a rapid internalization of the EGF-receptor complex (8, 9), and mitogenesis (10). Because the products of many oncogenes code for tyrosine kinases, including the v-erb B oncogene which codes for a truncated version of the EGF receptor (3), many studies have focused on understanding the relationship between tyrosine kinase activity and the control of cell growth and proliferation. However, other biochemical events may be important in the action of EGF. For example, activation of EGF receptors on A431 human epidermoid carcinoma cells has been reported to result in an increased rate of radiolabeling of plasma membrane phosphoinositides (11, 12), including PIP and PIP₃ (13), the production of diacylglycerol (13), the activation of protein kinase C (14), the uptake of Ca²⁺ (11, 12), a rapid but transient rise in cytoplasmic Ca²⁺ (15), and the activation of an amelioride-sensitive Na⁺/H⁺ exchange (13, 16).

Hormone-stimulated rises in intracellular Ca²⁺ concentration occur either by influx of extracellular Ca²⁺ and/or by release of stored Ca²⁺ from intracellular organelles. Only limited information is available on the molecular mechanisms that serve to link the activation of hormone receptors to the influx of extracellular Ca²⁺. In contrast, understanding of the molecular events underlying hormone-mediated mobilization of Ca²⁺ from intracellular sources has been advanced markedly in recent years. Stimulation of a variety of hormone receptors results in the rapid hydrolysis of plasma membrane PIP₂ to form the two second messengers Ins(1,4,5)P₃ and diacylglycerol (17). Ins(1,4,5)P₃ has been shown in many tissues to release Ca²⁺ from intracellular stores, most importantly the endoplasmic reticulum (17). The occurrence of a second InsP₃ isomer, Ins(1,3,4)P₃, has been demonstrated recently (18–20), although the cellular actions, if any, of this compound have not been defined.

Several recent reports provide evidence that activation of EGF receptors does not stimulate a measurable accumulation of inositol phosphates in BALB/c 3T3 fibroblasts (21) or a measurable change in the radiolabeling of polyphosphoinositides in rat hepatocyte membranes (22). However, data have been reported that suggest that EGF stimulates the hydrolysis of plasma membrane phosphoinositides to form inositol phosphates in A431 cells (11–14), although this response has not been shown directly. To this end, we have initiated studies to determine whether EGF as well as other Ca²⁺ mobilizing...
hormones stimulate the formation of inositol phosphates in A431 cells and to provide a detailed characterization of the inositol phosphates formed. We report that EGF, bradykinin, and histamine stimulate the rapid accumulation of InsP$_3$, InsP$_5$, and InsP$_2$ in A431 cells in a time- and concentration-dependent manner. Furthermore, data are presented to show that EGF and bradykinin stimulate the rapid but transient accumulation of both InsP$_3$, InsP$_5$, and InsP$_2$ (1,3,4,5)$_2$P, and that the observed increases in inositol phosphate levels in response to EGF and bradykinin are correlated with a rapid but transient rise in cytoplasmic Ca$^{2+}$ in the absence of extracellular Ca$^{2+}$. These data are consistent with the idea that EGF stimulates the formation of Ins(1,4,5)$_2$P to release Ca$^{2+}$ from intracellular stores in A431 cells.

**EXPERIMENTAL PROCEDURES**

**Materials**—Dubcco’s modified Eagle’s medium, Eagle’s minimal essential medium, and fetal calf serum were purchased from Grand Island Biological Co. (Grand Island, NY). EGF was prepared from mouse mammary glands as described previously (22). Bradykinin was purchased from Boehringer Mannheim, and orthophosphoric acid (HPLC grade) was purchased from American Radiolabeled Chemicals, Inc. (St. Louis, MO), and myo-[H]$^3$inositol, 1,4,5-trisphosphate (1 Ci/mmol) was obtained from Amersham Corp. A431 epidermoid carcinoma cells were obtained from the Lineberger Cancer Research Center Tissue Culture Facility at the University of North Carolina, Chapel Hill.

