Epidermal Growth Factor–stimulated Phosphoinositide Hydrolysis in Cultured Rat Inner Medullary Collecting Tubule Cells

Regulation by G Protein, Calcium, and Protein Kinase C

Isaac Teitelbaum, Alexis Strasheim, and Tomas Berl
Department of Medicine, University of Colorado School of Medicine, Denver, Colorado 80262

Abstract

Epidermal growth factor (EGF) exhibits specific saturable binding to cultured rat inner medullary collecting tubule cells and stimulates inositol trisphosphate (IP_3) production by these cells in a dose-dependent fashion. EGF-stimulated IP_3 production is enhanced by GTPγs or AlF_4^- and is inhibited by GDPβs or pertussis toxin. Alterations in extracellular Ca^{2+} have no effect on either basal or EGF-stimulated IP_3 production. Similarly, treatment with EGTA which decreases cytosolic Ca^{2+} is without effect. In contrast, treatment with ionomycin which increases cytosolic Ca^{2+} has no effect on basal IP_3 production but enhances the response to EGF. Activation of protein kinase C inhibits IP_3 production in response to either EGF or AlF_4^-. These studies demonstrate the occurrence of EGF-stimulated phospholipase C activity in the rat inner medullary collecting duct. Stimulation by EGF is transduced by a pertussis toxin–sensitive G protein, unaffected by alterations in extracellular Ca^{2+}, insensitive to a decrement in cytosolic Ca^{2+}, enhanced by an increase in cytosolic Ca^{2+}, and inhibited by protein kinase C. (*J. Clin. Invest.* 1990. 85:1044–1050.) epidermal growth factor • G protein • phospholipase C • protein kinase C • RIMCT cells • calcium

Introduction

Epidermal growth factor (EGF)[1] is a 53 amino acid peptide with potent mitogenic effects on many cell types (1). In addition, it has been demonstrated to exert effects apparently unrelated to its mitogenicity such as inhibition of gastric acid secretion and vasodilatation (2, 3). EGF has been demonstrated to stimulate phospholipase C (PLC) in several cell types, including a hepatocellular carcinoma line (4) and the A431 cell (5), a cell line that overexpresses the EGF receptor.

Studies aimed at assessing the ability of EGF to stimulate PLC in renal tissues have heretofore not been rewarding. EGF fails to stimulate PLC in either renal cortical slices (6) or glomerular mesangial cells (7). Yet it has been shown that EGF, via activation of protein kinase C, modulates the response to vasopressin in both rabbit cortical collecting tubule (8) and cultured rat inner medullary collecting tubule (RIMCT) cells (9). The present study was therefore undertaken to determine whether EGF is capable of stimulating PLC activity in cultured RIMCT cells and to examine the roles of guanine nucleotides, extra- and intracellular calcium, and protein kinase C in regulating EGF-stimulated phosphoinositide hydrolysis in this tissue.

Methods

Cell culture. RIMCT cells were prepared as previously described (10). However, to ensure that all studies were performed on confluent monolayers of cells, the wells were plated at three times the usual density.

^{125}I-EGF binding. ^{125}I-EGF (~ 1,000 Ci/mmol) was purchased from ICN Radiochemicals (Irvine, CA). Studies were performed in PBS at 4°C. Confluent monolayers of RIMCT cells were prepared as previously described. At 5 d media was aspirated and the cells were washed three times with ice-cold PBS. Cells were then incubated in ice-cold PBS containing ~ 100,000 disintegrations per minute ^{125}I-EGF per well in the absence or presence of varying concentrations (10^{-11}–10^{-7} M) of unlabeled recombinant human EGF (Boehringer Mannheim Biochemicals, Indianapolis, IN). After 2 h, which had been shown in preliminary studies to be adequate for equilibrium binding, the buffer was aspirated. The cells were then washed four times in ice-cold PBS, solubilized in 1% SDS, and aliquots taken for gamma counting. Binding in the presence of 1 μM unlabeled EGF was considered nonspecific. Data were calculated and plotted using the ENZFITTER program (11).

