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CID755673 enhances mitogenic signaling by phorbol esters, bombesin and EGF through a protein kinase D-independent pathway

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ABSTRACT

Recently, CID755673 was reported to act as a highly selective inhibitor of protein kinase D (PKD). In the course of experiments using CID755673, we noticed that it exerted unexpected stimulatory effects on [^{3}H]thymidine incorporation and cell cycle progression in Swiss 3T3 cells stimulated by bombesin, a Gq-coupled receptor agonist, phorbol 12,13-dibutyrate (PDBu), a biologically active tumor promoting phorbol ester and epidermal growth factor (EGF). These stimulatory effects could be dissociated from the inhibitory effect of CID755673 on PKD activity, since enhancement of DNA synthesis was still evident in cells with severely down-regulated PKD1 after transfection of siRNA targeting PKD1. A major point raised by our study is that CID755673 can not be considered a specific inhibitor of PKD and it should be used with great caution in experiments attempting to elucidate the role of PKD family members in cellular regulation, particularly cell cycle progression from G_1/G_o to S phase.

Introduction

Protein kinase D1 (PKD1) and two recently identified serine protein kinases termed PKD2 and PKD3, which are similar in overall structure and primary amino acid sequence to PKD1, constitute a new protein kinase family within the Ca^{2+}/calmodulin-dependent protein kinase group [1]. In non-stimulated cells, PKD1 is in a state of low kinase catalytic activity maintained by the N-terminal domain, which represses the catalytic activity of the enzyme by autoinhibition [1,2]. In response to cellular stimuli, PKD1 is converted from a low activity form into a persistently active form via a phosphorylation-dependent mechanism [3,4]. PKD1 activation has been demonstrated in response to many stimuli, including engagement of specific G protein-coupled receptors [5–10], signaling through Gq, G12, Gi, and Rho [9,11,12], activation of receptor tyrosine kinases [5,13,14], cross-linking of B-cell receptor and T-cell receptor in B and T lymphocytes, respectively [15,16], phorbol esters [3,4] and oxidative stress [17,18]. Our previous studies identified Ser^{744} and Ser^{748} in the PKD1 activation loop as phosphorylation sites critical for PKD1 activation [2,10,19,20]. Recent studies indicate that PKD1 can be a point of integration of sequential PKC-dependent and PKC-independent inputs [10,21].

PKD family members are implicated in the regulation of a variety of cellular functions, including signal transduction, chromatin organization, Golgi function, epithelial polarity, gene expression, immune regulation, inflammation and cell survival, adhesion, motility, differentiation, DNA synthesis and proliferation, [reviewed in Ref. [1]]. PKD1 has also been implicated in myocardial contraction, hypertrophy and remodeling [22], pancreatic inflammation [23], insulin secretion [24] angiogenesis [25] and cancer [7,26]. Consequently, the development of specific, cell-permeable PKD inhibitors would be extremely useful in helping to identify the physiological roles of PKD as well as for developing novel therapeutic approaches in a variety of pathological conditions.

Recently, CID755673 (2,3,4,5-tetrahydro-7-hydroxy-1H-benzofuro[2,3-c]azepin-1-one) has been reported to act as a highly selective inhibitor of the catalytic activity of members of the PKD family [27]. In Swiss 3T3 fibroblasts, a cell line used extensively as a model system to elucidate mechanisms of GPCR signaling [28], PKD1 expression potently enhances mitogenic responses induced by Gq-coupled receptor agonists, including increased DNA synthesis [6,8]. In the course of experiments using CID755673 in this system, we found that this compound exerted unexpected and potent stimulatory effects on [^{3}H]thymidine incorporation and cell cycle progression in cells stimulated with bombesin, a G protein-coupled

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receptor agonist, phorbol 12,13-dibutyrate (PDBu) or epidermal growth factor (EGF). The growth-enhancing actions of CID755673 could be dissociated from its inhibitory effects on PKD activity. Our results imply that CID755673 has other cellular target(s) in addition to PKD and therefore, experiments using this compound to elucidate the role of the PKD family in cell regulation should be interpreted with caution.

