Mechanism of Activation of Protein Kinase D2 (PKD2) by the CCKB/Gastrin Receptor

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Recently, we cloned a novel serine/threonine kinase termed protein kinase D2 (PKD2). PKD2 can be activated by phorbol esters both in vivo and in vitro but also by gastrin via the cholecystokinin/CCKB receptor in human gastric cancer cells stably transfected with the CCKB/gastrin receptor (AGS-B cells). Here we identify the mechanisms of gastrin-induced PKD2 activation in AGS-B cells. PKD2 phosphorylation in response to gastrin was rapid, reaching a maximum after 10 min of incubation. Our data demonstrate that gastrin-stimulated PKD2 activation involves a heterotrimeric Gs protein as well as the activation of phospholipase C. Furthermore, we show that PKD2 can be activated by classical and novel members of the protein kinase C (PKC) family such as PKCα, PKCe, and PKCγ. These PKCs are activated by gastrin in AGS-B cells. Thus, PKD2 is likely to be a novel downstream target of specific PKCs upon the stimulation of AGS-B cells with gastrin. Our data suggest a two-step mechanism of activation of PKD2 via endogenously produced diacylglycerol and the activation of PKCs.

We recently described the cloning of a novel human serine/threonine kinase termed PKD2 (1). This kinase exhibits a high homology to PKD/PKCμ and PKCβ but not to the classical, novel, and atypical members of the protein kinase C family. PKD2 has two cysteine-rich domains at its N terminus that bind phorbol esters and diacylglycerol and is potently activated by phorbol esters in vitro and in vivo. In addition, gastrin activates PKD2 via the CCKB/gastrin receptor (also termed the CCK2 receptor). However, the precise mechanism leading to the activation of PKD2 in response to gastrin has not been characterized.

Gastrin is implicated in a wide range of fundamental biological responses such as secretion, growth, and neoplastic transformation (2). Therefore, there is considerable interest in the elucidation of signaling pathways mediated by the CCKB/gastrin receptor in nontransformed and transformed cells. Gastrin, CCK, and CCK-related peptides exert their effects by binding to specific G protein-coupled receptor subtypes. The CCKB/gastrin receptor binds gastrin and CCK with similar affinity, whereas the CCKA (also called CCK1) receptor exhibits a 500-fold higher affinity for CCK than for gastrin (3). Upon binding to the CCKB/gastrin receptor, gastrin stimulates multiple signaling pathways. One of the earliest signal transduction pathways activated in response to gastrin is the stimulation of β isoforms of PLC, leading to IP3-mediated Ca2+-mobilization from internal stores and DAG-induced stimulation of classical and novel isoforms of the protein kinase C family.

In this manuscript we identify the precise signaling mechanism leading to activation of PKD2 in response to gastrin. We show that the activation of PKD2 by gastrin in AGS-B cells involves a Gs heterotrimeric G protein, PLC, and possibly various PKC isoforms, making PKD2 a novel downstream target of members of the PKC family.

EXPERIMENTAL PROCEDURES

Materials—[γ-32P]ATP (5000 Ci/mmol; 37 GBq = 1 mCi) was from Amer sham Biosciences. The polyclonal antibodies directed against the C terminus of PKD2 and the phosphorylated serine at position 876 (Ser(P)876) in PKD2 have been described previously (1). The polyclonal phospho-PKD1/PKC/PKCα Ser844/Ser846 antibody was obtained from Cell Signaling Tech. This antibody was raised against a synthetic phospho-Ser844/Ser846 peptide corresponding to residues surrounding Ser844/Ser846. This sequence is identical in PKD and PKD2, with the corresponding serine residues in PKD2 being Ser706 and Ser710. Antibodies directed against PKCα, PKCβ, PKCe, and PKCγ were from BD Transduction Laboratories. Phosphospecific antibodies against PKCα (Ser857) were from Upstate Biotechnology, and those against PKCe (Thr885) were from New England Biolabs. All other reagents were from standard suppliers as indicated in the text.

