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α_2-Adrenergic Receptors Activate Phospholipase C in Renal Epithelial Cells

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SUMMARY

The effects of α_2-adrenergic receptors are usually attributed to inhibition of adenylyl cyclase through pertussis toxin-sensitive G_i coupling. In kidney distal convoluted tubule (DCT) cells, stimulation of Na^+/K^+-ATPase by α_2 receptors involves activation of protein kinase C (PKC). To identify the signal pathways coupled to α_2 receptors, we measured cAMP production and show that the α_2 agonist B-HT 933 had no effect on basal or stimulated (forskolin, parathyroid hormone) cAMP accumulation in DCT cells but inhibited parathyroid hormone-stimulated cAMP accumulation in proximal tubule cells. I tested whether α_2 receptors on DCT cells stimulate PKC through second messengers generated from phospholipase C (PLC) activation. In DCT cells, B-HT 933 increased inositol-1,4,5-trisphosphate formation by 4–6-fold over control and increased diacylglycerol formation by 46%. Basal intracellular calcium concentration in single DCT cells averaged 114 nM and increased within 2 min to 196 nM with B-HT 933. Treatment with the PLC inhibitor U-73122 but not pertussis toxin blocked B-HT 933-induced rises in inositol-1,4,5-trisphosphate and intracellular calcium concentration. B-HT 933 increased PKC activity by 45% over control .in DCT cells. These findings provide evidence that α_2-adrenergic receptors activate PLC in DCT cells through a pertussis toxin-insensitive mechanism.

In many tissues, α_2-adrenergic receptors couple to G protein α subunits and inhibit adenylyl cyclase activity. The effects of α_2 receptors on adenylyl cyclase inhibition are affected by pertussis toxin, which ADP-ribosylates $G_{\alpha i}$. However, a number of physiological effects observed with stimulation of α_2-adrenergic receptors cannot be explained solely on the basis of inhibition of adenylyl cyclase and reductions in cellular cAMP (for a review, see Ref. 1).

α_2-Adrenergic receptors can be linked to alternative coupling mechanisms in addition to inhibition of adenylyl cyclase (2–5). Pertussis toxin-sensitive G proteins seem to couple most physiological signaling pathways linked to α_2-adrenergic receptors (1, 6). However, recent studies on α_2-adrenergic receptor/G protein coupling indicate that these receptors are very permissive in that (a) α_2 single receptor subtypes may activate multiple G protein subunits within a host cell, (b) some receptor subtypes prefer certain G protein α subunits, and (c) some G protein α subunits may substitute for preferred α subunits in particular cells (6).

In addition to coupling to pertussis toxin-sensitive G proteins, α_2 receptors have been shown to couple to G_s, G_z, and G_q proteins (2, 7–10). Expression of α_2-adrenergic receptors in cells lacking endogenous α_2 receptors demonstrate coupling to inhibition of adenylyl cyclase, and activation of PLC (4) and PLD (11), and stimulation of adenylyl cyclase (8). Therefore, a single receptor population is capable of interacting with multiple G proteins and initiating several second messenger pathways. A preliminary report suggests that the effects of α_2-adrenergic receptors in rat aorta cells are independent of adenylyl cyclase inhibition and mediated through PLD (12).

In the kidney, α_2-adrenergic receptors mediate several effects on tubular epithelial cells. Stimulation of α_2-adrenergic receptors on proximal tubule cells increase Na^+/H^+ exchange, Na^+/K^+-ATPase activity, and bicarbonate and water absorption (13–16); in collecting duct cells, they inhibit the hydro-osmotic effect of vasopressin (17–19). In both cell types, the α_2-adrenergic receptor effects on transport are attributed to G_i coupling, inhibition of adenylyl cyclase activity, and a decrease in cellular cAMP (14, 18, 20); the effects are attenuated by pertussis toxin. In DCT cells, activation of α_2-adrenergic receptors increases Na^+/K^+-ATPase activity through a PKC-dependent mechanism (21). The signaling pathways coupled to α_2 receptor activation and stimulation of PKC in DCT cells have not been identified.

The focus of this study was to identify α_2-adrenergic recep-

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ABBREVIATIONS: PLC, phospholipase C; PLD, phospholipase D; DCT, distal convoluted tubule; PKC, protein kinase C; Ins(1,4,5)P_3, inositol-1,4,5-trisphosphate; DMEM, Dulbecco's modified Eagle's medium; FCS, fetal calf serum; PSN, penicillin/streptomycin/neomycin; $([Ca^{2+}]_i$, intracellular Ca^{2+} concentration; Ins(1,4,5)P_3, inositol-1,4,5-trisphosphate; DAG, *sn*-1,2-diacylglycerol; PTH, parathyroid hormone; PI-PLC, phosphoinositide-specific phospholipase C; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

tor signaling pathways in distal tubule cells and determine whether similar coupling and signals exist with those of proximal tubule cells.[1] I report that stimulation of α_2-adrenergic receptors does not inhibit basal or hormone-stimulated cAMP accumulation, and the receptors are not sensitive to pertussis toxin in DCT cells. However, α_2-adrenergic receptor agonists cause a transient increase in intracellular calcium, diacylglycerol, inositol-1,4,5-trisphosphate formation, and PKC activity. I conclude that α_2 receptors are coupled to PLC in DCT cells and that cell-specific α_2-adrenergic receptor subtypes or differences in receptor coupling account for these distinct signaling pathways.

Experimental Procedures

Preparation of Primary Cell Cultures and Immortalized DCT Cells

Primary cultures of mouse distal tubule (composed of DCTs plus thick ascending limb cells) and proximal tubule (proximal convoluted and straight tubule) cells were prepared as reported previously (22). Immortalized DCT cells were prepared from primary cultures of mouse distal tubule cells using the double-antibody isolation technique noted above and subsequent exposure to a chimeric adenovirus 12-simian virus 40 (AD12/SV40) and were cloned by limiting cell dilution (22). Immortalized DCT cells express PTH receptors, thiazide- but not bumetanide-inhibitable Na^+ and Cl^- transport, and thiazide-stimulated Ca^{2+} uptake (23, 24). Cell passages 6–29 were used in the current study.

Measurement of Adenylyl Cyclase Activity in Renal Cells

Primary cultures of proximal and distal tubule cells and immortalized DCT cells were seeded onto 24-well plates at a density of 10^5 cells/well and grown to confluence in DMEM/F12, 10% FCS, and PSN (Sigma Chemical, St. Louis, MO). Cells were switched to receive DMEM/F12 without serum or antibiotics 16–18 hr before the assay. At the start of the experiment, cells were washed twice with 0.5 ml of buffer that contained 135 mM NaCl, 4 mM KCl, 1 mM KH_2PO_4, 1.2 mM $CaCl_2$, 1.2 mM $MgCl_2$, 10 mM HEPES, pH 7.4, and 5 mM glucose. Protein samples were obtained from three wells, and 0.5 ml of buffer containing 0.2% bovine serum albumin and 0.1 mM rolipram with or without drugs was added to each remaining well. The cells were incubated at 37° on a covered shaking water bath for 15 min. cAMP accumulation was terminated with the addition of 0.1 ml of 12% ice-cold trichloroacetic acid, and the cells were placed on ice for 1 hr. Samples were put into plastic tubes, the wells were rinsed once with 0.5 ml of 2% trichloroacetic acid, and the rinse was added to the sample and frozen at −20°. To assay for cAMP, accumulation samples were thawed and spun at 2370 × *g* at 4° for 25 min. A portion of the supernatant (0.9 ml) was removed, transferred to a glass tube, and extracted three times with an equal volume of water-saturated ether. Samples were evaporated to dryness in a SpeedVac (Savant Instruments, Farmingdale, NY) and reconstituted in 0.5 ml of Tris-EDTA buffer. cAMP was measured using a [^{3}H]cAMP assay kit (Diagnostic Products, Los Angeles, CA). The activity of adenylyl cyclase activity is expressed as pmol cAMP/mg of protein/5 min.

