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Cyclic Adenosine Monophosphate and Diacylglycerol
Mutually Inhibitory Second Messengers in Cultured Rat Inner Medullary Collecting Duct Cells

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Abstract

Studies were performed to examine interactions between the adenyl cyclase (AC) and phospholipase C (PLC) signaling systems in cultured rat inner medullary collecting duct cells. Stimulation of AC by either arginine vasopressin (AVP) or forskolin or addition of exogenous cAMP inhibits epidermal growth factor (EGF)-stimulated PLC. This inhibition is mediated by activation of cAMP-dependent kinase as it is prevented by pretreatment with the A-kinase inhibitor, N-[2-(methylamino)ethyl]-5-isoquinoline-sulfonamide (H8) but not by the C-kinase inhibitor, 1-(5-isoquinolylsulfonfyl)-2-methylpipеразине (H7). Exposure to EGF eliminates AVP-stimulated cAMP generation. This is not mediated by a cGMP-dependent protein kinase C as it is observed even in the presence of the cGMP-dependent protein kinase C, furbiprofen. Inhibition by EGF is not due to an increase in inositol trisphosphate (IP3) as exposure of saponin-permeabilized cells to exogenous IP3 is without effect. Inhibition by EGF is prevented by pretreatment with the C-kinase inhibitor, H7, but not by the A-kinase inhibitor, H8. Exposure to the synthetic diacglycerol (DAG), dioctanoylglycerol, also inhibits AVP-stimulated AC activity; therefore, inhibition by EGF is due to activation of protein kinase C. Thus, in cultured rat inner medullary collecting duct cells, cAMP and DAG function as mutually inhibitory second messengers with each impairing formation of the other. (J. Clin. Invest. 1990. 86:46-51.) Key words: adenyl cyclase • phospholipase C • protein kinase c • AVP • EOY • inner medullary collecting duct

Introduction

One of the major functions of the collecting duct is the stimulation of adenyl cyclase (AC) in response to arginine vasopressin (AVP). The ensuing increase in cAMP is responsible for mediating the enhanced hydraulic conductivity of the apical membrane. Recent studies from this laboratory have demonstrated that in addition to AC activity, cultured inner medullary collecting duct cells also exhibit hormonal stimulation of phospholipase C (PLC) activity upon exposure to epidermal growth factor (EGF) (1).

It is now well established that AC and PLC are not autonomous in their regulation of cellular events; rather, these two signaling systems may interact in either positive or negative fashion. For example, cAMP has been shown to inhibit phosphoinositide hydrolysis in platelets (2) and neutrophils (3), but it has no effect in monocytes (4). Conversely, activation of protein kinase C, one of the major consequences of phosphoinositide hydrolysis, has been reported to inhibit hormone-stimulated AC activity in some tissues (5-8), while it potentiates AC activity in other tissues (9-12).

Potential interactions between the AC and PLC signaling systems in renal tissues have not been extensively examined. cAMP has been shown to modestly attenuate α1-adrenergic-stimulated PLC activity in renal cortical slices (13), however, the cell responsible was not identified. Furthermore, the other signaling limb, i.e., the effect of stimulation of PLC on cAMP generation, was not examined. Similarly, while activation of protein kinase C has been shown to inhibit AVP-stimulated water flow in the toad bladder (14) and in the rabbit cortical collecting duct (15), in neither tissue has the effect of cAMP on hormonally stimulated PLC activity been examined. Accordingly, the present study was undertaken to examine bidirectional interactions between the AC and PLC signaling systems in a single cell type, the cultured rat inner medullary collecting tubule (RIMCT) epithelial cell.

Methods

Cell culture. Cultures of RIMCT cells were prepared as previously described (1, 16). For studies on cAMP production, tissue from two rats was used to plate one 24-well dish (Costar Data Packaging Corp., Cambridge, MA); for studies on inositol trisphosphate (IP3) production wells were plated at three times this density to obtain uniform confluent cultures.

Generation of samples for measurement of IP3. At 96 h the Hams F12/Liebovitz L15 medium in which the cells were initially grown was aspirated and the cultures washed twice with sterile PBS. The cells were then fed with inositol-free DME supplemented with 3H-myo-2-inositol (5 μCi/well, Amersham Corp., Arlington Heights, IL). Studies were performed after 24 h of labeling, as this time has been demonstrated to be sufficient for incorporation into the phospholipid pool. Media was aspirated from the cells and the cells were washed twice with 500 μl of PBS. The cells were then incubated for 15 min in PBS without or with desired test substances, e.g., AVP, forskolin, or CIpHeSCAMP. In studies employing the protein kinase inhibitors (17) 1-(5-isoquinolylsulfonfyl)-2-methylpipеразине (H7) or N-[2-(methylamino)ethyl]-5-isoquinoline-sulfonamide (H8) (Calbiochem-Behring Corp., La Jolla, CA), there was a 5-min preincubation with these compounds alone,

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followed by a 15-min incubation with these compounds plus any other desired agents.