Incubation with effectors. At 96 h the Ham's F12/Liebovitz L15 medium in which the cells were initially grown was aspirated and the cultures were washed twice with sterile PBS. The cells were then fed with inositol-free Dulbecco's MEM supplemented with [^{3}H]*myo*-2-inositol (5 μCi/well; Amersham Corp., Arlington Heights, IL). Studies were performed after 24 h of labeling, as this time was demonstrated to be sufficient for incorporation into the phospholipid pool. All studies were performed in PBS with the exception of those using guanine nucleotide analogues. As these studies require cell permeabilization, they were performed in a buffer designed to simulate the intracellular milieu (composition in millimoles: 20 NaCl, 100 KCl, 5 Mg sulfate, 1 NaH_2PO_4, 25 Na bicarbonate, 1 EGTA, pH 7.2) containing saponin (50 μg/ml). Such treatment of the cells does not interfere with their hormonal responsiveness (12).

Media was aspirated from the cells and the cells were washed twice with 500 μl PBS. The cells were then incubated for 15 min in PBS at the desired calcium concentration without or with desired test substances, e.g., aluminum fluoride, ionomycin (Calbiochem-Behring Corp., La Jolla, CA), or phorbol 12-myristate-13-acetate (PMA) (Sigma Chemical Co., St. Louis, MO). In studies using the protein kinase inhibitors (13), 1-(5-isoquinolinylsulfonyl)-2-methylpiperazine (H7) or *N*-(2-[methylamino]ethyl)-5-isoquinoline-sulfonamide (H8) (Calbiochem-Behring Corp.) there is a 5-min preincubation with these compounds alone, followed by a 15-min incubation with these compounds plus any other desired agents.

Address correspondence to Dr. Isaac Teitelbaum, C281, University of Colorado School of Medicine, 4200 E. 9th Ave., Denver, CO 80262.

Received for publication 3 May 1989 and in revised form 17 October 1989.

1. *Abbreviations used in this paper:* EGF, epidermal growth factor; IP_3, inositol trisphosphate; PLC, phospholipase C; RIMCT, rat inner medullary collecting tubule.

J. Clin. Invest.

0021-9738/90/04/1044/07 $2.00
Volume 85, April 1990, 1044–1050

Upon completion of all incubations the buffer was aspirated and replaced with 500 μl of PBS without or with purified murine EGF (Sigma Chemical Co.) or recombinant human EGF at the desired concentration. After exposure for the desired length of time the reaction was terminated by the addition of 500 μl of ice-cold 20% TCA. The cells were scraped off of the dish and centrifuged at 1,000 *g* for 10 min. The supernate containing inositol phosphates was washed four times with an equal volume of ether and stored at −20°C until analysis.

Determination of inositol phosphates. Inositol phosphates were separated by anion exchange chromatography as described by Berridge et al. (14). In brief, the samples were thawed and brought to a pH greater than or equal to 6.0 with 50 mM Tris base. They were then applied to Dowex 1x-8 colums (formate form). Serial elutions are performed with water, 5 mM sodium tetraborate/60 mM sodium formate, 0.1 M formic acid/0.2 M ammonium formate, 0.1 M formic acid/0.4 M ammonium formate, and 0.1 M formic acid/1 M ammonium formate, which elute, respectively, inositol, glyceryl phosphoryl inositol, inositol phosphate, inositol bisphosphate, and inositol trisphosphate (IP_3). The results are expressed as counts per minute of IP_3 per well. When grown to confluence, the protein content of the wells was 100±3.86 μg with interwell variation of < 11%.

Statistical analysis. Each well was considered an *n* of 1. Comparisons between two treatment groups were by the unpaired *t* test. Comparisons between three or more treatment groups were done by one-way analysis of variance followed by appropriate contrasts of a priori assumptions (15). In all circumstances $P < 0.05$ was considered significant. Data are presented as mean±SEM.

Results

^{125}I-EGF binding. EGF exhibits specific saturable binding to its receptor on RIMCT cells. Fig. 1 depicts the displacement of radiolabeled EGF by increasing concentrations of unlabeled peptide. The calculated K_d is 18.7±3.8 nM (n = 4).