Assessment of PLC Activity

The capacity of α_2-adrenergic receptors to stimulate PLC was tested by measuring their ability to (a) produce a rapid and transient increase in $[Ca^{2+}]_i$, (b) increase Ins(1,4,5)P_3 formation, and (c) enhance DAG formation.

Measurement of $[Ca^{2+}]_i$. Intracellular fluorescence measurements of calcium were performed as described previously in detail (23, 24). Primary distal and proximal tubule cells and immortalized DCT cells were grown to near-confluence on 25-mm glass coverslips and rinsed three times with Krebs-Ringer-HEPES buffer before a 60-min incubation at 37° with Fura-2 AM (10^{-5} M; Molecular Probes, Eugene, OR). The coverslips were mounted in a chamber of a microincubation system that was temperature controlled to 37° (MS-C; Narishige, Greenvale, NY) and was affixed to the stage of a Nikon Diaphot inverted microscope. Fluorescence excitation and emission intensities were measured with a Nikon Photoscan-2 (Nikon, Natick, MA).

Measurement of Ins(1,4,5)P_3 formation. Primary cultures of proximal and distal tubule cells and immortalized DCT cells were grown to confluence in six-well plates. Cells were serum and antibiotic starved overnight before assay. Cells were rinsed with a modified Kreb-Ringer-HEPES buffer that contained 140 mM NaCl, 4.6 mM KCl, 1 mM $CaCl_2$, and 1 mM $MgCl_2$ and was buffered to pH 7.40 ± 0.01 with 28 mM HEPES and 18 mM Tris base and adjusted to 295 ± 2 mOsmol/kg H_2O. A second rinse consisted of a buffer containing 50 mM LiCl, which replaced an equivalent amount of NaCl. Lithium has been commonly used in phosphoinositide hydrolysis assays to uncompetitively inhibit inositol monophosphatase and inositol polyphosphate-1-phosphatase and prevent the subsequent recycling steps of phosphatidylinositol (25, 26). Lithium inhibition of recycling combined with the selective radioreceptor assay provides an accurate index of agonist-induced hydrolysis of phosphatidylinositol-4,5-bisphosphate by PLC to Ins(1,4,5)P_3. Cells were treated in the wells with a total of 500 μl of buffer with or without hormone/drugs for 2 min at 37° on a shaking incubator. Although receptor-mediated Ins(1,4,5)P_3 formation is generally increased within 30 sec, several reports indicate that significant Ins(1,4,5)P_3 are not observed within 2 min (27, 28); preliminary time course studies indicated that 2 min was required for significant Ins(1,4,5)P_3 formation to be detectable. The treatment was terminated by the addition of 100 μl of ice-cold 20% perchloric acid. Wells were then scraped, cells/buffer was placed in a 1.5-ml conical centrifuge tube, and proteins pelleted by centrifugation at 2000 × *g* for 15 min at 4°. The supernatant was neutralized to pH ~7.5 by the addition of 175 μl of buffer consisting of 1.5 M KOH and 120 mM HEPES and recentrifuged as above to remove $KClO_4$ precipitate. The supernatant (~500 μl) was removed and placed in a 1.5 ml-conical centrifuge tube. All samples were evaporated at medium heat (43°) to dryness in a SpeedVac. Samples were reconstituted in 100 μl of distilled H_2O. Samples and standards (100 μl) were analyzed with Amersham (Arlington Heights, IL) [^{3}H]Ins(1,4,5)P_3 assay system. Protein-bound samples and standards were placed in 5 ml of scintillation fluid and counted in a β-scintillation counter. Results are reported as pmol/well/2 min.

Determination of DAG accumulation. Primary cultures of distal tubule cells or immortalized DCT cells were grown to 80–100% confluence on 100-mm tissue culture dishes in DMEM/F12 containing 5% FCS and PSN. Cells were rinsed twice with a phosphate-buffered saline solution. Cells were then treated for 5 min with agonist or vehicle in phosphate-buffered saline; at 5 min, the reaction was terminated by the removal of buffer (with or without drug), and 2.5 ml of ice-cold distilled H_2O was added. Cells were then scraped, a sample was retained for protein normalization, and the remainder of the cells were placed in a glass centrifuge tube for extraction with chloroform/methanol/H_2O (1:2:1.8, v/v/v). The lipid extract obtained from the cells was dried under NO_2 and subsequently assayed for DAG using a *sn*-1,2-diacylglycerol radioenzymatic assay kit (Amersham). Results are reported as pmol/5 min/mg of protein.

[1] In this report, the term "distal tubule cells" refers to primary cultures of DCT and cortical thick ascending mouse limb cells; "proximal tubule cells" denotes cells from primary cultures of proximal convoluted and straight mouse segments; and "immortalized DCT cell" refers to AD12/SV40 exposed distal mouse tubule cells cloned by limiting cell dilution and exhibiting a DCT cell phenotype.

Determination of PKC Activity

The activity of PKC was measured as the phosphorylation of a synthetic fragment of *N*-acetylated myelin basic protein[4–14] (29). Cells were grown on 100-mm tissue culture dishes to 80–100% confluence in DMEM/F12, 5% FCS, and PSN. Monolayers were serum-starved for 12 hr before the addition of agonists or vehicle. Activation of PKC was measured in DEAE-purified total PKC using the PKC assay system kit (GIBCO, Grand Island, NY). Measurements were performed in the presence of 100 nM okadaic acid and in the absence of phorbol esters. PKC activity is expressed as pmol/min/mg of protein.

Materials and Preparation of Drug Solutions

Adrenergic receptor agonists were prepared so that the molar concentration indicated in the text was the final concentration to which cells were exposed. Solutions containing drugs were prepared fresh daily. Rolipram was purchased from BIOMOL Research Laboratories (Plymouth Meeting, MA), and B-HT 933 was a gift from Boehringer Ingelheim Pharmaceuticals (Ridgefield, CT). Other adrenergic agonists and antagonists were purchased from Research Biochemicals (Natick, MA). Bovine PTH[1–84] was obtained through the National Hormone and Pituitary Program, National Institute of Diabetes and Digestive and Kidney Disorders, National Institute of Child Health and Human Development, and the United States Department of Agriculture (Bethesda, MD).

Statistical Analysis

All cAMP, Ins(1,4,5)P_3, and PKC activity measurements were made in triplicate and DAG measurements were made in duplicate within an individual experiment. The data are presented as mean ± standard error. Comparisons between control and drug-treated groups were examined by post-hoc analysis of multiple comparisons with the Bonferonni or Newman-Keuls multiple-comparisons tests using the statistical software Instat for MacIntosh (GraphPAD Software, San Diego, CA). Probability values of $p = 0.05$ were considered statistically significant.