Cell Culture—Stock cultures of HEK293, AGS-B, and CX-1 cells were maintained in DMEM supplemented with 10% fetal bovine serum in a humidified atmosphere containing 10% CO2 at 37 °C. AGS-B cells are AGS human gastric cancer cells (American Type Culture Collection) stably transfected with the expression construct CCKB-pcDNA1-neo comprising the full-length coding region of the human CCKB/gastrin receptor and the neomycin resistance gene as described previously (4). cDNA Expression Vectors and AGS-B and HER293 Cell Transfection—The constitutively active PKC clones α, β, δ, ε, and η subcloned into the pCAG vector were obtained from Dr. P. J. Parker, Protein Phosphorylation Laboratory, Imperial Cancer Research Fund. Briefly, the constitutively active mutants were generated by the deletion of critical amino acids from the autoinhibitory pseudosubstrate region of the full-length PKC clones as described previously (5, 6). Exponentially growing AGS-B or HEK293 cells (5 × 106 cells/35-mm dish) were transfected with minigene constructs targeting Gαq, Gα11, Gα12, Gα13, Gα20, or Gαi3 (7), the respective PKC isoform or FLAG-tagged PKD2 as indicated in the Figs. 2, 4, and 5 legends, using FuGENE as described in detail by the manufacturer (Roche Molecular Biochemicals). Briefly,

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1.5 μg of DNA was used for each 35-mm dish. FuGENE was diluted with 150 μl of DMEM and, after 5 min, added to the DNA. After another 15 min of incubation with gentle mixing, the DNA-FuGENE complex in DMEM was added to AGS-B or HEK293 cells in DMEM. The cells were used for experimental purposes 48 h later.

Western Blotting and Immunoprecipitations—For detection of the PKD2 protein, membranes were blocked using 3% nonfat dried milk in phosphate-buffered saline (pH 7.2) and incubated for 2 h with anti-FLAG monoclonal antibody (1:200), or polyclonal PKD2 antibody (1:200), or polyclonal Ser(P)876 antibody (1:2000) in phosphate-buffered saline containing 3% nonfat dried milk. For detection of PKD2 phosphorylated at Ser706 and Ser710, the PKD2 antisera (1:100 dilution), the Ser(P)876 PKD2 antisera (1:200), or the monoclonal FLAG antibody (1:440) was used according to the manufacturer’s instructions (1:1000). Immunoreactive bands were visualized using horseradish peroxidase-conjugated anti-mouse IgG (for the anti-FLAG antibody) or anti-rabbit IgG (for the anti-PKD2 or Ser(P)876 antibodies) and subsequent enhanced chemiluminescence. For immunoprecipitations, HEK293 or AGS-B cells in 35-mm dishes transfected with various plasmids as described in the Figs. 2, 4, and 5 legends were washed twice in ice-cold PBS and lysed in a solution containing 50 mM Tris/HCl, pH 7.6, 2 mM EGTA, 2 mM EDTA, 2 mM dithiothreitol, protease inhibitors (aprotinin (10 μg/ml), leupeptin (100 μg/ml), pepstatin (0.7 μg/ml), and 1 mM 4-(2-aminoethyl)-benzenesulfonyl fluoride hydrochloride) and 1% Triton X-100 (lysis buffer I). Proteins were immunoprecipitated with the PKD2 antisera (1:100 dilution), the Ser(P)876 PKD2 antisera (1:200), or the monoclonal FLAG antibody precoupled to agarose (Sigma) or polyclonal antibodies directed against PKCa, PKCe, and PKγ as described in the Figs. 1–5 legends. The immune complexes were recovered using protein A-Sepharose where appropriate. For the immunoprecipitations of endogenous PKD2 we used 1 μg of protein per condition. Chemiluminescence signals were quantified using the LAS-1000 system (Fujifilm).

Elution of PKD2 from Immunocomplexes—Immunoprecipitates were prepared as described above and washed once with lysis buffer I and twice with lysis buffer II (lysis buffer I was without Triton X-100). PKD2 was then eluted at 4°C for 30 min by batchwise incubation of the immunoprecipitates with 0.5 mg/ml immunizing peptide in lysis buffer II (elution buffer; 4 volumes of elution buffer to 1 volume of protein A-agarose).