Effect of EGF on IP_3 formation: dose response and time course. The dose-response curve and time dependence of EGF-stimulated IP_3 formation are depicted in Fig. 2. As seen in Fig. 2 *A*, at 1 and 5 nM EGF inositol phosphate formation is not higher than baseline. A significant increase is first detected at 10 nM EGF and the response increases further at 50 and 100 nM EGF. To assess the purity of the murine EGF used in this study, it was compared with recombinant human EGF; at both 5 nM (2,523±135 vs. 2,573±585 cpm/well) and 100 nM (4,067±641 vs. 3,740±761 cpm/well) the response was similar. As seen in Fig. 2 *B*, EGF-stimulated IP_3 formation was very rapid, increasing three- to fivefold within the first 10 s and gradually decreasing to baseline over the ensuing 2–5 min. Therefore, all subsequent studies were performed using murine EGF at a concentration of 100 nM with a 10-s exposure.

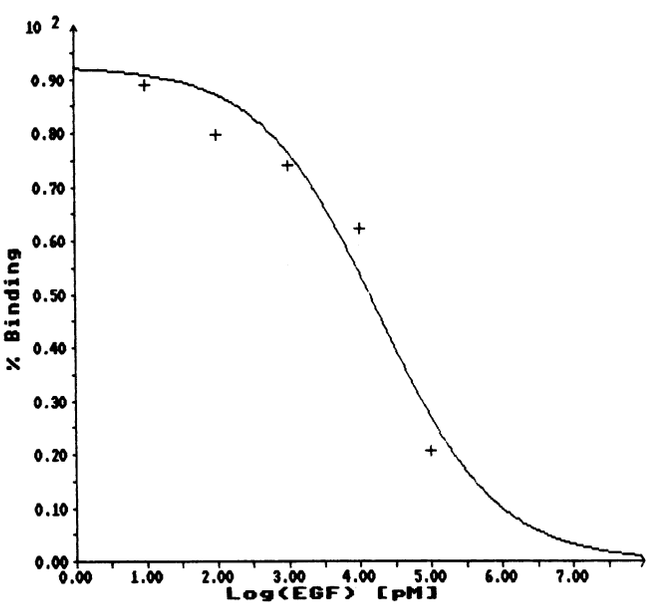

Figure 1. Displacement of ^{125}I-EGF by increasing concentrations of unlabeled recombinant human EGF (n = 4). The calculated K_d is 18.7±3.8 nM.

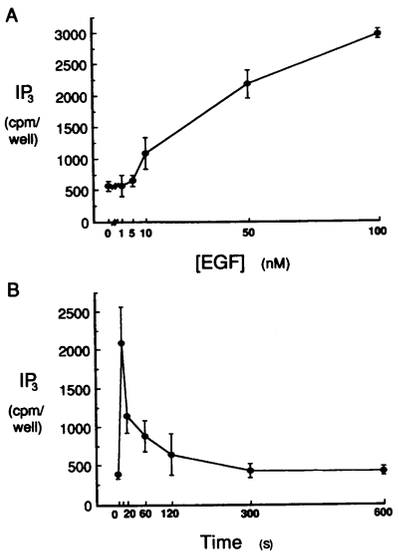

Figure 2. A, Dose-response curve for EGF-stimulated IP_3 production (n = 3). Cells were exposed to varying concentrations of EGF for 10 s. IP_3 production is first detected at 10 nM EGF. *B,* Time course of EGF-stimulated IP_3 production (n = 3). Cells were exposed to 100 nM EGF for varying lengths of time. IP_3 production peaks within 10 s and declines rapidly thereafter.