Results

α_2-Adrenergic Receptor Effects on cAMP Accumulation

The selective α_2 receptor agonist B-HT 933 had no effect on basal levels of cAMP accumulation in primary cultures of proximal or distal tubule cells or immortalized DCT cells (Fig. 1 and Table 1). B-HT 933 did not modify PTH-stimulated cAMP accumulation in primary cultures of distal tubule cells (Fig. 1) or immortalized DCT cells (Table 1). Other selective α_2 receptor agonists (e.g., UK 14,304, clonidine, and guanabenz) had no effect on PTH-, isoproterenol-, or forskolin-stimulated cAMP accumulation in DCT cells) (Table 1). Consistent with the lack of effect of α_2 agonists on hormone-stimulated cAMP accumulation was the absence of an inhibitory response with pertussis toxin treatment (Fig. 1). In contrast to the negative effects in distal tubule cells, B-HT 933 significantly reduced PTH-stimulated cAMP accumulation in primary cultures of proximal cells (Fig. 1). Proximal tubule cells pretreated with pertussis toxin (10 ng/ml; 18 hr) exhibited no difference in basal levels of cAMP accumulation (basal level, 31 ± 4 pmol/mg of protein/5 min; pertussis toxin treated, 33 ± 3 pmol/mg of protein/5 min). However, pertussis toxin attenuated the α_2-receptor-mediated reduction in PTH-stimulated cAMP accumulation (Fig. 1).

Activation of α_2 Receptors Stimulate PLC in DCT Cells

I previously reported (21) that stimulation of α_2-adrenergic receptors increased Na^+/K^+-ATPase activity in DCT cells through a PKC-dependent pathway. I determined whether stimulation of α_2-adrenergic receptors activates PLC and liberates second messengers, which, in turn, increase PKC activity. Second messengers that result from activation of PI-PLC include increases in $[Ca^{2+}]_i$, Ins(1,4,5)P_3, and DAG.

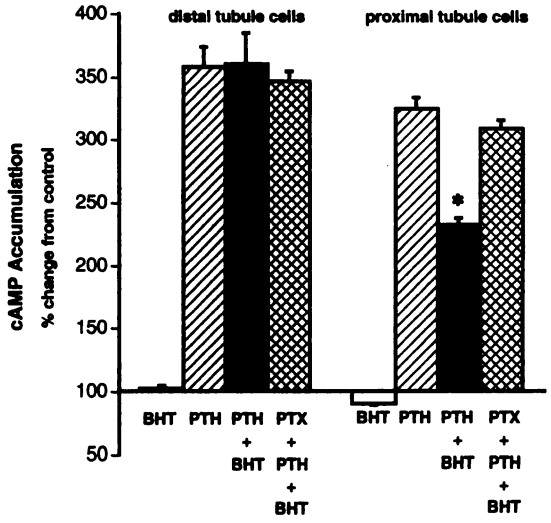

Fig. 1. α_2-Adrenergic receptors inhibit PTH-stimulated cAMP accumulation in primary proximal but not distal tubule cells. Basal rates of cAMP accumulation in primary distal (26 ± 4 pmol/mg of protein/5 min) or proximal (33 ± 4 pmol/mg of protein/5 min) tubule cells were unaffected by the α_2 agonist B-HT 933 (*BHT*; 1 μM). B-HT 933 had no effect on PTH-stimulated cAMP accumulation in distal tubule cells but reduced accumulation in proximal tubule cells. Pretreatment with pertussis toxin (*PTX*; 10 ng/ml, 18 hr) abolished the BHT effect in proximal cells but had no effect in distal tubule cells. *Bars*, mean values of five separate experiments with triplicate determinations. *, $p < 0.05$ compared with PTH-stimulated level.

α_2-Adrenergic receptor agonists increase $[Ca^{2+}]_i$ in DCT cells. Fig. 2A depicts the response of a single primary cultured distal tubule cell exposed to the selective α_1 agonist cirazoline, the mixed agonist norepinephrine, and the α_2 agonist B-HT 933. Cirazoline had no effect on $[Ca^{2+}]_i$, whereas both norepinephrine and B-HT 933 produced a rapid and transient rise in $[Ca^{2+}]_i$. Similar observations were made in immortalized DCT cells and are summarized in Table 2. The transient rise of $[Ca^{2+}]_i$ induced by B-HT 933 was abolished by pretreatment with yohimbine, an α_2 receptor antagonist, but not with the α_1 antagonist prazosin or nifedipine, a Ca^{2+} channel blocker. When cells were placed in Ca^{2+}-free buffer and then treated with B-HT 933, a transient rise in $[Ca^{2+}]_i$ was observed that was equivalent to the increase noted in cells bathed in Ca^{2+}-containing buffer. These findings are consistent with the view that α_2 receptor-mediated rises in $[Ca^{2+}]_i$ are due to mobilization of Ca^{2+} from intracellular stores and not from entry through dihydropyridine-sensitive Ca^{2+} channels (30). In contrast to the response from distal tubule and immortalized DCT cells, the α_1 agonist cirazoline and norepinephrine resulted in a spiking and transient increase in $[Ca^{2+}]_i$ in proximal tubule cells (Fig. 2B). No detectable response was observed on addition of the α_2 receptor agonist B-HT 933.

α_2-Adrenergic receptor activation increases Ins(1,4,5)P_3 formation in DCT cells. As further evidence for α_2 receptor activation of PI-PLC in DCT cells, agonist-induced Ins(1,4,5)P_3 and DAG formations were measured. As depicted in Fig. 3, B-HT 933 and mixed agonists norepineph-

TABLE 1

α_2-Adrenergic receptor agonists do not alter basal or hormone-stimulated cAMP accumulation in immortalized DCT cells

Values represent mean ± SEM with triplicate determinations in 3–5 separate experiments. Cells were treated UK 14,304, clonidine, guanabenz, and B-HT 933 at 1 μM.

	cAMP accumulation				
	Control	UK 14,304	Clonidine	Guanabenz	B-HT 933
	pmol · mg protein^{-1} · 5 min^{-1}				
pmol · mg protein^{-1} · 5 min^{-1}	12 ± 1	13 ± 2	13 ± 1	12 ± 2	15 ± 3
PTH[1-84] 10 nM	41 ± 8	41 ± 13	41 ± 13	43 ± 14	45 ± 7
Isoproterenol 1 μM	62 ± 13	70 ± 11	62 ± 10	67 ± 6	60 ± 9
Forskolin 1 μM	359 ± 10	319 ± 45	334 ± 16	362 ± 33	336 ± 38