In Vitro Kinase Assay—For experiments investigating the effect of GF I on the in vitro kinase activity of PKD2, the kinase was eluted from the immunocomplexes as described above. 20 μl of the eluate were mixed with 20 μl of phosphorylation mix containing 100 μM [γ-32P]ATP (specific activity 400–600 cpm/pmol), 30 mM Tris, pH 7.4, 30 mM MgCl2, 1 mM dithiothreitol, and 250 mM PDBu and 100 μg/ml PS. The mixture was subsequently incubated for 10 min at 30°C. Reactions were terminated by adding an equal amount of 2× SDS-PAGE sample buffer (1 M Tris/HCl, pH 6.8, 0.1 mM Na3VO4, 6% sodium dodecyl sulfate, 0.5 M EDTA, pH 8.0, 4% 2-mercaptoethanol, and 10% glycerol) and were further analyzed by SDS-PAGE. To examine PKD2 autokinase activity, histone phosphorylation by PKD2, and the autokinase activity of the various PKC isoforms, the respective kinase was not eluted. Immunocomplexes were washed twice with lysis buffer I, twice with lysis buffer II, and twice with kinase buffer (30 mM Tris/HCl, pH 7.4, 10 mM MgCl2, and 1 mM dithiothreitol) and then resuspended in 30 μl of kinase buffer in the presence (for histone phosphorylation) or absence (for autokinase activity) of 0.5 mg/ml histone H1 and 100 μM [γ-32P]ATP. Reactions were incubated for 10 min at 30°C, terminated by adding an equal amount of 2× SDS-PAGE sample buffer, and further analyzed by SDS-PAGE followed by autoradiography. Autoradiographs were further analyzed by scanning densitometry using the MacBAS V2.5 software (Fuji Photo Film, 1996).

RESULTS

The CCKB/Gastrin Receptor Mediates Rapid Activation of PKD2—We have demonstrated that the activation of PKD2 can be efficiently monitored by examining serine phosphorylation at pSer876 in PKD2 (1). To determine the kinase of gastrin-induced PKD2 activation, AGS-B cells were transfected with 100 nM gastrin for various times and further analyzed by SDS-PAGE and Western blotting using the Ser(P)876 antibody. As shown in Fig. 1A, gastrin induced a rapid phosphorylation of PKD2 at Ser876 detectable as early as 2.5 min after the addition of gastrin to the cells and still detectable after 60 min of stimulation. The increase in Ser876 phosphorylation was paralleled by an increase in histone phosphorylation in PKD2 kinase assays. A maximum 8-fold increase in histone phosphorylation of PKD2 at Ser876 in AGS-B cells. Serum-starved AGS-B cells were treated with 100 nM gastrin for the times indicated and lysed, and the lysates were further analyzed by Western blotting with the Ser(P)876 antibody as described under “Experimental Procedures.” Right panel, the activation of PKD2 by gastrin in human CX-1 colon carcinoma cells. CX-1 cells were transfected with 100 nM gastrin (G) or 200 nM PDBu (P) for 10 min. Cells were lysed, and the lysates were further analyzed using the Ser(P)876 (pSer876) antibody. The position of PKD2 phosphorylated at Ser876 is indicated by an arrowhead. B, the kinetics of PKD2-induced histone phosphorylation in response to gastrin in AGS-B cells. Serum-starved AGS-B cells were treated for various times with 100 nM gastrin and lysed. The lysates were immunoprecipitated with anti-PKD2 antibody, and PKD2 activity was measured by a histone kinase assay followed by SDS-PAGE and autoradiography. The position of phosphorylated histone (Hist) is indicated by an arrowhead. The level of histone phosphorylation was quantified by scanning densitometry as described under “Experimental Procedures.” Values shown are expressed as fold increase in PKD2 kinase activity above controls and are the means ± S.E. of three independent experiments.