As EGF stimulates phospholipase A_2 in RIMCT cells (12) and arachidonic acid metabolites may themselves stimulate PLC (16, 17), care was taken to ensure that EGF-stimulated phosphatidylinositol 4,5-bisphosphate hydrolysis was not mediated via increased PGE_2. Over the range of 10^{-11}–10^{-5} M, PGE_2 did not stimulate IP_3 production in RIMCT cells (1,077±290 cpm/well, 10^{-5} M PGE_2 vs. 1,084±227, basal, n = 6, NS). Also, the cyclooxygenase inhibitor flurbiprofen (5 μM; Sigma Chemical Co.) which inhibits ionomycin-stimulated PGE_2 production by ~ 95% (64.87±12.69 vs. 3.98±1.79 pg/μg protein, n = 5, $P < .01$) has no effect on EGF-stimulated IP_3 production (5,615±645 vs. 4,505±610 cpm/well, n = 5, NS). Therefore, EGF stimulates phosphoinositide hydrolysis directly, not via an arachidonic acid metabolite.

Studies on the role of a guanine nucleotide-binding protein (G protein). The activity of G proteins is, by definition, subject to modulation by guanine nucleotide analogues. The activity of G proteins is enhanced by the GTP analogue, GTPγs, and is inhibited by the GDP analogue GDPβs (18). To assess whether the formation of IP_3 is modulated by a G protein, these analogues were used. As shown in Fig. 3, neither of these compounds affects basal IP_3 levels. However, exposure to 10 μM GTPγs results in marked enhancement of EGF-stimulated IP_3 production from 1,667±116 to 3,156±528 cpm per well (P < .05); conversely, exposure to GDPβs completely prevents EGF stimulation of IP_3 production, as the IP_3 level is 451±16, which is similar to the control value of 495±35, P = NS.

Aluminum fluoride has been shown to mimic the activity of GTPγs in several cell systems (19, 20). It has the additional

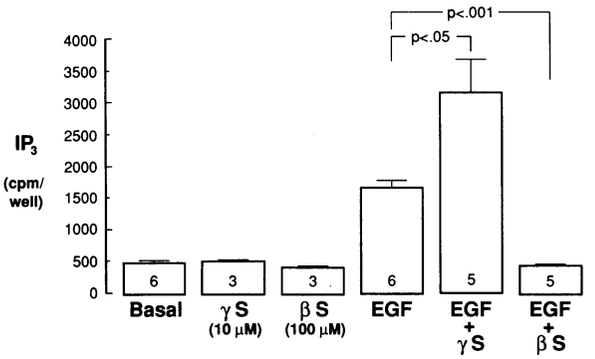

Figure 3. Effect of guanine nucleotides on basal and EGF-stimulated IP_3 production in saponin-permeabilized cells. γs = 10 μM GTPs; βs = 100 μM GDPβs. The number at the base of each bar is the n for that setting. Basal IP_3 levels are not affected by guanine nucleotides. EGF-stimulated IP_3 production is enhanced by γs and inhibited by βs.

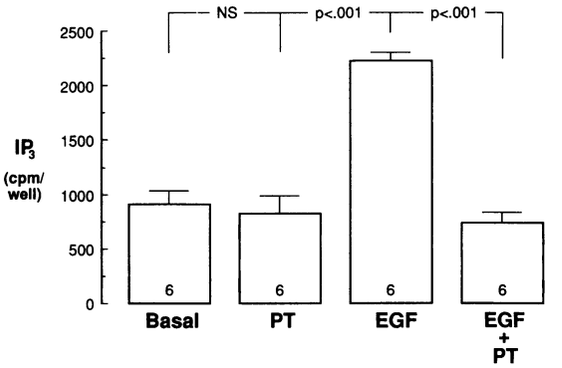

Figure 4. Effect of treatment with pertussis toxin (*PT;* 100 ng/ml for 16 h) on basal and EGF-stimulated IP_3 production (n = 6). PT has no effect on basal IP_3 levels but eliminates stimulation by EGF.

advantage of permeating the cell and can therefore be used in intact cells. As shown in Table I, a 15-min exposure to aluminum fluoride results in significant enhancement of both basal and EGF-stimulated IP_3 formation.

Having established that a G protein is involved in the transduction of EGF receptor-stimulated PLC activity, we sought to determine whether this G protein is pertussis toxin sensitive. We examined the ability of EGF to stimulate IP_3 formation in cells that had been treated overnight with pertussis toxin (100 ng/ml), which fully ADP-ribosylates susceptible G proteins (12). While pertussis toxin has no effect on basal IP_3 formation as shown in Fig. 4, it mimics the effect of GDPβs to eliminate stimulation by EGF.