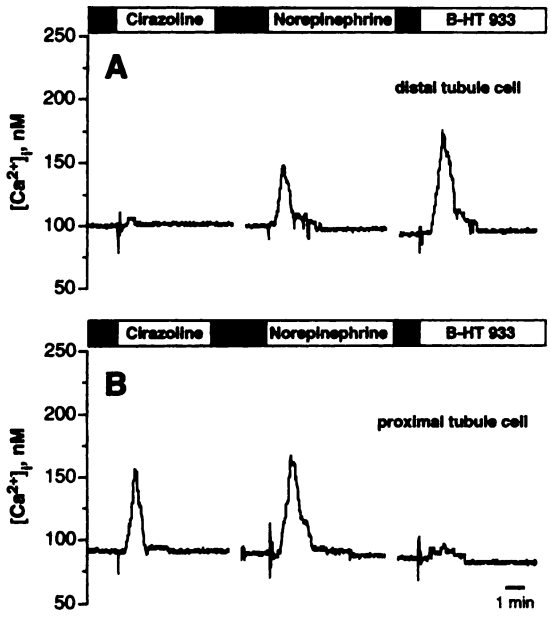

Fig. 2. α_2-Adrenergic receptor agonists increase intracellular Ca^{2+} activity in primary distal but not proximal tubule cells. A, Measurement of $[Ca^{2+}]_i$ in a distal tubule cell treated with cirazoline (α_1 agonist; 1 μM), the mixed agonist norepinephrine (1 μM), or B-HT 933 (α_2 agonist; 1 μM). *Bars*, treatment periods. B, Response in a proximal tubule cell to cirazoline, norepinephrine, and B-HT 933. After agonist exposure, drugs were washed from the chamber for 5–10 min before recording of basal levels and introduction of the next agonist. *Traces*, representative of three to five separate experiments.

TABLE 2

α_2-Adrenergic receptor induced rise of $[Ca^{2+}]_i$ is blocked by α_2 antagonists but not α_1 antagonists, Ca^{2+} channel blockers, or Ca^{2+}-free buffer in immortalized DCT cells

Values indicate mean ± standard error for separate experiments (in parentheses) measured in single immortalized DCT cells. Cells were exposed to 10 μM prazosin, yohimbine, or nifedipine and 1 μM B-HT 933.

	$[Ca^{2+}]_i$	
	Basal	B-HT 933
	nM	
Control	114 ± 5 (5)	196 ± 8 (5)
Prazosin	113 ± 6 (3)	189 ± 7 (3)
Yohimbine	106 ± 6 (3)	123 ± 10[a] (3)
Nifedipine	118 ± 7 (4)	203 ± 8 (4)
Ca^{2+}-free buffer	104 ± 8 (3)	179 ± 12 (3)

[a] $p < 0.01$ compared with B-HT 933-stimulated increase of $[Ca^{2+}]_i$.

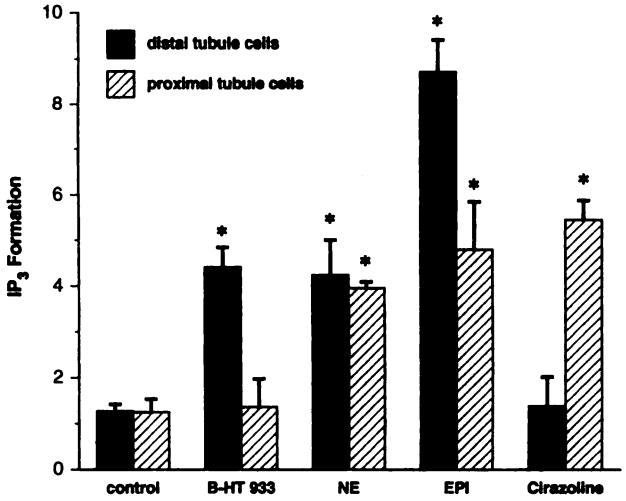

Fig. 3. α_2 Agonists increase Ins(1,4,5)P_3 (IP_3) formation in primary distal but not proximal tubule cells. Basal rates of Ins(1,4,5)P_3 formation averaged 1.3 ± 0.3 in primary distal and 1.3 ± 0.2 pmol/well/2 min in proximal tubule cells. *Bars*, mean ± standard error of triplicate measurements in four to six independent experiments. *, $p < 0.05$ compared with control rate.

rine and epinephrine significantly increased Ins(1,4,5)P_3 formation in primary cultures of distal tubule cells. The α_1-selective agonist cirazoline had no effect on Ins(1,4,5)P_3 formation in distal tubule cells. A similar increase was noted in immortalized DCT cells treated with norepinephrine and selective α_2 agonists (Table 3). In contrast to the rise of $[Ca^{2+}]_i$ observed with α_2 agonists in distal cells, proximal tubule cells exhibited no response to the α_2 agonist B-HT 933 (Fig. 3). Treatment of proximal tubule cells with norepinephrine, epinephrine, or an α_1 agonist increased Ins(1,4,5)P_3 formation.

α_2-Adrenergic receptor activation increases DAG formation in DCT cells. Activation of PI-PLC results in the hydrolysis of several phosphoinositides, including phosphatidylinositol-4,5-bisphosphate, to Ins(1,4,5)P_3 and DAG (31, 32). To test whether α_2 receptors activate PLC with attendant increases in DAG formation in DCT cells, we directly measured DAG formation (Fig. 4A). B-HT 933 increased DAG formation by 46%. A similar increase was observed in primary cultures of distal tubule cells (37%; data not shown).

α_2 Agonist-Induced PI-PLC Activation Increases PKC Activity

As previously reported, the increase in Na^+/K^+-ATPase activity in DCT cells mediated by α_2 receptors was abolished by the PKC inhibitor calphostin C (21). To determine whether receptor-mediated PI-PLC second messengers lead

TABLE 3

α_2-Adrenergic receptor-stimulated Ins(1,4,5)P_3 formation is blocked by the PLC inhibitor U-73122 but not pertussis toxin treatment in immortalized DCT cells

Cells were pretreated with 10 μM concentration of the PLC inhibitor U-73122 (active form) or U-73343 (inactive form) for 10 min or pertussis toxin (10 ng/ml; 18 hr) before treatment with 1 μM B-HT 933, norepinephrine, or clonidine. Values represent mean ± standard error of triplicate determinations in four separate experiments.

	Ins(1,4,5)P_3 formation			
	Control	U-73122	U-73343	Pertussis toxin
	pmol · well^{-1} · 2 min^{-1}			
Control	0.8 ± 0.2	1.3 ± 0.4	1.2 ± 0.4	1.2 ± 0.2
B-HT 933	5.6 ± 0.7	1.0 ± 0.4[a]	5.6 ± 1.7	5.7 ± 0.5
Norepinephrine	8.1 ± 2.5	1.1 ± 0.4[a]	6.4 ± 1.4	6.8 ± 1.4
Clonidine	5.6 ± 1.4			5.8 ± 0.6

[a] $p < 0.01$ compared with α_2 receptor-stimulated level.

to increased PKC activity, basal and α_2 agonist-treated cell levels of PKC activity were measured directly. Fig. 4B shows that an α_2 receptor agonist increased PKC activity in DCT cells by 45%.