Gastrin-induced Activation of PKD2 Involves a Heterotrimeric Go Protein in AGS-B Cells—The CCKB receptor can couple to various heterotrimeric G proteins such as Gα11, Gα12, and Gα16 (8). The coupling of a G protein-coupled receptor to specific heterotrimeric G proteins can be examined by the expression of minigene plasmid constructs that encode oligonucleotide sequences corresponding to the C-terminal undecapeptide of distinct α subunits (7). Transfection of the vectors leads to an inability of a receptor to couple to that G protein via an autoinhibitory mechanism. To directly assess which G protein subunit was involved in the gastrin-induced activation of PKD2, AGS-B cells were transfected with various minigenes to block the activation of specific endogenous Go subunits prior to incubation of the cells with gastrin for 10 min. Activation of PKD2 was further analyzed using the Ser(P)876 antibody. As shown in Fig. 2A (upper panel), only the minigene targeting
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Fig. 2. A, Go heterotrimeric G protein is involved in gastrin-induced PKD2 activation. Serum-starved AGS-B cells were transfected with empty vector (−) or with expression plasmids containing cDNAs encoding the various C-terminal G protein α-subunit minigenes as indicated. These minigene vectors express a peptide that specifically blocks receptor activation of the G protein. 48 h after transfection cells were treated with 100 nM gastrin for 10 min or received an equivalent amount of solvent (−), they were subsequently lysed, and the lysates were further analyzed by Western blotting with the Ser(P)876 (pSer876) antisera (upper panel) as described under “Experimental Procedures.” Equal expression of PKD2 protein in each condition was determined using the anti-PKD2 antiserum (lower panel). The positions of PKD2 phosphorylated at Ser876 and the PKD2 protein are indicated by arrowheads. Similar results were obtained in three independent experiments. B, a Go heterotrimeric G protein is involved in the gastrin-induced activation of PKCa. AGS-B cells were treated as described above and lysed. Lysates were further analyzed by Western blotting using a PKCa Ser(P)657 (pSer657) antisera (upper panel) or an anti-PKCα antibody (lower panel). The positions of PKCa phosphorylated at Ser657 and the PKCa protein are indicated by arrowheads.

Go reduced gastrin-induced activation of PKD2. Thus, PKD2 activation in response to gastrin involves the activation of a Go heterotrimeric G protein.

Go regulates the activation of various PKC isoforms including PKCa (9). To compare the role of Go as an upstream regulator of PKD2 and PKC activation, AGS-B cells were transfected with the Go minigene construct, and the activation of PKCa was subsequently analyzed using an activation-specific antibody that selectively detects active PKCa phosphorylated at Ser657 (10, 11). As shown in Fig. 2B (upper panel), the minigene targeting Go prevented gastrin-induced phosphorylation of PKCa at Ser657. Thus, Go is an upstream regulator of both PKCa and PKD2.

Activation of PKD2 by Gastrin Is Dependent on Phospholipase C Activity—One of the earliest events in the signal transduction pathways mediated by the CCK2 receptor after the activation of a heterotrimeric G protein is the activation of the β-isofrom of PLC producing two second messengers, IP3 and DAG. IP3 binds to its intracellular receptor and triggers the release of Ca2+ from internal stores. DAG, the other second messenger, activates members of the PKC family but also members of the PKD family (12). Because the same heterotrimeric G protein can regulate the activation of PKCs and PKD2, we wanted to examine whether gastrin-induced activation of PKD2 in AGS-B cells also requires phospholipase C activation.

AGS-B cells were incubated with increasing concentrations of the selective phospholipase C inhibitor ET18OCH3 (13) prior to stimulation with gastrin. As shown in Fig. 3A (upper panel), the PLC inhibitor blocked PKD2 phosphorylation at Ser876 in a concentration-dependent manner. Half-maximum and maximal inhibition of PKD2 activation were achieved at 6.4 and 20 μM ET18OCH3, respectively.

Recently, Ser744 and Ser748 were identified as in vivo transphosphorylation sites within the activation loop of PKD using a phosphospecific antibody that recognizes activated PKD dually phosphorylated at Ser744 and Ser748 (14). Interestingly, the sequence used to generate this antibody is identical in PKD and PKD2, with the corresponding serine residues being Ser706 and Ser710 in PKD2. Because AGS-B cells express very little PKD/PKCμ as compared with PKD2, we wanted to

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examine whether gastrin could stimulate phosphorylation of Ser766/Ser710 in PKD2 using this antibody. As shown in Fig. 3A (lower panel), only a single band of phosphorylated proteins running at 105 kDa (the molecular mass of PKD2) was detectable in lysates of AGS-B cells stimulated with gastrin. In parallel experiments, the antibody also detected FLAG-tagged PKD2 transfected into HEK293 cells after stimulation with PDBu (data not shown and Fig. 5B). Gastrin-stimulated phosphorylation of PKD2 on Ser766/Ser710 was also prevented by the incubation of the cells with the PLC inhibitor at concentrations comparable with those required to inhibit PKD2 autokinase activity and Ser766 phosphorylation of PKD2. Accordingly, ET18OCH3 inhibited PKD2 autokinase activity at similar concentrations (Fig. 3B, upper panel). At the concentrations used, ET18OCH3 did not affect the expression of PKD2 protein and had no effect on the viability of AGS-B cells (Fig. 3B, lower panel, and data not shown).