Studies on the role of Ca^{2+}. Studies were performed to examine the potential effects of alterations in both extracellular and intracellular Ca^{2+} on EGF-stimulated PLC activity. The Ca^{2+} concentration of the PBS routinely used in generating inositol phosphate samples is 0.6 μM. To determine whether an elevation in extracellular Ca^{2+} could alter the effect of EGF, the concentration of the cation was elevated to 1 or 2 mM. As shown in Fig. 5, EGF-stimulated IP_3 production is unaffected by increasing the extracellular Ca^{2+} concentration to either a physiologic level of 1 mM or to a supraphysiologic level of 2 mM. Since this insensitivity to extracellular Ca^{2+} by no means precludes a sensitivity to alterations in cellular Ca^{2+}, we next assessed the effect of alterations in intracellular Ca^{2+}. Cytosolic Ca^{2+} was decreased by a 15-min exposure to 0.6 μM extracellular Ca^{2+} in the presence of 1 mM EGTA (10). To increase cytosolic Ca^{2+} we exposed cells for 15 min to an extracellular Ca^{2+} concentration of 1 mM in conjunction with 100 nM ionomycin. Neither of these maneuvers alone altered basal IP_3 production, as IP_3 levels were 854±112 cpm/well control, 655±74 (n = 4, NS) in the presence of EGTA, and 948±158 (n = 6, NS) in the presence of 1 mM Ca^{2+} + ionomycin. As shown in Fig. 6, decreasing cytosolic Ca^{2+} with EGTA has no effect on EGF-stimulated IP_3 production. However, increasing cytosolic Ca^{2+} by exposure to 1 mM extracellular Ca^{2+} in the presence of 100 nM ionomycin does result in significant potentiation of EGF-stimulated IP_3 production (1,672±110 vs. 3,389±529, n = 5, $P < 0.05$); this potentiation by ionomycin of the response to EGF is dependent on the entry of calcium from the extracellular compartment as it is not observed when ionomycin is used in the virtual absence of extracellular Ca^{2+} (Fig. 6).

Table I. Effect of AlF_4^- (25 mM NaF + 10 μM $AlCl_3$) on Basal and EGF-stimulated IP_3 Production

	Control	+AlF_4^-	*P* value
Basal (n = 9)	690±99	1,805±187	<0.001
EGF (n = 5)	1,467±62	2,677±368	<0.01

Studies on the role of protein kinase C. Several studies have suggested a role for protein kinase C to modulate the activity of PLC (21–24). To determine whether activation of protein kinase C is involved in the regulation of EGF-stimulated PLC activity in the inner medullary collecting duct, we assessed IP_3 production in the absence and presence of the phorbol ester, PMA (1 μM). As seen in Fig. 7, a 15-min preincubation with PMA completely eliminates subsequent EGF-stimulated IP_3 production; the structurally similar compound 4-α phorbol, which does not activate protein kinase C, has no effect on EGF-stimulated IP_3 production. PMA similarly inhibits AlF_4^--stimulated IP_3 production (6,522±525 vs. 894±297 cpm/well, n = 5, $P < 0.001$), suggesting a postreceptor site of inhibition.

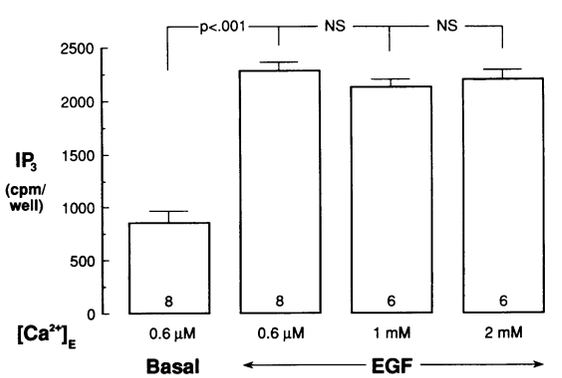

Figure 5. Effect of alterations in extracellular Ca^{2+} ($[Ca^{2+}]_E$) on EGF-stimulated IP_3 production. The number at the base of each bar is the n for that setting. EGF-stimulated IP_3 production is constant at $[Ca^{2+}]_E$ ranging from 0.6 μM to 2 mM.