α_2-Adrenergic Receptor Stimulations of Ins(1,4,5)P_3 and $[Ca^{2+}]_i$ Are Abolished by the PLC Inhibitor U-73122

Together, the findings that stimulation of α_2 receptors in DCT cells result in increased Ins(1,4,5)P_3 and DAG formation and a transient rise in $[Ca^{2+}]_i$ are consistent with activation of PI-PLC. U-73122, a selective PLC inhibitor, was used to characterize further the participation of PI-PLC in mediating the generation of these second messengers (33, 34). As presented in Table 3, pretreatment with U-73122 abolished the increase of Ins(1,4,5)P_3 formation in response to α_2 receptor agonists. In comparison, pretreatment with U-73343, an inactive structural analog of U-73122, had no effect. Pertussis toxin had no effect on α_2 receptor-induced increases in Ins(1,4,5)P_3 formation (Table 3). Similar results were observed with α_2 receptor-mediated increases in $[Ca^{2+}]_i$ in immortalized DCT cells (Fig. 5). The transient rise of $[Ca^{2+}]_i$ induced by B-HT 933 was blocked by U-73122, whereas pertussis toxin had no effect on either the basal or agonist-stimulated levels of $[Ca^{2+}]_i$. As an independent measure of U-73122 inhibition of PLC, immortalized DCT cells and primary cultures of distal cells were treated with 100 nM bradykinin (35). PLC-coupled bradykinin receptors have been localized to distal nephron sites (36, 37). Exposure to bradykinin produces a rapid and transient elevation of intracellular $[Ca^{2+}]_i$ (from a basal level of 111 ± 7 to 215 ± 13 nM) that was reduced to 131 ± 14 nM with U-73122 pretreatment but not with U-73343 (203 ± 15 nM; three independent experiments). Similar results were observed for the effects of bradykinin on Ins(1,4,5)P_3. Basal levels of Ins(1,4,5)P_3 formation in primary cultures of distal cells averaged 0.9 ± 0.3 pmol/well/2 min and increased to 4.4 ± 0.5 pmol/well/2 min with bradykinin treatment; pretreatment with U-73122 reduced bradykinin-stimulated Ins(1,4,5)P_3 formation to 1.3 ± 0.4 pmol/well/2 min. Inhibition of α_2 receptor-mediated Ins(1,4,5)P_3 and $[Ca^{2+}]_i$ increases with U-73122 provide further evidence that α_2 receptors stimulate PI-PLC in DCT cells.

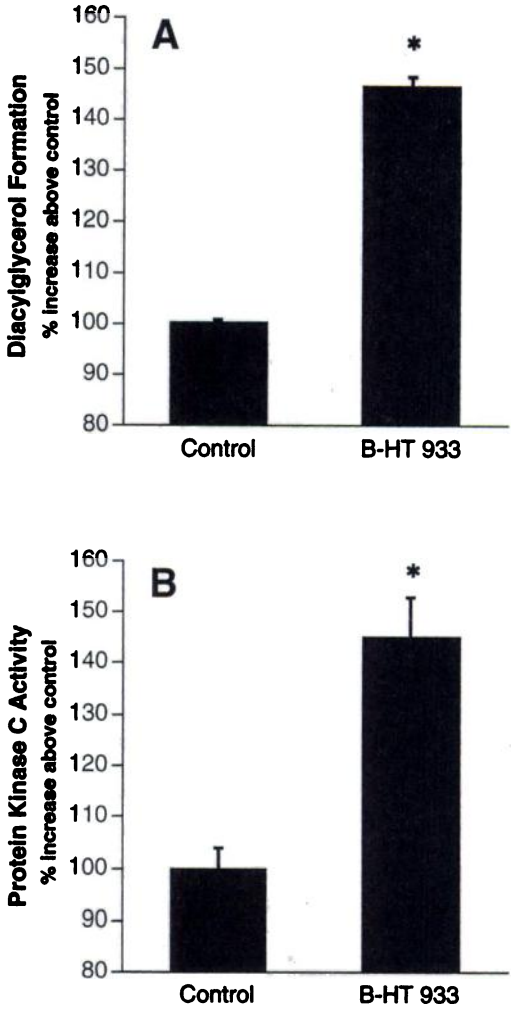

Fig. 4. α_2-Adrenergic receptor activation increases DAG formation and PKC activity in immortalized DCT cells. A, The basal rate of DAG formation averaged 160 ± 4 pmol/5 min/mg of protein. B, Basal PKC activity averaged 163 ± 8 pmol/min/mg of protein. *Bars*, mean ± standard error of duplicate determinations in five separate experiments. *, $p < 0.05$ compared with control level.

Discussion

The postulated transduction mechanism coupling α_2 receptors in most cells is inhibition of adenylyl cyclase (for a review, see Ref. 38). α_2-Adrenergic receptor-mediated inhibition of adenylyl cyclase is mediated by G_i. Treatment with pertussis toxin inactivates G_i by ADP ribosylating the α subunit. The blockade of α_2-mediated responses by pertussis toxin is often used as a demonstration for the coupling of α_2 receptors to inhibition of adenylyl cyclase (18, 20, 38). In the kidney, the physiological effects of α_2 receptors in proximal tubule (14, 20) and collecting duct (18, 39) are mediated through pertussis toxin-sensitive inhibition of adenylyl cyclase.

These results provide strong evidence that α_2-adrenergic receptors couple to PI-PLC in distal tubule cells. This is not surprising in lieu of recent evidence that suggests that α_2 receptors interact with other G proteins that are insensitive to pertussis toxin and activate signaling pathways independent of G_i coupling to adenylyl cyclase (2, 4, 5, 8, 11). Activation of α_2-adrenergic receptors stimulates PLC and PLD

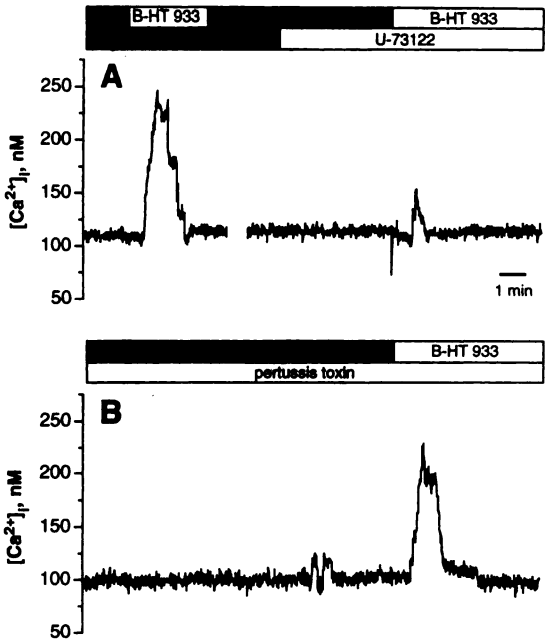

Fig. 5. α_2 Agonist-induced increase of $[Ca^{2+}]_i$ is blocked by the PLC inhibitor U-73122 but not pertussis toxin. A, 1 μM B-HT 933 causes a transient rise in $[Ca^{2+}]_i$ in an immortalized DCT cell that on washout and treatment with 10 μM U-73122 is virtually abolished. B, B-HT 933 results in a prompt rise in $[Ca^{2+}]_i$ in a DCT cell pretreated with pertussis toxin (10 ng/ml; 18 hr). *Traces*, representative of three independent experiments for each treatment.

and adenylyl cyclase as well as inhibiting adenylyl cyclase (3, 8, 11). The transient coexpression of α_{2A} receptors and $G_{\alpha i}$, $G_{\alpha q}$, and $G_{\alpha s}$ subunits demonstrates that α_2 receptor agonists not only inhibit adenylyl cyclase but also stimulate PLC and adenylyl cyclase activity (2). Using transient and stable expression systems, Cotecchia *et al.* (4) showed that α_{2A} and α_{2C} receptors can couple to inhibition of adenylyl cyclase and activation of PLC as demonstrated by phospholipid metabolism.