Role of PKCs in Gastrin-induced PKD2 Activation in AGS-B Cells—DAG is endogenously produced after the stimulation of cells with gastrin and activates members of the PKC family. Interestingly, the search of a novel data base (website: scansite.mit.edu; Ref. 15) for sequence motifs within PKD2 likely to be phosphorylated by specific protein kinases revealed putative phosphorylation sites in PKD2 for PKCα (Ser2090) and PKCε (Thr1117). Thus, PKD2 could be a downstream target of PKCs. To determine whether PKCs could be involved in the activation of PKD2 in response to gastrin, AGS-B cells were treated with various concentrations of the PKC inhibitors GF I and Ro 318220 prior to stimulation of the cells with gastrin. As shown in Fig. 4A, PKD2 autokinase activity and histone phosphorylation in response to gastrin were inhibited in a concentration-dependent manner by both inhibitors. Half-maximum and maximum inhibition of PKD2 autokinase activity as well as histone phosphorylation were achieved at 0.8 and 5 μM Ro 318220 and 2 and 5 μM GF I, respectively. 5 μM GF I inhibited PKD2 kinase activity by about 70%, whereas in cells treated with 5 μM Ro 318220, PKD2 kinase activity was virtually abolished. GF I and Ro 318220 also inhibited gastrin-induced phosphorylation of PKD2 at Ser576 at similar concentrations.

Both inhibitors, however, can inhibit other protein kinases in addition to PKCs (16) and could therefore directly inhibit PKD2. To determine the effect of both inhibitors on the kinase activity of PKD2 stimulated in vitro, HEK293 cells were transfected with a FLAG-tagged PKD2 expression plasmid, and the immunoprecipitated kinase was activated in vitro with PS and PDBu in the presence or absence of Ro 318220 or GF I. As shown in Fig. 4C, both GF I and Ro 318220 indeed inhibited the autokinase activity of PKD2 by about 75% and 70%, respectively. Interestingly, histone phosphorylation by PS/PDBu-activated PKD2 was virtually abolished by the treatment of cells with the same concentration of GF I or Ro 318220. Thus, using these inhibitors we could not differentiate between activation of PKC and PKD2.

PKCs α, ε, and η Mediate the Activation of PKD2—We therefore wanted to assess directly whether PKC isoforms could activate PKD2. cDNAs corresponding to PKD2 and constitutively activated PKC isoforms were transiently coexpressed in HEK293 cells, and PKD2 kinase activity was determined in the cell lysates 48 h after transfection. Our results demonstrate that PKCα, PKCε, and PKCη potently activate PKD2 autokinase activity (Fig. 5A). Similarly, these PKC isoforms also stimulated phosphorylation of Ser576 and Ser766/Ser710 in PKD2 (Fig. 5B). The activation of PKD2 by PKCη and PKCε was comparable with the effect of a maximum efficient concentration of PDBu. PKCα-induced PKD2 autokinase activity was
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Gastrin induces multiple intracellular signaling pathways (17) that mediate various biological functions including growth and neoplastic transformation (2). Indeed, gastrin has been implicated in the development of colorectal cancers that produce gastrin-related peptides and express CCKB receptors (18–20). Therefore, there is considerable interest in the elucidation of signaling pathways mediated by the CCKB/gastrin receptor in tumor cells.

Recently, we cloned a novel serine/threonine kinase termed PKD2 because of the high homology of this kinase with PKD/ PKCμ. In the present manuscript we have identified signaling pathways mediating PKD2 activation in response to the binding of gastrin to the CCKB receptor in human gastric cancer cells. Using AGS-B cells, a human gastric cancer cell line stably transfected with the CCKB/gastrin receptor (4), we demonstrate that gastrin rapidly activates PKD2 by a Gαq-dependent signaling pathway. Our data show that endogenously expressed CCKB receptors also mediate the activation of PKD2, e.g. in CX-1 colorectal cancer cells. Further-