Materials and methods

Cell culture. Swiss 3T3-PKD.GFP cells, which overexpress wild type PKD and control Swiss 3T3-GFP cells were generated and propagated as previously described [6,8].

Immunoblotting and detection of PKD. Immunoblotting was performed as previously described [21]. Autoluminograms were scanned using a Fujifilm LS4000 (Life Science), and the labeled bands were quantified using the Multigauge software program (Fujifilm).

In vitro kinase assay of PKD. Purified PKD1 (20 ng/reaction) was incubated with ATP (100 μM, 1 μCi/reaction [γ-32P]ATP) and syntide-2 (0.7 mM) either in the absence or presence of CID755673. After 10 min at 30 °C, the reactions were terminated by adding 100 μl of 75 mM H_3PO_4, and 75 μl of the mixed supernatant was spotted to Whatman P-81 phosphocellulose paper. Papers were washed thoroughly in 75 mM H_3PO_4, dried, and radioactivity incorporated into syntide-2 was determined by Cerenkov radiation in a scintillation counter.

Assay of DNA Synthesis-Confluent cultures of Swiss 3T3, Swiss 3T3-PKD.GFP and Swiss 3T3-GFP cells were washed twice with Dulbecco's modified Eagle's medium (DMEM) and incubated with 2 ml DMEM/Waymouth's medium (1:1, v/v) containing [^{3}H]-thymidine (0.2 μCi/ml, 1 μM) and various agonists as described in the figure legends. After 40 h of incubation at 37 °C, acid-insoluble radioactivity was determined as described previously [21].

Transfection with siRNA-Subconfluent cultures (~40–60% confluence) of Swiss 3T3 cells were transfected with small interfering RNA (siRNA) targeting PKD1 from Dharmacon (Chicago, IL), as recently described [21]. Seven days after transfection, cells were used for experiments and subsequent Western blot analysis.

Flow cytometric analysis. Confluent cultures of Swiss 3T3 cells were washed two times with DMEM and incubated for 40 h with DMEM–Waymouth's medium (1:1 vol/vol) containing various additions as described in each experiment. After 18 h of incubation at 37 °C, 1 μM colchicine was added to accumulate in G_2/M all cells that progressed through the cell cycle. After an additional 22 h of incubation, the cultures were washed three times with PBS containing 4 mM EDTA. Cells were detached by treatment with trypsin (0.025%), suspended in DMEM containing 10% fetal bovine serum, centrifuged at 1000*g* for 5 min and washed three times in PBS. Cells (10^6; 200 μl) were stained by adding 800 μl of a solution containing propidium iodide (50 μg/ml), sodium citrate (1 mg/ml), and Triton X-100 (0.1%). The stained chromosomal DNA was kept on ice for 15 min and analyzed on a FACScalabar (Becton–Dickinson).

Materials. CID755673 was obtained from two different sources: A custom made synthesis from AsisChem Inc (Ma, USA) and a commercially available source TOCRIS (Mo, USA), with purities of 99.25% and >99%, respectively. We used two different antibodies to detect the phosphorylated state of either Ser^{744} or Ser^{748} in the PKD activation loop. One antibody (anti-pS744/pS748), obtained from Cell Signaling Technology, Beverly, MA, predominantly detects the phosphorylated state of Ser^{744} [20]. A second antibody, obtained from Abcam (ab17945), detects the phosphorylated state of Ser^{748} [10]. Bombesin, PDGF, TGFβ and EGF were obtained from Sigma, St. Louis MO. All other reagents were from standard suppliers and were of the highest grade commercially available.