Several independent lines of evidence demonstrate that α_2 receptors in DCT cells couple to PI-PLC. The stimulation of PLC catalyzes the hydrolysis of membrane phosphatidylinositol-4,5-bisphosphate (and other inositol lipids) to Ins(1,4,5)P_3 and DAG. First, when either primary cultures of distal tubule cells or immortalized DCT cells were treated with α_2 receptor agonists, there was a significant increase in Ins(1,4,5)P_3 formation (Table 3 and Fig. 3). This effect was specific for α_2 but not α_1 receptor agonists. In comparison, there was no effect of α_2 agonists in primary cultures of proximal tubule cells, but α_1 agonists markedly increased Ins(1,4,5)P_3 formation in this segment, which is consistent with published data (40, 41). Second, α_2 receptor activation significantly increased DAG accumulation in distal tubule cells (Fig. 4A). Taken together, the α_2 receptor-induced increases in Ins(1,4,5)P_3 and DAG indicate the presence of both hydrolysis products because of activation of PI-PLC. Third, α_2 agonists transiently increase $[Ca^{2+}]_i$ in primary cultures of distal tubule cells and immortalized DCT cells (Fig. 2 and Table 2). This effect is not observed with α_1 agonists, and the α_2 agonist-stimulated increase of $[Ca^{2+}]_i$ is blocked by α_2 but not α_1 receptor antagonists. The rapid and transient nature of this response is due to mobilization from intracellular stores because the Ca^{2+} channel blocker nifedipine did not abolish the α_2 receptor-induced increase in $[Ca^{2+}]_i$ and a similar rise was observed when experiments were performed in Ca^{2+}-free buffer (30). Fourth, the use of the PLC inhibitor U-73122 abolished α_2 receptor-mediated increases in Ins(1,4,5)P_3 and $[Ca^{2+}]_i$ (Table 3 and Fig. 5). The increase in Ins(1,4,5)P_3 and DAG formation and $[Ca^{2+}]_i$, together with the block by U-73122, provides compelling evidence that α_2 receptors activate PI-PLC in DCT cells. In contrast, stimulation of α_2-adrenergic receptors on proximal tubule cells reduced PTH-stimulated cAMP accumulation, and the receptor-mediated stimulation was sensitive to pertussis toxin (Fig. 1); α_2 receptor agonists did not increase Ins(1,4,5)P_3 formation or produce a transient rise in $[Ca^{2+}]_i$ (Figs. 2B and 3) These findings are in accord with those reported in collecting duct cells (39), thin descending limb of Henle (42), proximal tubule (43), and OK cells (20, 44, 45) for pertussis toxin-sensitive G_i coupling and inhibition of adenylyl cyclase.

Several reports demonstrate the interaction of α_2 receptors with multiple G proteins (2, 4, 8, 11). When high levels of α_2 receptors are expressed with either transient or stable transfection into cultured cell lines, it seems that α_2 receptors are capable of activating PLC, PLD, and phospholipase A_2 in addition to inhibiting adenylyl cyclase (2, 4, 5, 11). In each of these studies, the coupling to phospholipases was pertussis toxin sensitive and mediated by G_i. Preliminary data from work in rat aorta suggest that α_2 receptors increase PLD activity independent of inhibition of adenylyl cyclase (12). The data demonstrate that activation of α_2 receptors on DCT cells does not inhibit either basal or hormone-stimulated adenylyl cyclase (Table 1 and Fig. 1) and does not express pertussis toxin-sensitive coupling to PLC (Table 3 and Fig. 5). This response is very different from that observed with α_2 receptors on proximal tubule cells in this and other reports in which α_2 receptor effects are attributed to inhibition of adenylyl cyclase and attenuated with pertussis toxin treatment (18, 20, 39, 43–46).

Several possibilities may be used to interpret the findings in DCT cells compared with α_2 receptor coupling in proximal tubule, collecting duct, and other cells. One explanation is that DCT cells express high levels of a particular α_2 receptor subtype that facilitates interaction with PLC. At present, the identification of α_2 receptors subtypes in DCT cells has not determined. However, nearly all G_i-coupled receptors, when expressed in high levels with transient or stable transfection, are capable of stimulating PLC to some degree (47). To determine the level of α_2 receptors expressed on DCT cells, binding studies were performed on immortalized DCT cell membranes with [^{3}H]rauwolscine. Preliminary data indicate comparable levels of α_2 receptor expression on DCT cells (74 fmol/mg of protein) with membranes from primary cultures of distal and proximal cells (114 and 146 fmol/mg of protein, respectively; data not shown). These levels of α_2 receptor expression are similar to those reported with enriched fractions of proximal and distal tubule cells (170 and 250 fmol/mg of protein, respectively; Ref. 48), rat cortical tubules (310 fmol/mg of protein; Ref. 49), and rat renal cortical membranes (340 fmol/mg of protein; Ref. 50). Alternatively, the levels of α_2 receptors expressed in DCT cells are 10–100-fold less than α_2 receptor expression and coupling to PLD in fibroblasts (2.8 pmol/mg of protein; Ref. 11), the activation of adenylyl cyclase in Chinese hamster ovary cells (1.5 pmol/mg

of protein; Ref. 51), and for simultaneous coupling to G_i and G_s (~5–10 pmol/mg of protein; Ref. 8).

Regardless of the level of α_2 receptor expression in DCT cells, the lack of pertussis toxin sensitivity is not consistent with coupling of the receptor to G_i. One could envision a deficiency of α_i subunits in DCT cells, but preliminary data obtained from Western blotting indicate the presence of α_i subunits 1–3 in these cells.[2] Because only $\alpha_{i/o}$ subunits have sites for ADP ribosylation by pertussis toxin, it is unlikely that G_i is responsible for α_2 receptor coupling in these cells. There is limited direct evidence that PLC can be activated by G_i; however, the recent finding that G protein $\beta\gamma$ subunits activate PLC suggests an alternative mechanism (3, 52).

A second possibility is that pertussis toxin-insensitive coupling of the α_2 receptor in DCT cells is mediated by a subunit of the G_q family of G proteins (53). Experiments using recombinant α and $\beta\gamma$ subunits show that α subunits of G_q and G_{11} stimulate PLC isozymes through a pertussis toxin-insensitive mechanism (52). The observations in DCT cells would be consistent with such a mechanism. Preliminary data obtained with Western and Northern blots indicate the presence of α_q and α_{11} subunits in DCT cells.[3] The lack of pertussis toxin sensitivity, activation of PLC, and presence of $G_q/G_{\alpha 11}$ in DCT cells would agree with this coupling mechanism. Alternatively, Blank *et al.* (54) reported that $\beta\gamma$ subunits of pertussis toxin-sensitive or -insensitive G proteins can activate PLC. With receptor occupation, G_i, G_q/G_{11}, or other G proteins are activated and release $\beta\gamma$ subunits that are responsible for the stimulation of PLC.