Upon completion of all preincubations, the buffer was aspirated and replaced with 500 μl of PBS without or with 100 nM EGF (Sigma Chemical Co., St. Louis, MO). Previous studies have demonstrated that EGF-stimulated IP$_3$ production in RIMCT cells peaks at 10 s (1). Therefore, the reaction was terminated after 10 s 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. (18). In brief, the samples were thawed and brought to a pH of 6.0 or greater with 50 mM Tris base. They were then applied to Dowex 1x-8 columns (formate form) (Dow Corning Corp., Midland, MI). Serial elutions were 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/1.0 M ammonium formate which elute, respectively, inositol, glycerophosphorylinositol, inositol phosphate, inositol bisphosphate (IP$_2$), and IP$_3$. Results are expressed as counts per minute of IP$_3$ per well.

**Determination of DAG levels.** At 96 h after plating cells were fed with Ham’s/Leibovitz medium containing [14C]arachidonic acid (6 μCi/ml) and 5% fetal calf serum. After 16–18 h the medium was aspirated and cells were washed twice with PBS. Cells exposed to PBS containing the desired test substances for 15 min; PBS was aspirated and the cells scraped into 1 ml of ice-cold methanol under N$_2$. After 60 min at 4°C 2 ml of ice-cold formic acid was added, the N$_2$ was sonicated, gaseous with N$_2$, and extracted overnight at 4°C. 750 μl of H$_2$O was added, samples were vortexed for 2–3 min, then centrifuged at 1,200 rpm for 6 min. The lower phase that contained lipids was aspirated, dried under N$_2$, and re-suspended in 50 μl of chloroform/methanol, 2:1.

Samples were spotted on to standard silica plates and subjected to thin layer chromatography using a petroleum ether/diethyl ether/glacial acetic acid (70:30:1) solvent system. The plates were subjected to autoradiography and the diacylglycerol (DAG) containing bands were scraped and quantitated by liquid scintillation counting (19). DAG was expressed as a percentage of total phospholipids.

**Determination of cAMP production.** Studies were performed in Krebs-Ringer’s buffer, pH 7.4, at 300 mosM/kg H$_2$O in the presence of 0.5 mM isobutylmethylxanthine to inhibit phosphodiesterase. Studies using exogenous IP$_3$ require cell permeabilization. These studies were performed in a buffer designed to simulate the intracellular milieu (composition in millimoles: 20 NaCl, 100 KCl, 5 Mg sulfate, 1 NaH$_2$PO$_4$, 25 Na bicarbonate, and 1.0 EGTA, pH 7.2) containing saponin (50 μg/ml). Incubation with effector solutions and subsequent densitometry of cAMP and protein were performed as reported previously (16, 20). Results are expressed as femtomoles cAMP per microgram protein.

**Statistical analysis.** In studies on cAMP production, values obtained in triplicate wells were means for an n of one; in studies on IP$_3$ or DAG production each well was considered an n of one. 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 (ANOVA) followed by appropriate contrasts of a priori assumptions (21). In all circumstances P < 0.05 was considered significant. Data are presented as mean±SEM.

**Results**

**Effect of cAMP on EGF-stimulated PLC activity.** The effect of cAMP on EGF-stimulated IP$_3$ production is portrayed in Fig. 1. EGF significantly increases IP$_3$ production from 975±102 to 2,371±63 cpm/well (P < 0.001). Stimulation by EGF is not observed in cells pretreated with 100 nM AVP (1,024±158),

10 μM forskolin (686±57), or 0.1 mM CIPheScAMP (774±108). Pretreatment with CIPheScAMP results in a decrease in inositol monophosphate (IP$_1$) (1,658±169 vs. 893±149 cpm/well; n = 4, P < 0.02) and IP$_2$ (906±142 vs. 513±49 cpm/well; n = 4, P < 0.05) as well. Similarly, as seen in Fig. 2, pretreatment with CIPheScAMP decreases EGF-stimulated DAG production from 4.20±0.48 to 2.85±0.04% of total phospholipid (n = 3; P < 0.05). Thus cAMP, whether added exogenously or stimulated endogenously, inhibits subsequent stimulation of PLC.