**Cell Cultures**—A431 human epidermoid carcinoma cell stock cultures were maintained at 37°C in an 8% CO$_2$, humidified atmosphere in Dulbecco’s modified Eagle’s medium containing high glucose (4500 mg/l) supplemented with 5% fetal calf serum and antibiotics. All stock plates of cells were confluent and 6–8 days old at the time of subculture. Subculture was accomplished by aspirating the medium and detaching the cells by the addition of 3 mM EGTA in isotonic NaCl-citrate buffer (pH 7.8) for 30 min. Cells were seeded at a 1:5 time course of agonist-stimulated accumulation of inositol phosphates as described previously (22). Samples were injected into a Waters HPLC system equipped with a Whatman Partisil SAX 10 (4.6 x 250 mm) anion exchange column equilibrated with H$_2$O and maintained at a flow rate of 1.25 ml/min. ATP, ADP, and AMP (5 nmol of each) were used as UV markers. After the sample was injected, a linear gradient from ammonium formate (4000 x g) and the trichloroacetic acid was extracted from each sample by three washes with 2 ml of diethyl ether. Residual ether was removed by aspiration and exposure to nitrogen gas. Samples used for HPLC analysis of inositol phosphates were frozen at −20°C and saved for analysis at a later time. [H]$^3$inositol-labeled inositol phosphates in the aqueous sample were determined by anion exchange chromatography as described previously (22).

**Determination of InsP$_3$ Isomers by HPLC—InsP$_3$ isomers were analyzed by the method of Irvine et al. (19) and as described previously with minor modifications (22, 30). EGF (100 ng/ml; 16 pM) or bradykinin (1 pM) was added to Eagle’s minimal essential medium-HEPES (25 mm) containing 5% fetal calf serum and antibiotics in the presence of extracellular Ca$^{2+}$. These data are consistent with the idea that EGF stimulates the formation of Ins(1,4,5)$_2$P to release Ca$^{2+}$ from intracellular stores in A431 cells.

**RESULTS**

EGF (100 ng/ml; ~17 nM), bradykinin (1 μM), and histamine (1 μM; data not shown) each stimulated a rapid rise in the level of total inositol phosphates, i.e., InsP$_3$ + InsP$_5$ + InsP$_2$, that was readily measurable within 15 s (Fig. 1). Inositol phosphate levels continued to rise for up to 10 min in the presence of each hormone (data not shown). EGF, bradykinin,
EGF-stimulated Accumulation of Inositol (1,4,5)-Trisphosphate

**FIG. 1.** Time course of accumulation of inositol phosphates (InsP) in the presence of EGF and bradykinin (BK). A431 cells were grown to confluency and incubated for 2 days with 2 μCi/ml [3H]inositol in inositol-free medium in the absence of serum. Cells were incubated with EGF (○), bradykinin (■), or vehicle (□, ▲) for the indicated times in the presence of 10 mM LiCl and were assayed for the accumulation of inositol phosphates as described under "Experimental Procedures." Each point represents the mean of three dishes ± S.E., and the data are representative of three experiments.

**FIG. 2.** Concentration-dependent formation of inositol phosphates (InsP) by EGF, bradykinin (BK), and histamine (HA) in A431 cells. A431 cells were grown to confluency and incubated for 2 days with 2 μCi/ml [3H]inositol in inositol-free medium in the absence of serum. Cells were treated with the indicated various concentrations of EGF (○), bradykinin (■), or histamine (▲) for 10 min in the presence of 10 mM LiCl and were assayed for the accumulation of inositol phosphates as described under "Experimental Procedures." The control level of radioactivity accumulated in the absence of hormone in the presence of 0.1% BSA over the same period was 992 ± 22 cpm; this value was subtracted from each value presented. Each point represents the mean of three determinations, and the data are representative of three experiments.