Results and discussion

In order to evaluate the inhibitory effect of CID755673 on PKD activation induced by GPCR agonists in Swiss 3T3 cells, quiescent cultures of these cells overexpressing PKD (Swiss 3T3-PKD.GFP cells) were pretreated with various concentrations of this compound for 1 h and then stimulated with 10 nM bombesin for 10 min. Cell lysates were used to determine PKD phosphorylation at Ser^{744} and Ser^{748}, located in the activation loop, and Ser^{916}, an autophosphorylation site [2,10,20,29]. As shown in Fig. 1, cell exposure to CID755673 reduced PKD autophosphorylation on Ser^{916} but did not suppress the phosphorylation of this residue even at a concentration as high as 50 μM (Fig. 1A, blots; B, scanning densitometry). In contrast, CID755673 did not interfere with PKD phosphorylation on Ser^{744}. These results are consistent with a model of PKD regulation that anticipates PKC-mediated transphosphorylation of Ser^{744} and PKD-mediated autophosphorylation on Ser^{916} [10,21]. The intermediate inhibitory effect of CID755673 on the phosphorylation of Ser^{748} (Fig. 1A, blots; C, scanning densitometry) is consistent with the notion that this residue is modified through both transphosphorylation and autophosphorylation mechanisms [10]. Similar results were obtained when Swiss 3T3-PKD.GFP cells were stimulated with PDBu instead of bombesin (results not shown). We verified that CID755673 directly inhibits recombinant PKD1 activity in a concentration-dependent manner (Fig. 1D).

CID755673 enhances DNA synthesis induced by bombesin or PDBu

In Swiss 3T3 cells, PKD1 overexpression potently and selectively enhances DNA synthesis and cell proliferation induced by Gq-coupled receptor agonists, including bombesin, or phorbol esters, such as PDBu [6,8]. Furthermore, siRNA-mediated knockdown of endogenous PKD1 attenuates the mitogenic effect of either GPCR agonists or PDBu in these cells [21]. Consequently, we anticipated that treatment of Swiss 3T3 cells overexpressing PKD1 with CID755673 should abrogate the enhanced DNA synthesis induced by bombesin in these cells. Unexpectedly, we found that CID755673 did not produce any inhibitory effect on bombesin-induced [^{3}H]thymidine incorporation into Swiss 3T3-PKD.GFP cells, even at a concentration as high as 50 μM (Fig. 2A, *closed circles*). On the contrary, our results reproducibly showed that exposure to CID755673 (5–50 μM) further enhanced [^{3}H]thymidine incorporation induced by the Gq-coupled receptor agonist in these cells.

We also tested whether CID755673 exerted any effect on bombesin-induced DNA synthesis in Swiss 3T3 cells expressing GFP but endogenous PKD1 [Swiss 3T3-GFP cells, Ref. [6]]. We found that treatment with CID755673 produced a dramatic, dose-dependent increase in [^{3}H]thymidine incorporation in Swiss 3T3-GFP cells stimulated with 10 nM bombesin (Fig. 2A, *open circles*). CID755673 did not produce any significant effect in cells that were not stimulated with bombesin (Fig. 2A, *triangles*). Interestingly, CID755673-induced potentiation of DNA synthesis was evident at lower concentrations (e.g. 10 μM) than those necessary to produce substantial inhibition of PKD autophosphorylation on Ser^{916} (indicative of PKD1 activation) within cells. Furthermore, exposure to CID755673 markedly enhanced bombesin-induced DNA synthesis in Swiss 3T3 cells that were not subjected to any previous transfection or selection (Fig. 2B).

As mentioned above, PKD1 plays a critical role in mediating stimulation of DNA synthesis induced by phorbol esters, including PDBu [21]. As shown in Fig. 2C, treatment with CID755673 produced a striking increase in [^{3}H]thymidine incorporation in Swiss 3T3 cells stimulated with PDBu. Similar results were obtained with preparations of CID755673 obtained from two independent sources. In view of the established role of PKD1 signaling in DNA