**Mechanism of inhibition by cAMP.** To determine whether the effect of cAMP is mediated by stimulation of protein kinase A, the effect of H$_8$, a relatively specific inhibitor of cyclic nucleotide-dependent kinases (17), was examined. As seen in Fig. 3, 50 μM H$_8$ itself has no effect on subsequent EGF-stimulated IP$_3$ production. However, inhibition of PLC activity by CIPheScAMP is not observed in the presence of H$_8$ (2,124±158 vs. 2,183±162, NS). In contrast, H$_7$, a relatively selective inhibitor of protein kinase C (17), which itself has no effect on EGF-stimulated IP$_3$ production, fails to prevent inhibition by CIPheScAMP (2,206±115 vs. 842±19, P < 0.001). This indicates that inhibition of EGF-stimulated PLC activity by cAMP is mediated by activation of A-kinase.

**Figure 1.** Effect of cAMP on epidermal growth factor (EGF)-stimulated IP$_3$ production (n = 4). Cells are exposed to AVP, forskolin, or CIPheScAMP for 15 min before a 10-s incubation with 100 nM EGF. Exposure to cAMP prevents stimulation by EGF.

**Figure 2.** Effect of cAMP on EGF-stimulated DAG production (n = 3). Cells are exposed to CIPheScAMP for 15 min before and during incubation with EGF. CIPheScAMP inhibits EGF-stimulated DAG production.
Effect of stimulation of PLC on AVP-stimulated AC activity. The effect of EGF on basal and AVP-stimulated cAMP accumulation is depicted in Fig. 4. EGF has no effect on basal cAMP accumulation; however, simultaneous addition of EGF eliminates the response to either a submaximal (10\(^{-7}\) M, 56.2±3.30 vs. 28.92±3.14 fmol/μg protein, \(P < 0.001\)) or a maximal (10\(^{-5}\) M, 151.1±23.32 vs. 37.05±4.91, \(P < 0.001\)) concentration of AVP.

Though all studies were performed with 100 nM EGF, AVP-stimulated AC activity was, in fact, inhibited at much lower concentrations of EGF. As seen in Fig. 5, inhibition by EGF was observed at a concentration as low as 0.1 nM with a calculated IC\(_{50}\) of 0.93 nM. AVP-stimulated AC activity was completely inhibited by 5 nM EGF.

**Mechanism of inhibition by EGF.** In addition to stimulating PLC, EGF also stimulates phospholipase A\(_2\) (PLA\(_2\)) in RIMCT cells with resultant PGE\(_2\) production (22). PGE\(_2\) has been suggested to inhibit AC in the rabbit cortical collecting duct (23). Stimulation of PLC results in the formation of DAG and IP\(_3\). IP\(_3\) increases cytosolic Ca\(^{2+}\) by releasing Ca\(^{2+}\) from nonmitochondrial pools (24). DAG activates protein kinase C (25). Previous studies have demonstrated inhibition of AVP-stimulated cAMP accumulation (16, 26) or water flux (27) upon increasing cytosolic Ca\(^{2+}\). Activation of protein kinase C is known to inhibit stimulation of AC in a variety of tissues (5–8). Studies were undertaken, therefore, to determine which of these second messengers, PGE\(_2\), IP\(_3\), or DAG, mediates EGF inhibition of AVP-stimulated AC activity.

![Graph showing effect of EGF on AVP-stimulated cAMP generation](image)

**Figure 4.** Effect of EGF on AVP-stimulated cAMP generation (\(n = 6\)). EGF (100 nM) is added simultaneously with AVP. Exposure to EGF eliminates AVP-stimulated cAMP generation.

**The role of PGE\(_2\).** To examine a potential role for EGF-stimulated PGE\(_2\) production in mediating inhibition of AC, the effect of EGF was examined in the presence of the cyclooxygenase inhibitor, flurbiprofen (5 μM; Sigma Chemical Co.), which inhibits ionomycin-stimulated PGE\(_2\) production >95% (1). Inhibition of AC by EGF is not mediated by a cyclooxygenase product as even in the presence of flurbiprofen, EGF eliminates the response to 10\(^{-7}\) M AVP (156.27±27.05 vs. 38.9±10.20 fmol/μg protein; \(n = 3\), \(P < 0.02\)).

**The role of IP\(_3\).** RIMCT cells were permeabilized with saponin as described above and the ability of AVP to stimulate cAMP accumulation was assessed in the absence or presence of 10 μM IP\(_3\). Saponization itself had no effect on AC, as AVP still increased cAMP accumulation from 58.19±4.94 to 146.10±24.57 fmol/μg protein (\(n = 5\); \(P < 0.01\)). Exposure to IP\(_3\) had no effect on either basal (49.47±4.87 vs. 58.18±4.94; \(n = 5\); NS) or AVP-stimulated cAMP accumulation (166.15±28.76 vs. 146.10±24.57; \(n = 5\); NS). Therefore, inhibition of AVP-stimulated AC activity by EGF was not a consequence of an IP\(_3\)-induced increase in cytosolic Ca\(^{2+}\).