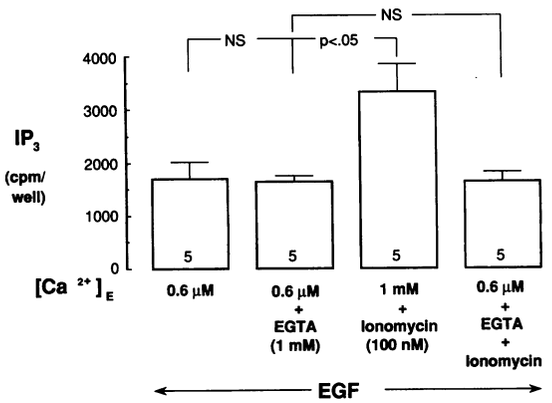

Figure 6. Effect of alterations in cytosolic Ca^{2+} on EGF-stimulated IP_3 production (n = 5). Decreasing cytosolic Ca^{2+} has no effect but increasing it potentiates the response to EGF.

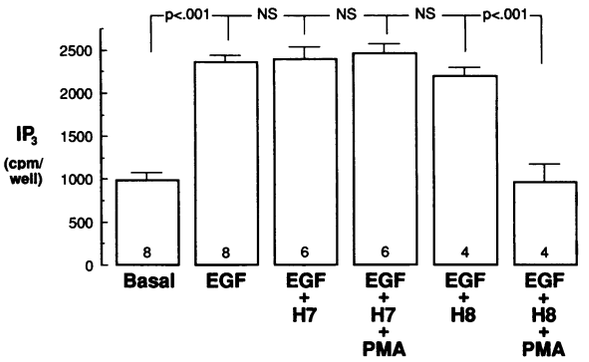

Figure 8. Effect of protein kinase inhibitors to prevent PMA inhibition of IP_3 production. The number at the base of each bar is the n for that setting. 1-(5-Isoquinolinylsulfonyl)-2-methylpiperazine (*H7*) and *N*-(2-[methylamino]ethyl)-5-isoquinoline-sulfonamide (*H8*) are both used at 50 μM. Inhibition by PMA is prevented by H7 but not by H8.

To further establish a role for protein kinase C in the modulation of EGF-stimulated PLC activity, we examined the ability of relatively selective protein kinase inhibitors to prevent PMA-induced inhibition of EGF-stimulated IP_3 production. H7 is a relatively specific inhibitor of protein kinase C; H8 is a relatively specific inhibitor of cyclic nucleotide-dependent protein kinases (13). As seen in Fig. 8, treatment with H7 for 20 min before exposure to EGF has no effect on EGF-stimulated IP_3 production; yet, exposure to H7 for 5 min before and during the incubation with PMA completely prevents PMA-induced inhibition of the EGF signal. In contrast, exposure to H8, which also has no effect by itself, does not prevent subsequent inhibition by PMA.

Discussion

EGF is known to stimulate phosphoinositide hydrolysis in several tissues (4, 5). However, studies in renal tissues have failed to detect EGF-stimulated PLC activity in either glomerular mesangial cells (7) or rat cortical slices (6). While EGF has been reported to inhibit arginine vasopressin–stimulated water transport in the rabbit cortical collecting duct by activation of protein kinase C (8), the ability of EGF to promote phosphoinositide hydrolysis in the collecting duct has not been directly examined. The studies described herein are the first to clearly demonstrate EGF-stimulated phosphoinositide hydrolysis in cultured RIMCT cells.