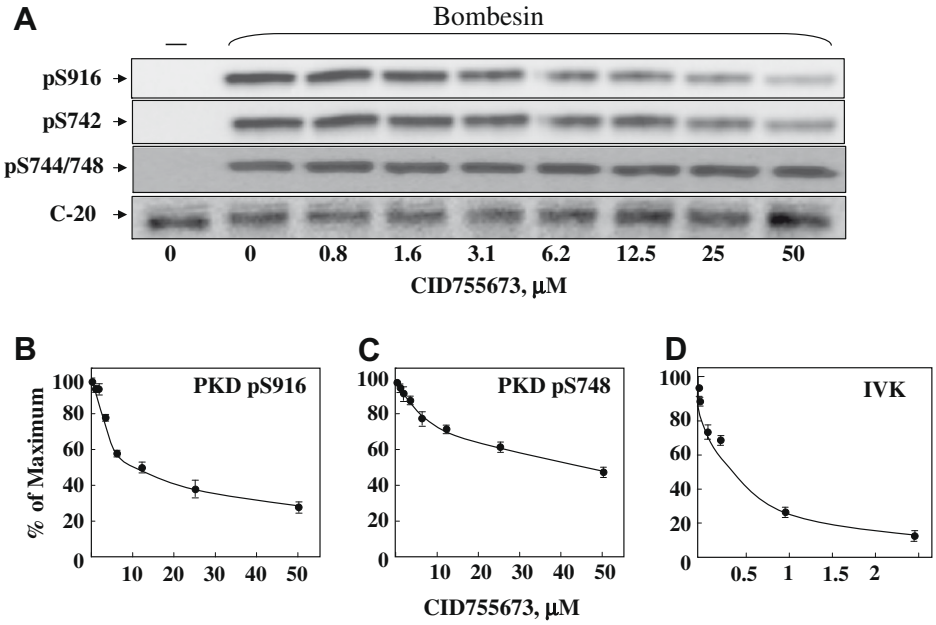

Fig. 1. Effect of increasing concentrations of CID755673 on PKD1 phosphorylation on Ser^{916}, Ser^{744} and Ser^{748} induced by bombesin stimulation. Swiss 3T3 PKD1.GFP cells were incubated without (−) or with (+) increasing concentrations of CID755673 for 1 h prior to stimulation with 10 nM bombesin for 10 min and then lysed with 2× SDS–PAGE sample buffer. (A) Samples were analyzed by SDS–PAGE and immunoblotting with the following antibodies; phospho PKD1 pS^{916}, pS^{744}, pS^{748} and PKD-C20 to verify equal loading. Shown here are representative autoluminograms; similar results were obtained in three independent experiments. (B, C) Autoluminograms of PKD1 Ser^{916} and PKD1 Ser^{748} were quantified by scanning densitometry. The results shown are the mean ± SEM ($n = 3$) and are expressed as percentage of the maximum increase induced by treatment with bombesin. (D) Purified PKD1 activity was measured by syntide-2 phosphorylation. The results shown are the mean ± SEM ($n = 3$) and are expressed as a percentage of the maximum activity.