**The role of DAG.** To examine a potential role for DAG-mediated increase in the activity of protein kinase C in inhibition of AC by EGF, the effect of protein kinase C inhibition was examined. As seen in the upper panel of Fig. 6, pretreatment with the C-kinase inhibitor, H7, prevented inhibition of AVP-stimulated AC activity by EGF. In contrast, in a separate set of experiments shown in the lower panel of Fig. 6, the A-kinase inhibitor, H8, conferred no protection against inhibition by EGF.

To confirm that activation of protein kinase C results in inhibition of AVP-stimulated AC activity, the effect of the synthetic highly permeable compound sn-1,2-dioctanoylglycerol (DOG) (28) was examined. As shown in Fig. 7, simultaneous addition of 100 nM DOG inhibited AVP-stimulated AC activity from 100.89±9.33 to 58.29±7.21 (\(n = 3\), \(P < 0.02\)). Inhibition by DOG was not observed in cells pretreated with H7, as AVP-stimulated cAMP generation remained at 100.08±7.03 fmol/μg even upon exposure to DOG.

**Discussion**

The role of phosphoinositide hydrolysis in the function of the collecting duct and its potential interactions with AC have not
Inhibitory Messengers in the Renal Medulla

Stimulation of AC by either AVP or forskolin or the provision of exogenous cAMP all result in a decrease in EGF-stimulated IP₃ production (Fig. 1). It has been suggested that production of DAG, the endogenous activator of protein kinase C, may result not only from the hydrolysis of polyphosphoinositides but of other phospholipids (e.g., phosphatidylethanolamine) as well (30). It is important to note, therefore, that pretreatment with CIPheScAMP inhibits EGF-stimulated DAG production (Fig. 2) as well as IP₃ formation. While the source of the DAG is unknown, it is clear that inhibition of PLC by cAMP results in a decrease in the levels of both second messengers, IP₃ and DAG. The exact locus of inhibition by cAMP is not known. However, it is likely that cAMP decreases IP₃ levels by impairing the formation of IP₃ rather than by enhancing its degradation, as the levels of IP, and IP₂ are also decreased. The decrease in PLC activity upon exposure to cAMP is a consequence of an A-kinase–mediated phosphorylation event as inhibition by cAMP is not observed in the presence of the A-kinase inhibitor, H8 (Fig. 3). Although the Kᵢ of H8 for cAMP-dependent protein kinase is only modestly lower than that of H7 (1.2 vs. 3.0 µM) (17), the specificity of H8 for A-kinase is demonstrated by the inability of H7 to prevent inhibition of PLC by cAMP. Conversely, inhibition of AC by DOG, which is known to function by activation of protein kinase C, is prevented by H7 but not by H8 despite the only modest difference in their respective Kᵢs for protein kinase C (6.0 vs. 15.0 µM).

Similar to its ability to inhibit PLC, cAMP has also been shown to inhibit PLA₂ in cultured RIMCT cells (31) as well as other tissues (32, 33). The concentration of CIPheScAMP that inhibits PLC (0.1 mM) is the same as that which inhibits PLA₂ in this same tissue (31) and is comparable to that which stimulates water flux in the toad bladder (34).

Exposing RIMCT cells to AVP in the presence of EGF results in abolition of AVP-stimulated cAMP generation (Fig. 4). As EGF eliminates the response to AVP even in the background of cyclooxygenase inhibition, it is clear that the effect of EGF is not a consequence of its stimulation of PGE₂ production (22). This is in accord with previous studies in RIMCT cells that do not support an inhibitory effect of PGE₂ on AC in this tissue. Sato and Dunn have reported that PGE₂ at high concentration enhances cAMP accumulation in RIMCT cells but observed no inhibition at any concentration (35). Similarly, previous studies from this laboratory have shown that in RIMCT cells exposed to the calcium ionophore A23187, which very potently stimulates PGE₂ production, AVP-stimulated cAMP accumulation is not enhanced upon inhibition of cyclooxygenase with meclofenamate (16).