In summary, α_2 receptors in DCT cells couple to PI-PLC and increase Ins(1,4,5)P_3, DAG, and $[Ca^{2+}]_i$ levels. Second messenger formation is blocked by U-73122, a PLC inhibitor. In contrast to α_2 receptors located on proximal tubule cells, α_2 receptors on DCT do not inhibit adenylyl cyclase activity and are not sensitive to pertussis toxin. The lack of pertussis toxin sensitivity indicates that α_2 receptors on DCT cells are not coupled to traditional G_i proteins that have sites for ADP-ribosylation by pertussis toxin. For the present, it is unclear whether differences in α_2 receptor subtypes or G protein subunit coupling or abundance confer the characteristic signaling mediated by α_2 receptors in DCT cells.

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References

1. Limbird, L. E. Receptors linked to inhibition of adenylate cyclase: additional signaling mechanisms. *FASEB J.* **2**:2686–2695 (1988).
2. Chabre, O., B. R. Conklin, S. Brandon, H. R. Bourne, and L. E. Limbird. Coupling of the α_{2A}-adrenergic receptor to multiple G-proteins: a simple approach for estimating receptor-G-protein coupling efficiency in a transient expression system. *J. Biol. Chem.* **269**:5730–5734 (1994).
3. Milligan, G. Mechanisms of multifunctional signalling by G protein-linked receptors. *Trends Pharmacol. Sci.* **14**:239–244 (1993).
4. Cotecchia, S., B. K. Kobilka, K. W. Daniel, R. D. Nolan, E. Y. Lapetina, M. G. Caron, R. J. Lefkowitz, and J. W. Regan. Multiple second messenger pathways of α-adrenergic receptor subtypes expressed in eukaryotic cells. *J. Biol. Chem.* **265**:63–69 (1990).
5. Jones, S. B., S. P. Halenda, and D. B. Bylund. α_2-Adrenergic receptor stimulation of phospholipase A_2 and of adenylate cyclase in transfected Chinese hamster ovary cells is mediated by different mechanisms. *Mol. Pharmacol.* **39**:239–245 (1991).
6. Raymond, J. R. Multiple mechanisms of receptor-G protein signaling specificity. *Am. J. Physiol.* **269**:F141–F158 (1995).
7. Conklin, B. R., O. Chabre, Y. H. Wong, A. D. Federman, and H. R. Bourne. Recombinant $G_{q\alpha}$: mutational activation and coupling to receptors and phospholipase C. *J. Biol. Chem.* **267**:31–34 (1992).
8. Eason, M. G., H. Kurose, B. D. Holt, J. R. Raymond, and S. B. Liggett. Simultaneous coupling of α_2-adrenergic receptors to two G-proteins with opposing effects: subtype-selective coupling of α_2C10, α_2C4, and α_2C2 adrenergic receptors to G_i and G_s. *J. Biol. Chem.* **267**:15795–15801 (1992).
9. Gesek, F. A., and P. A. Friedman. Calcitonin stimulates calcium transport in distal convoluted tubule cells. *Am. J. Physiol.* **264**:F744–F751 (1993).
10. Wong, Y. H., B. R. Conklin, and H. R. Bourne. G_z-mediated hormonal inhibition of cyclic AMP accumulation. *Science (Washington D. C.)* **255**: 339–342 (1992).
11. MacNulty, E. E., S. J. McClue, I. C. Carr, T. Jess, M. J. O. Wakelam, and G. Milligan. α_2-C10 Adrenergic receptors expressed in rat 1 fibroblasts can regulate both adenylylcyclase and phospholipase D-mediated hydrolysis of phosphatidylcholine by interacting with pertussis toxin-sensitive guanine nucleotide-binding proteins. *J. Biol. Chem.* **267**:2149–2156 (1992).
12. Jinsi, A., and R. C. Deth. α_2-Adrenergic receptor coupling to phospholipase D in rat aorta and PC 12 cells. *FASEB J.* **9**:A105 (1995).
13. Nord, E. P., D. Goldfarb, N. Mikhail, P. Moradeshagi, A. Hafezi, S. Vaystub, and P. A. Insel. *Alpha*$_2$ adrenergic agonists stimulate Na^+-H^+ antiport activity in the rabbit proximal tubule. *J. Clin. Invest.* **80**:1755–1762 (1987).
14. Gesek, F. A., and J. W. Strandhoy. Dual interactions between α_2-adrenoceptor agonists and the proximal Na^+/H^+ exchanger. *Am. J. Physiol.* **258**:F636–F642 (1990).
15. Beach, R. E., S. J. Schwab, P. C. Brazy, and V. W. Dennis. Norepinephrine increases Na^+-K^+-ATPase and solute transport in rabbit proximal tubules. *Am. J. Physiol.* **252**:F215–F220 (1987).
16. Chan, Y. L. Adrenergic control of bicarbonate absorption in the proximal convoluted tubule of the rat kidney. *Pfluegers Arch. Eur. J. Physiol.* **388**: 159–164 (1980).
17. Stanton, B. A., E. Puglisi, and M. Gellai. Localization of α_2-adrenoceptor-mediated increase in renal Na^+, K^+, and water excretion. *Am. J. Physiol.* **252**:1016–1021 (1987).
18. Ribeiro, C. P., C. Pedrosa, F. Ribeiro-Neto, J. B. Field, and W. N. Suki. Prevention of α_2-adrenergic inhibition on ADH action by pertussis toxin in rabbit CCT. *Am. J. Physiol.* **253**:C105–C112 (1987).
19. Clarke, D., and L. C. Garg. *Alpha*-2 adrenergic receptors in inner medullary collecting duct cells of the rabbit kidney. *J. Pharmacol. Exp. Ther.* **264**:879–888 (1993).
20. Clarke, J. D., E. J. J. Cragoe, and L. E. Limbird. α_2-Adrenergic receptors regulate Na^+-H^+ exchange via a cAMP-dependent mechanism. *Am. J. Physiol.* **259**:F977–F985 (1990).
21. Gesek, F. A. Stimulation of α_2-adrenergic receptors increases Na^+-K^+-ATPase activity in distal convoluted tubules. *Am. J. Physiol.* **265**:F561–F568 (1993).
22. Pizzonia, J. H., F. A. Gesek, S. M. Kennedy, B. A. Coutermarsh, B. J. Backsai, and P. A. Friedman. Immunomagnetic separation, primary culture and characterization of cortical thick ascending limb plus distal convoluted tubule cells from mouse kidney. *In Vitro Cell Dev. Biol.* **27A**: 409–416 (1990).
23. Gesek, F. A., and P. A. Friedman. Mechanism of calcium transport stimulated by chlorothiazide in mouse distal convoluted tubule cells. *J. Clin. Invest.* **90**:429–438 (1992).
24. Gesek, F. A., and P. A. Friedman. On the mechanism of parathyroid hormone stimulation of calcium uptake by mouse distal convoluted tubule cells. *J. Clin. Invest.* **90**:749–758 (1992).
25. Nahorski, S. R., C. I. Ragan, and R. A. J. Challiss. Lithium and the phosphoinositide cycle: an example of uncompetitive inhibition and its pharmacological consequences. *Trends Pharmacol. Sci.* **12**:297–303 (1991).
26. Wojcikiewicz, R. J. H., A. B. Tobin, and S. R. Nahorski. Desensitization of cell signalling mediated by phosphoinositidase C. *Trends Pharmacol. Sci.* **14**:279–285 (1993).
27. Abdel-Latif, A. A. Calcium-mobilizing receptors, polyphosphoinositides, and the generation of second messengers. *Pharmacol. Rev.* **38**:227–272 (1986).
28. Berridge, M. J., and R. J. Irvine. Inositol triphosphate, a novel second messenger in cellular transduction. *Nature (Lond.)* **312**:315–321 (1984).
29. Yasuda, I., A. Kishimoto, S. Tanaka, M. Tominaga, A. Sakurai, and Y. Nishizuka. A synthetic peptide substrate for selective assay of protein kinase C. *Biochem. Biophys. Res. Commun.* **166**:1220–1227 (1990).
30. Putney, J. W., Jr., and G. St. J. Bird. Calcium mobilization by inositol phosphates and other intracellular messengers. *Trends Endocrinol. Metab.* **5**:256–260 (1994).
31. Dennis, E. A., S. G. Rhee, M. Billah, and Y. A. Hannun. Role of phospholipases in generating lipid second messengers in signal transduction. *FASEB J.* **5**:2068–2077 (1991).
32. Exton, J. H. Phosphoinositide phospholipases and G proteins in hormone action. *Annu. Rev. Physiol.* **56**:349–369 (1994).
33. Martin, K. J., C. L. McConkey, A. K. Jacob, E. A. Gonzalez, M. Khan, and J. J. Baldassare. Effect of U-73,122, an inhibitor of phospholipase C, on