**Fig. 5.** A, the effect of constitutively activated PKCa, PKCβ, PKCe, and PKCγ on PKD2 activity in cotransfected HEK293 cells. Left panel, HEK293 cells were cotransfected with FLAG-tagged PKD2 in pcDNA3 and either empty vector (−) or expression plasmids containing the cDNAs encoding the constitutively activated PKC isoforms as indicated. 48 h after transfection, cells were treated with 200 nM PDBu for 10 min (PDBu, +) or received an equivalent amount of solvent (−). The cells were then lysed, and the lysates were immunoprecipitated with antibody. B, the effect of constitutively activated PKCa, PKCβ, PKCe, and PKCγ on phosphorylation of PKD2 at Ser876 and Ser706/Ser710 in cotransfected HEK293 cells. HEK293 cells were cotransfected with FLAG-tagged PKD2 in pcDNA3 and either empty vector (−) or expression plasmids containing the cDNAs encoding the constitutively active PKC isoforms as indicated. 48 h after transfection, cells were treated with 200 nM PDBu for 10 min (PDBu, +) or received an equivalent amount of solvent (−). The cells were then lysed, and the lysates were immunoprecipitated with antibody. C, the effect of constitutively activated PKCa, PKCβ, PKCe, and PKCγ on phosphorylation of PKD2 at Ser876 and Ser706/Ser710 in cotransfected HEK293 cells. HEK293 cells were cotransfected with FLAG-tagged PKD2 in pcDNA3 and either empty vector (−) or expression plasmids containing the cDNAs encoding the constitutively active PKC isoforms as indicated. 48 h after transfection, cells were treated with 200 nM PDBu for 10 min (PDBu, +) or received an equivalent amount of solvent (−). The cells were then lysed, and the lysates were immunoprecipitated with antibody.

**Fig. 6.** Model of activation of PKD2 by the CCKB/gastrin receptor. Binding of gastrin to its receptor activates a heterotrimeric Gαq protein, which in turn activates phospholipase C leading to the production of IP3 and DAG. DAG in turn activates PKCs α, ε, and γ, which induce activation of PKD2 in AGS-B cells. Thus, PKD2 is a novel mediator in the signal transduction network induced by the CCKB/gastrin receptor and a novel downstream target of certain members of the PKC family.

**PKCγ**

The phosphorylation of Ser876 was markedly more pronounced than the phosphorylation of Ser706/Ser710.

The activation of PKD2 by the PKC isoforms was specific. Constitutively activated PKCδ did not stimulate PKD2 autokinesis activity and had no effect on the phosphorylation of Ser876 and Ser706/Ser710 in PKD2 (Fig. 5B). This demonstrates that specific conventional and novel PKCs are potential upstream activators of PKD2.

**Gastrin Activates Various PKC Isoforms in AGS-B Cells**—To clarify whether PKCa, PKCe, and PKCγ could be mediators of gastrin-stimulated PKD2 activation, it was important to determine whether gastrin activates these PKCs in AGS-B cells. Therefore, we examined the kinase activity of these PKCs in response to gastrin. As shown in Fig. 5C, gastrin could activate PKCa, PKCe, and PKCγ in AGS-B cells. Activation of these PKCs by gastrin was comparable with the effect of a maximum efficient concentration of PDBu. Thus, these PKC isoforms could mediate gastrin-induced PKD2 activation, establishing a novel signaling cascade elicited by the CCKB/gastrin receptor (Fig. 6). Interestingly, gastrin also activated PKDδ in AGS-B cells. The effect of gastrin on PKCδ activation was also comparable with that of PDBu (Fig. 5C, lower panel). This shows that gastrin can activate multiple PKC isoforms in AGS-B cells, but only specific isoforms of the PKC family mediate gastrin-induced activation of PKD2.

**DISCUSSION**

The phosphorylation of Ser876 and Ser706/Ser710 was comparable upon stimulation of cells with PDBo. However, in cells transfected with PKCe or