The present work demonstrates that in A431 epidermoid carcinoma cells, EGF and other Ca2+ mobilizing hormones stimulate the rapid formation of Ins(1,4,5)P3, and a concomitant rise in cytosolic Ca2+, apparently mobilized from intra-
EGF-stimulated Accumulation of Inositol (1,4,5)-Trisphosphate

**TABLE I**

<table>
<thead>
<tr>
<th>Hormone-stimulated accumulation of InsP,, InsP,, and InsP, in A431 cells</th>
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<tbody>
<tr>
<td>A431 cells were labeled overnight with 3 μCi/ml of [H]inositol in inositol-free medium in the absence of fetal calf serum. Cells were treated with vehicle (0.1% BSA), EGF, bradykinin, or histamine at the indicated concentrations for 5 min. Individual inositol phosphates were separated and collected by anion exchange chromatography as described under &quot;Experimental Procedures.&quot; The data are the mean ± S.E. of four determinations and are representative of three experiments. Initial (t = 0) levels of radioactivity, in cpm, for InsP,, InsP,, and InsP, were 1457 ± 187, 771 ± 5, and 1894 ± 90, respectively, and were subtracted from the values presented.</td>
</tr>
</tbody>
</table>

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<thead>
<tr>
<th>Addition</th>
<th>InsP, cpm ± S.E.</th>
<th>Control %</th>
<th>InsP, cpm ± S.E.</th>
<th>Control %</th>
<th>InsP, cpm ± S.E.</th>
<th>Control %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vehicle</td>
<td>1731 ± 280</td>
<td>100</td>
<td>1731 ± 280</td>
<td>100</td>
<td>1731 ± 280</td>
<td>100</td>
</tr>
<tr>
<td>EGF (500 ng/ml)</td>
<td>3239 ± 402</td>
<td>107</td>
<td>1277 ± 227</td>
<td>282*</td>
<td>117 ± 76</td>
<td>100</td>
</tr>
<tr>
<td>Histamine (1 mM)</td>
<td>3229 ± 238</td>
<td>185*</td>
<td>1373 ± 66</td>
<td>233*</td>
<td>708 ± 4</td>
<td>605*</td>
</tr>
<tr>
<td>Bradykinin (1 μM)</td>
<td>4654 ± 432</td>
<td>267*</td>
<td>1341 ± 240</td>
<td>296*</td>
<td>765 ± 45</td>
<td>655*</td>
</tr>
</tbody>
</table>

*The increase in inositol phosphate formation due to hormone was significantly greater than control with p < 0.05. Statistical analysis of the data was performed using a two-tailed Student's t test.

Fig. 3. Elution profile of InsP, isomers isolated from A431 cells. HPLC elution of [H]inositol phosphates and characterization of the radioactive peaks as Ins(1,4,5)P, and Ins(1,3,4)P, were as described under "Experimental Procedures." Data are presented as fractions collected at 0.3-min intervals. A431 cells were labeled for 2 days with 3 μCi/ml [H]inositol in inositol-free medium in the absence of serum. Top panel, initial (T = 0) amounts of [H] radioactivity in the cells were measured (O), or cells were treated with 300 ng/ml EGF for 15 s in the absence of LiCl ( ). Bottom panel, initial (t = 0) amounts of [H] radioactivity in the cells were measured (O), or cells were treated with 1 μM bradykinin (BK) for 15 s in the absence of LiCl ( ). The data are single determinations and are representative of seven experiments.

Fig. 4. Time course of EGF and bradykinin receptor-stimulated accumulation of Ins(1,3,4)Ps and Ins(1,4,5)Ps in A431 cells. Cells were labeled for 48 h with 3 μCi/ml [H]inositol in inositol-free medium in the absence of serum. The cells were incubated with 300 ng/ml EGF (A and B) or 1 μM bradykinin (C and D) for 5-320 s in the absence of LiCl and were assayed for InsP, isomers as described under "Experimental Procedures." The time-dependent accumulations are presented for Ins(1,4,5)Ps in the presence of EGF ( ) or vehicle ( ), and Ins(1,3,4)Ps in the presence of EGF ( ) or vehicle ( ). The data are the mean ± S.E. of four determinations and are representative of four experiments.

*The increase in inositol phosphate formation due to hormone was significantly greater than control with p < 0.05. Statistical analysis of the data was performed using a two-tailed Student's t test.

Cellular stores. This conclusion is based on the observations that EGF stimulates the accumulation of total inositol phosphates in a time- and concentration-dependent manner, stimulating the rapid accumulation of Ins(1,4,5)Ps and Ins(1,3,4)Ps, and stimulates a rapid, albeit transient, rise in cytosolic Ca++. Bradykinin produced a qualitatively similar response.