Studies were then performed to determine whether one of the second messengers produced upon stimulation of PLC in response to EGF, IP₃ or DAG, is responsible for mediating inhibition of AC by EGF. Exposure of saponin-permeabilized cells to exogenous IP₃ had no effect on AVP-stimulated cAMP accumulation. Release of Ca²⁺ from cellular stores has been reported to occur in response to the concentration of IP₃ (10 µM) used in this study (36, 37). The magnitude of the increase in cytosolic Ca²⁺ in response to IP₃ in RIMCT cells was not determined. Therefore, the present study does not unequivocally exclude a role for Ca²⁺ in mediating inhibition by EGF.
However, as inhibition of protein kinase C (see below) results in complete restoration of AVP-responsiveness in the presence of EGF, the role of any other mediator is likely to be minimal. The lack of inhibition by IP<sub>3</sub> differs from previous studies in the collecting duct in which increased cytosolic Ca<sup>2+</sup> inhibited the response to AVP (16, 27). This most likely reflects the fact that the amount of Ca<sup>2+</sup> released by IP<sub>3</sub> and, therefore, the subsequent increase in cytosolic Ca<sup>2+</sup> is much less than that obtained with a calcium ionophore (36, 37), as had been employed in earlier studies (16, 27).

Rather than an effect of IP<sub>3</sub>, inhibition of AC by EGF appears to be due to activation of protein kinase C as EGF has no effect in the presence of the C-kinase inhibitor, H7 (Fig. 6). We have previously reported that EGF-stimulated IP<sub>3</sub> production in RIMCT cells is first detected at 10 nM EGF (1). Yet, as seen in Fig. 5, inhibition of AVP-stimulated AC activity is observed with as little as 0.1 nM EGF and is complete at 5 nM. As activation of PLC results in hydrolysis of not only phosphatidylinositol bisphosphate but of phosphatidylinositol monophosphate and phosphatidylinositol as well (and perhaps other phospholipids, e.g., phosphatidycholine) production of DAG, on a molar basis, is likely to exceed production of IP<sub>3</sub> (30). Therefore, inhibition of AC by concentrations of EGF below the threshold for detection of IP<sub>3</sub> production itself suggests that the inhibition is a consequence of DAG production.

Activation of protein kinase C has been shown to inhibit AVP-stimulated water flow in the toad bladder (14) and in the isolated perfused rabbit cortical collecting tubule (15). Though a pre-cAMP site appears operant in the toad bladder (14), the predominant site of action of protein kinase C in both tissues is post-cAMP. Likewise, the ability of EGF to inhibit AVP- and cAMP-stimulated water flux in the rabbit cortical collecting tubule to a similar extent (38) suggests a predominantly post-cAMP site of action. The studies reported herein demonstrate that, apart from any post-cAMP effects, EGF, via activation of protein kinase C, profoundly inhibits the generation of cAMP in response to AVP. This finding is consistent with other studies that have shown protein kinase C-mediated inhibition of AVP-stimulated AC activity in Madin-Darby canine kidney cells (39) or cultured rabbit cortical collecting ducts (40). However, in marked contrast to these studies in which inhibition of AC is observed only after more prolonged exposure (15 min to 6 h) to activators of protein kinase C, in the present study inhibition by EGF or DOG requires no preincubation but is observed upon addition of these agents simultaneously with AVP. This indicates a direct inhibitory effect of protein kinase C rather than one mediated by a secondary phenomenon such as stimulation of cell proliferation, as had been previously suggested (40). Furthermore, unlike the other studies in which activation of protein kinase C attenuates but does not eliminate AVP-stimulated cAMP generation, exposure of RIMCT cells to EGF abolishes the response to AVP. DOG is less potent an inhibitor of AC, perhaps reflecting the need for permeation of the cell membrane. Finally, in none of the previous studies on the effect of protein kinase C was the other signaling limb, i.e., the effect of cAMP on phosphoinositide hydrolysis, examined.

The physiologic correlates of the biochemical observations described herein require further examination. As the role of phosphoinositide hydrolysis in the function of the collecting duct is itself unclear, one can only speculate upon the significance of its modulation by cAMP. In contrast, the profound inhibition of AVP-stimulated cAMP generation by activation of protein kinase C suggests a possible role for protein kinase C in “turn off” of the AVP signal.

In summary, the data presented herein demonstrate for the first time the existence of this bidirectional signaling system in cultured inner medullary collecting tubule cells (Fig. 8). Stimulation of AC results in the formation of cAMP which, via activation of A-kinase, inhibits PLC; conversely, stimulation of PLC results in the formation of DAG which, via activation of C-kinase, inhibits AC. The exact loci of inhibition by these respective kinases remain to be determined.

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Figure 8. Schematic representation of the interactions between the AC and PLC signaling systems in the cultured rat inner medullary collecting duct.
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