[2] F. A. Gesek, unpublished observations.
[3] F. A. Gesek, unpublished observations.

actions of parathyroid hormone in opossum kidney cells. *Am. J. Physiol.* **266:**F254–F258 (1994).
34. Tatrai, A., S. K. Lee, and P. H. Stern. U-73122, a phospholipase C antagonist, inhibits effects of endothelin-1 and parathyroid hormone on signal transduction in UMR-106 osteoblastic cells. *Biochim. Biophys. Acta* **1224:** 575–582 (1994).
35. Weiss, B. A., and P. A. Insel. Intracellular Ca^{2+} and protein kinase C interact to regulate α_1-adrenergic- and bradykinin receptor-stimulated phospholipase A_2 activation in Madin-Darby canine kidney cells. *J. Biol. Chem.* **266:**2126–2133 (1991).
36. Aboolian, A., M. Vander Molen, and E. P. Nord. Differential effects of phorbol esters on PGE_2 and bradykinin-induced elevation of $[Ca_i^{2+}]$ in MDCK cells. *Am. J. Physiol.* **256:**F1135–F1143 (1989).
37. Figueroa, C. D., C. B. Gonzalez, S. Grigoriev, S. A. Abd Alla, M. Haasemann, K. Jarnagin, and W. Muller-Esterl. Probing for the bradykinin B2 receptor in rat kidney by anti-peptide and anti-ligand antibodies. *J. Histochem. Cytochem.* **43:**137–148 (1995).
38. Ruffolo, R. R., A. J. Nichols, J. M. Stadel, and J. M. Hieble. Structure and function of α-adrenoceptors. *Pharmacol. Rev.* **43:**475–505 (1991).
39. Gellai, M., and R. M. Edwards. Mechanism of α_2-adrenoceptor agonist-induced diuresis. *Am. J. Physiol.* **255:**F317–F323 (1988).
40. Wirthensohn, G., and W. G. Guder. Stimulation of phospholipid turnover by angiotensin II and phenylephrine in proximal convoluted tubules microdissected from mouse nephron. *Pfluegers Arch. Eur. J. Physiol.* **404:** 94–96 (1985).
41. Neylon, C. B., and R. J. Summers. Simulation of α_1-adrenoceptors in rat kidney mediates increased inositol phospholipid hydrolysis. *Br. J. Pharmacol.* **91:**367–376 (1987).
42. Umemura, S., D. D. Smyth, and W. A. Pettinger. Regulation of renal cellular cAMP levels by prostaglandins and α_2-adrenoceptors: microdissection studies. *Kidney Int.* **29:**703–707 (1986).
43. Umemura, S., D. Marver, D. D. Smyth, and W. A. Pettinger. α_2-adrenoceptors and cellular cAMP levels in single nephron segments from the rat. *Am. J. Physiol.* **249:**F28–F33 (1985).
44. Murphy, T. J., and D. B. Bylund. Characterization of *alpha*-2 adrenergic receptors in the OK cell, an opossum kidney cell line. *J. Pharmacol. Exp. Ther.* **244:**571–578 (1988).
45. Cheng, L., T. Liang, P. Precht, and B. Sacktor. *Alpha*-2-adrenergic modulation of the parathyroid-hormone-inhibition of phosphate uptake in cultured renal (OK) cells. *Biochem. Biophys. Res. Commun.* **155:**74–82 (1988).
46. Strandhoy, J. W., and F. A. Gesek. Stimulatory and inhibitory interactions between α-adrenoceptors and the renal Na/H antiporter (Abstract). *Kidney Int.* **35:**488A (1989).
47. Zhu, X., S. Gilbert, M. Birnbaumer, and L. Birnbaumer. Dual signaling potential is common among G_s-coupled receptors and dependent on receptor density. *Mol. Pharmacol.* **46:**460–490 (1994).
48. Sundaresan, P. R., T. L. Fortin, and S. L. Kelvie. α- and β-adrenergic receptors in proximal tubules of rat kidney. *Am. J. Physiol.* **253:**F848–F856 (1987).
49. Sundaresan, P. R., M. Barac-Nieto, G. W. Stambo, and S. L. Kelvie. Glomerular and tubular α_1- and α_2-adrenoceptors in the rat kidney: distribution in basolateral and brush border membranes of tubular cells. *J. Cardiovasc. Pharmacol.* **13:**16–24 (1989).
50. Insel, P. A., M. D. Snavely, D. P. Healy, P. A. Munzel, C. L. Potenza, and E. P. Nord. Radioligand binding and functional assays demonstrate postsynaptic *alpha*$_2$-receptors on proximal tubules of rat and rabbit kidney. *J. Cardiovasc. Pharmacol.* **7** (Suppl. 8)**:**S9–S17 (1985).
51. Fraser, C. M., S. Arakawa, W. R. McCombie, and J. C. Venter. Cloning, sequence analysis, and permanent expression of a human α_2-adrenergic receptor in Chinese hamster ovary cells. *J. Biol. Chem.* **264:**11754–11761 (1989).
52. Boyer, J. L., S. G. Graber, G. L. Waldo, T. K. Harden, and J. C. Garrison. Selective activation of phospholipase C by recombinant G-protein α- and $\beta\gamma$-subunits. *J. Biol. Chem.* **269:**2814–2819 (1994).
53. Wilkie, T. M., P. A. Scherle, M. P. Strathmann, V. Z. Slepak, and M. I. Simon. Characterization of G-protein α subunits in the G_q class: expression in murine tissues and in stromal and hematopoietic cell lines. *Proc. Natl. Acad. Sci. USA* **88:**10049–10053 (1991).
54. Blank, J. L., K. A. Brattain, and J. H. Exton. Activation of cytosolic phosphoinositide phospholipase C by G-protein $\beta\gamma$ subunits. *J. Biol. Chem.* **267:**23069–23075 (1992).

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