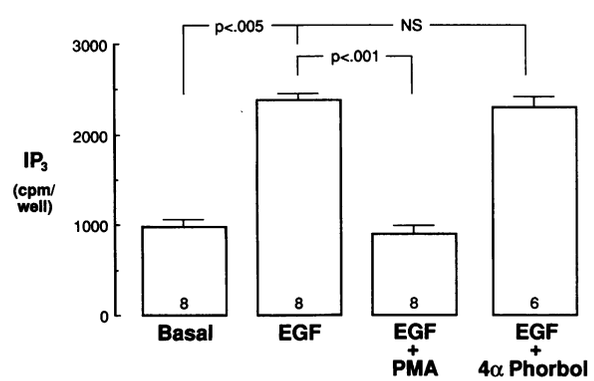

Figure 7. Effect of phorbol esters on EGF-stimulated IP_3 production. The number at the base of each bar is the n for that setting. PMA (1 μM) activates protein kinase C and inhibits IP_3 production; 4-α phorbol (1 μM), a structurally similar compound that does not activate protein kinase C, has no effect.

An IP_3 signal is first detectable at an EGF concentration of 10 nM and increases thereafter. The dose response is the same whether purified murine or recombinant human EGF is used. As seen in Fig. 1, maximal displacement of ^{125}I-EGF from its receptor requires ~ 100 nM unlabeled EGF. The calculated K_d of 18.7 nM is similar to that of the low affinity binding site reported in several tissues (25–27), including a preliminary report in the rat renal papillary collecting duct (28). The further increase in IP_3 production at concentrations of EGF significantly higher than the K_d may reflect the fact that equilibrium binding is not achieved during the very short exposure time (10 s) used in this study. The time course of EGF-stimulated IP_3 production (Fig. 2 *B*) is extremely rapid, with IP_3 production peaking within 10 s and falling swiftly thereafter, returning to a level indistinguishable from baseline at 2 min.

Recent studies from this laboratory have demonstrated that EGF-stimulated phospholipase A_2 activity is transduced by a guanine nucleotide-binding regulatory (G) protein (12). The present experiments reveal that a G protein is also involved in the transduction of receptor-stimulated phospholipase C activity. This is demonstrated by the observation that EGF-stimulated PLC activity in RIMCT cells is enhanced by GTPγs (Fig. 3) or AlF_4^- (Table I) and is inhibited by GDPβs (Fig. 3). It should be noted that while GTPγs has no effect on basal IP_3 production, AlF_4^- does. This probably reflects the fact that fluoroaluminates are capable of complexing with the GDP bound form of G proteins, whereas binding of GTPγs requires release of the previously bound GDP; this release occurs only upon receptor activation (29). While there is precedent for a role for G proteins in EGF receptor-stimulated PLC activity in other cell types, this is the first such demonstration in renal epithelial cells.

The G protein that transduces stimulation of PLC has been shown to serve as a substrate for pertussis toxin–catalyzed ADP-ribosylation in many (30, 31) but not all (32, 33) systems. In contrast to EGF-stimulated PLC activity in the A431 cell line, which is not inhibited by pertussis toxin (34), the G

protein described herein is pertussis toxin sensitive (Fig. 4). As pertussis toxin ADP-ribosylates only a single 41-kD protein in plasma membranes prepared from RIMCT cells (12), the $G\alpha$ subunit responsible for transducing EGF-stimulated phosphoinositide hydrolysis is presumed to be an α_i similar (or identical) to those previously described (18).

Although PLC is generally recognized as a Ca^{2+}-stimulatable enzyme (35), it exhibits a broad range of Ca^{2+} sensitivities in varying tissues. The role of Ca^{2+} in PLC activity in renal cells has not been previously investigated. In cultured RIMCT cells both basal and EGF-stimulated PLC activity are insensitive to alterations in extracellular Ca^{2+} ranging from 600 nM to 2 mM (Fig. 5). Similarly, chelation of extracellular Ca^{2+} with a resulting fall in cytosolic Ca^{2+} (10) has no effect on either basal or EGF-stimulated IP_3 levels. In fact, stimulation of IP_3 production by EGF in saponin-permeabilized cells (Fig. 3) when ambient Ca^{2+} is only 10 nM (12) suggests that PLC has no absolute requirement for Ca^{2+}. The occurrence of significant phosphoinositide hydrolysis in the presence of exceedingly low Ca^{2+} suggests that renal inner medullary PLC resembles the type I form of brain and liver (36). Increasing cytosolic Ca^{2+} by exposure to ionomycin has no effect on basal IP_3 levels but potentiates the response to EGF. This contrasts with the observations of Wahl et al. (5) in A431 cells in which A23187 inhibited the response to EGF. However our results are in concert with most studies that have demonstrated Ca^{2+} sensitivity of PLC (35, 37–39). Ionophores have cellular effects other than increasing cell Ca^{2+}, such as alterations in cell pH. The failure to observe potentiation of the EGF effect by ionomycin in the absence of extracellular Ca^{2+} strongly suggests that the observed effect of the ionophore is in fact due to its Ca^{2+} mobilizing action.