Previous findings with A431 cells indirectly suggested that EGF activates phospholipase C. Sawyer and Cohen (11) reported that EGF stimulated the incorporation of [3P] into phosphatidylinositol and produced a 10-fold increase in the [3P] labeling of phosphatidic acid. Consistent with these findings, Smith et al. (12) reported that EGF stimulated a 3- to 4-fold increase in [H]inositol-labeled phosphatidylinositol that was accompanied by a 60-70% increase in the levels of [14C]-labeled diacylglycerol. Macara (13) reported recently that EGF did increase the turnover of the polyphosphoinositides, PIP and PIP2, and phosphatidic acid as determined by nonequilibrium labeling with [3P]. In contrast to the current work, EGF had no apparent effect on inositol phosphate levels. However, the earliest time point measured was 2.5 min, and perhaps, based on the response to EGF observed in the current study, a measurable elevation could have been observed at earlier times of hormone challenge. Alternatively, a different clone of A431 cells may express a different response to EGF.

Several reports have demonstrated that EGF increases Ca++ fluxes and cytoplasmic Ca++ levels in A431 cells. Sawyer and Cohen (11) observed, and Macara (13) recently confirmed, that EGF stimulates the rapid uptake and accumulation of Ca++. These reports, however, did not further test the capac-
Fig. 5. Effect of extracellular Ca\(^{2+}\) on changes in cytosolic free Ca\(^{2+}\) levels in response to EGF and bradykinin in A431 cells. Cells were grown on glass coverslips (22-mm square, Corning) and loaded with Fura-2 as described under "Experimental Procedures." Individual cells were monitored for changes in Fura-2 fluorescence by digital fluorescence video microscopy and analyzed by digital video image processing as described under "Experimental Procedures." A, A431 cells were treated with 300 ng/ml EGF in the presence of 1.8 mM extracellular Ca\(^{2+}\) (C, n = 4), or extracellular Ca\(^{2+}\) was chelated by the addition of 3 mM EGTA (pH 7.4, 37°C) (●, n = 3); B, A431 cells were treated with 1 μM bradykinin in the presence of extracellular Ca\(^{2+}\) (C, n = 2), or extracellular Ca\(^{2+}\) was chelated by the addition of EGTA (●, n = 2). The mean (±S.E.) resting level of cytosolic Ca\(^{2+}\) was calculated to be 48 ± 3 nM (n = 11).

The mechanism(s) whereby the activation of hormone receptors results in the conversion of PIP\(_2\) to Ins\(_{1,4,5}P_3\) has not been delineated. Bradykinin and histamine have been shown to stimulate the accumulation of Ins\(_{1,4,5}P_3\) in several tissues, and recent work using washed membranes from 1321N1 human astrocytoma cells has shown that carbachol (31), bradykinin, and histamine\(^3\) regulate inositol phosphate formation in a guanine nucleotide-dependent manner. Studies with a variety of other tissues and hormone receptors also strongly suggest the involvement of a guanine nucleotide regulatory protein in hormone-stimulated activation of phospholipase C (32–35). Consistent with this idea is the recent observation of guanine nucleotide-stimulated formation of inositol phosphates in washed membrane preparations from A431 cells.\(^4\) The mechanism(s) whereby EGF and other peptide growth factor receptors that are tyrosine-specific protein kinases, such as the platelet-derived growth factor receptor, regulate the breakdown of PIP\(_2\) are undefined; to date, a guanine nucleotide regulatory protein has not been implicated. A431 cells should provide a useful model system for comparison of the mechanisms whereby EGF and other non-tyrosine kinase receptors regulate phosphoinositide metabolism and Ca\(^{2+}\) mobilization.

Acknowledgments—We are indebted to Elaine Lloyd for her excellent work in preparation of the manuscript. J. R. H. would also like to thank J. M. English for thoughtful discussion, patience, and constant support and encouragement.

REFERENCES

3. J. R. Hepler, T. K. Harden, and H. S. Earp, unpublished observations.
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