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more, in addition to Ser\textsuperscript{876}, we have identified Ser\textsuperscript{706} and Ser\textsuperscript{710} as in vivo phosphorylation sites in PKD2 in response to gastrin. These residues correspond to Ser\textsuperscript{744} and Ser\textsuperscript{748} in PKD. The sequence within the activation loop surrounding Ser\textsuperscript{706} and Ser\textsuperscript{710} in PKD2 (1GEKpsFRpRSVGGT) is identical to the corresponding sequence in PKD. In PKD the phosphorylation of Ser\textsuperscript{744} and Ser\textsuperscript{748} is mediated through a PKC-dependent pathway (14). In addition, a search for sequence motifs within PKD2 likely to be phosphorylated by specific protein kinases in a novel data base (cansite.mit.edu; Ref. 15) revealed putative phosphorylation sites for PKC\textit{C} and PKC\textit{E}. Treatment of intact cells with two different PKC inhibitors, GF I and Ro 318220, prevented gastrin-induced PKD2 autokinase activity, histone phosphorylation, and the phosphorylation of PKD2 at Ser\textsuperscript{706} and Ser\textsuperscript{708}/Ser\textsuperscript{710}. However, both inhibitors also prevented PKD2 activation in vitro by PS/PDBu. This is in marked contrast to PKD; PS/PDBu-stimulated PKD activity is not affected by these inhibitors (21, 22). Thus, despite the high 98% sequence homology of the kinase domains of PKD2 and PKD (1), there seems to be a functional difference in the catalytic domains with regard to their sensitivity toward inhibitors. In addition, there also seems to be a difference in the substrate specificity of PKD2 and PKD; histone is only poorly phosphorylated by PKD/PKC\textit{\mu} (23) but very efficiently phosphorylated by PKD2 (1). At present, we are trying to clarify this point by three-dimensional structural analysis of the catalytic domains of PKD and PKD2, respectively.

Despite the fact that PKC inhibitors were not suitable to differentiate between PKC and PKD2 kinase activity, we provide direct evidence that PKC\textit{\alpha}, PKC\textit{\epsilon}, and PKC\textit{\eta} are potential upstream activators of PKD2; in particular, the activation of PKD2 in response to PKC\textit{\alpha} and PKC\textit{\eta} was comparable with the activation by PDBu. Interestingly, PKC\textit{\alpha}, like PDBu, induced a similar level of phosphorylation of PKD2 at Ser\textsuperscript{706} and Ser\textsuperscript{708}/Ser\textsuperscript{710}, respectively. In contrast, the phosphorylation of Ser\textsuperscript{706} in response to PKC\textit{\epsilon} or PKC\textit{\eta} was more pronounced than the phosphorylation of Ser\textsuperscript{706}/Ser\textsuperscript{710}. This suggests that the phosphorylation of Ser\textsuperscript{706}/Ser\textsuperscript{710} in PKD2 reflects the activation of the kinase, but the degree of Ser\textsuperscript{706}/Ser\textsuperscript{710} phosphorylation does not necessarily correspond to the level of PKD2 activation. The effect of PKCs on PKD2 activation is specific. Constitutively activated PKC\textit{\beta} failed to stimulate PKD2 autokinase activity. This does not exclude the fact that PKD2 could be phosphorylated by PKC\textit{\beta} as suggested by the scansite results without affecting PKD2 kinase activity. Whereas PKC\textit{\epsilon} and PKC\textit{\eta} are also activating PKD/PKC\textit{\mu} (21, 22), PKC\textit{\alpha} has not been shown to induce PKD/PKC\textit{\mu} activation, suggesting that there could be differences in the upstream regulators of PKD2 and PKD/PKC\textit{\mu}. Finally, our data demonstrate that gastrin activates PKC\textit{\alpha}, PKC\textit{\epsilon}, and PKC\textit{\eta} in AGS-B cells. Interestingly, we could detect an increase in phosphorylation/activation of PKCs after treatment of AGS-B cells with gastrin or PDBu not only by using activation-specific antibodies but also in immune complex kinase assays. This finding contrasts somewhat with studies (24) indicating that some PKCs exist already in the fully phosphorylated state in resting cells. This discrepancy could be partly explained by the fact that in our serum-starved AGS-B cells we find a rather low basal level of PKC phosphorylation, which can be markedly increased upon the incubation of cells with gastrin or PDBu. Indeed, similar results have been demonstrated for PKC\textit{\delta} in serum-starved HEK 293 cells (25). Furthermore, there is evidence that the phosphorylation of some PKCs such as PKC\textit{\epsilon} is primarily regulated rather than constitutive (26). It is interesting to note that both gastrin and PDBu also activated PKC\textit{\delta} in the AGS-B cells as demonstrated in immune complex kinase assays or by using an activation-specific antibody that selectively detects PKC\textit{\delta} phosphorylated at Thr\textsuperscript{505}. This suggests that only certain isoforms of the PKC family mediate gastrin-induced activation of PKD2. In conclusion, we envisage a novel signaling cascade downstream of the CCK\textsubscript{\textit{B}} receptor involving Ga\textsubscript{q} and phospholipase C and PKC\textit{\epsilon}, PKC\textit{\eta}, or PKC\textit{\delta}, leading to the activation of the novel serine/threonine kinase PKD2.

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