Activation of protein kinase C has been shown to provide negative feedback control to inhibit further receptor-stimulated phosphoinositide hydrolysis in many (40–43), but not all, tissues (44). A potential role for protein kinase C in the regulation of PLC has not been investigated in renal epithelial cells. In RIMCT cells, exposure to the protein kinase C–stimulating phorbol ester, PMA, but not the inactive analogue, 4-α phorbol, inhibits EGF-stimulated IP_3 production. This inhibition is prevented by pretreatment with the relatively specific C-kinase inhibitor, H7, but not by H8, which is more specific for cyclic nucleotide-dependent kinases.

The manner in which activation of protein kinase C decreases EGF-stimulated IP_3 accumulation in RIMCT cells is not known. Possible mechanisms include: phosphorylation of the EGF receptor which, in other systems, has been shown to decrease both the ligand binding and tyrosine kinase activity of the receptor (45); phosphorylation of the G protein involved in transduction of the signal (46, 47); or phosphorylation of PLC itself (48). The observation that activation of protein kinase C also inhibits AlF_4^--stimulated phosphoinositide hydrolysis argues strongly for a postreceptor site of inhibition. Alternatively, IP_3 accumulation could decrease due to phosphorylation of IP_3 5′-phosphomonoesterase which has been shown to increase the activity of the phosphatase (49), thereby accelerating degradation of IP_3. The observation that inositol monophosphate and inositol bisphosphate are both slightly decreased after exposure to PMA (data not shown) argues against the latter possibility.

The mRNA for prepro EGF is found in abundance in the thick ascending limb and early distal convoluted tubule of the murine kidney (50). Immunocytochemical studies have localized EGF to the apical membrane of these nephron segments (51). The stimulus to secretion or release of EGF and the manner in which it is presented to more distal segments of the nephron (i.e., luminal or peritubular membrane) are unknown. It is noteworthy, however, that specific EGF binding has recently been demonstrated in the rabbit cortical collecting duct (8) and has been reported in preliminary fashion in the rabbit outer medullary (52) and cultured rat inner medullary collecting ducts as well (28). Binding of EGF to its receptor has been reported to inhibit the hydroosmotic response to AVP in the rabbit cortical collecting duct at a post-cAMP site (8). Inhibition of protein kinase C eliminated the effect of EGF, but the ability of EGF to promote phosphoinositide hydrolysis and thereby stimulate protein kinase C was not assessed. Apart from a post-cAMP site of inhibition, studies from this laboratory have demonstrated that EGF, via stimulation of protein kinase C, eliminates AVP-stimulated cAMP generation in the cultured rat inner medullary collecting duct (9). It is apparent, therefore, that EGF-stimulated phosphoinositide hydrolysis plays an important role in the modulation of the hydroosmotic response to vasopressin. This study is the first to describe some of the factors involved in regulating this important signal transduction process.

In summary, EGF-stimulated PLC activity is transduced by a pertussis toxin–sensitive G protein. Stimulation of PLC by EGF does not require and is insensitive to alterations in extracellular Ca^{2+}. While decreasing cytosolic Ca^{2+} is without effect, increasing intracellular Ca^{2+} markedly enhances the response to EGF. EGF-stimulated PLC activity is inhibited upon activation of protein kinase C. The mechanism of this inhibition will require further investigation.

Acknowledgments

The authors would like to thank Ms. Linda M. Benson for expert secretarial assistance.

This work was supported by National Institutes of Health grants DK-38464 and PPG DK-19928.

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