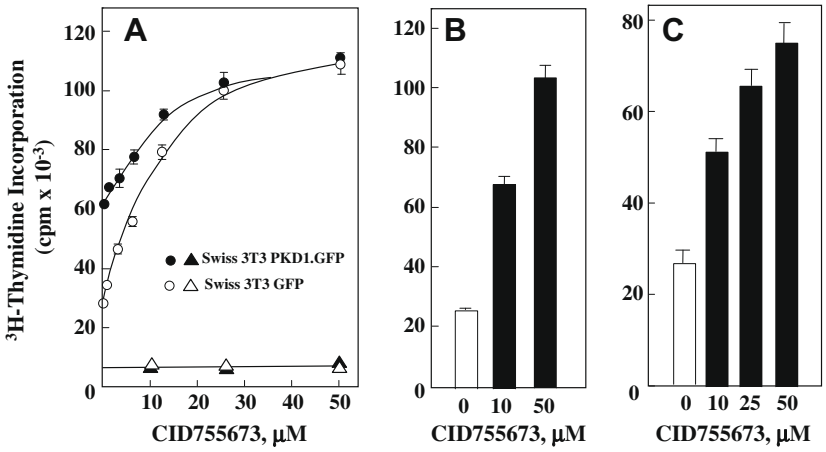

Fig. 2. CID755673 potentiates DNA synthesis in response to bombesin and PDBu. (A) Confluent Swiss 3T3 PKD1.GFP cells (closed symbols) and Swiss 3T3-GFP cells (open symbols) were washed and incubated in DMEM/Waymouth's medium containing [^{3}H]thymidine and increasing concentrations of CID755673 for 1 h prior to stimulation with 10 nM bombesin (circles). Parallel cultures were incubated with CID755673 but without bombesin (triangles). After 40 h, DNA synthesis was assessed by measuring the [^{3}H]thymidine incorporated into acid-precipitable material. Results are expressed as cpm/culture × 10^{-3}, mean ± SE ($n = 3$); maximal stimulation, obtained with 10% fetal bovine serum (FBS), was 120 × 10^{-3} cpm/culture. (B) Confluent Swiss 3T3 cells were washed and incubated in DMEM/Waymouth's medium containing [^{3}H]thymidine and increasing concentrations of CID755673 for 1 h prior to stimulation of the cells with 10 nM bombesin. After 40 h, DNA synthesis was assessed by measuring [^{3}H]thymidine incorporated into acid-precipitable material. Results are expressed as cpm/culture × 10^{-3}, mean ± SE ($n = 3$); maximal stimulation, obtained with 10% FBS, was 105 × 10^{-3} cpm/culture. (C) Confluent Swiss 3T3 cells were washed and incubated in DMEM/Waymouth's medium containing [^{3}H]thymidine and increasing concentrations of CID755673 for 1 h prior to stimulation with 100 nM PDBu. After 40 h, DNA synthesis was assessed by measuring the [^{3}H]thymidine incorporated into acid-precipitable material. Results are expressed as cpm/culture × 10^{-3}, mean ± SE ($n = 3$); maximal stimulation, obtained with 10% FBS, was 95 × 10^{-3} cpm/culture.

synthesis in response to bombesin or phorbol esters [6,8,21], the results obtained with CID755673 were surprising since rather than abrogate DNA synthesis, this compound produced a striking stimulation of [^{3}H]thymidine incorporation in Swiss 3T3, Swiss 3T3-GFP and in Swiss 3T3-PKD.GFP cells.

CID755673 enhances DNA synthesis induced by EGF, PDGF or TGFβ

EGF does not stimulate either PKC or PKD1 activation [6,8] in intact Swiss 3T3 cells and PKD1 overexpression does not enhance EGF-induced DNA synthesis in these cells [6,8]. Furthermore, siRNA-mediated knockdown of PKD1 did not attenuate DNA synthesis in response to EGF in Swiss 3T3 cells [21]. All these studies indicate that EGF induces its growth-promoting effects through PKC/PKD-independent pathways in Swiss 3T3 cells [28]. Consequently, if all the effects of CID755673 were due to PKD inhibition, we expected that exposure to this compound should not have any detectable effect on EGF-stimulated [^{3}H]thymidine incorporation in Swiss 3T3 cells. In contrast, we found that treatment with CID755673 induced a marked increase in [^{3}H]thymidine incorpora-

tion promoted by EGF in these cells (Fig. 3A). In parallel cultures, we verified that EGF, at the concentration used in these experiments (5 ng/ml), did not induce any detectable PKD1 autophosphorylation on Ser^{916} (Fig. 3A, upper).

If CID755673 potentiated EGF-induced DNA synthesis independently of PKD1 in Swiss 3T3 cells, PKD1 knockdown should not prevent the enhancement of DNA synthesis induced by this compound in EGF-treated cells. As shown in Fig. 3B, siRNAs targeting PKD1 produced striking depletion of PKD1 protein (~90%) but did not prevent the increase in DNA synthesis induced by CID755673 in EGF-stimulated Swiss 3T3 cells (Fig. 3C). These results strongly support the conclusion that the stimulatory effects of CID755673 on cell cycle progression of 3T3 cells are not mediated by inhibition of PKD1 activity.

Recently, we demonstrated that PKD1 overexpression does not enhance the mitogenic response induced by PDGF or TGFβ, implying that the growth-promoting effects of these factors are mediated by PKD1-independent pathways [21]. Similar to the results obtained with EGF, exposure to CID755673 enhanced [^{3}H]thymidine incorporation induced by either PDGF or TGFβ (Fig. 3D). These results strengthened the notion that the potentiating effects of CID755673 on [^{3}H]thymidine incorporation can be dissociated from its ability to alter PKD activity and therefore imply that this compound acts via additional target(s) in Swiss 3T3 cells.

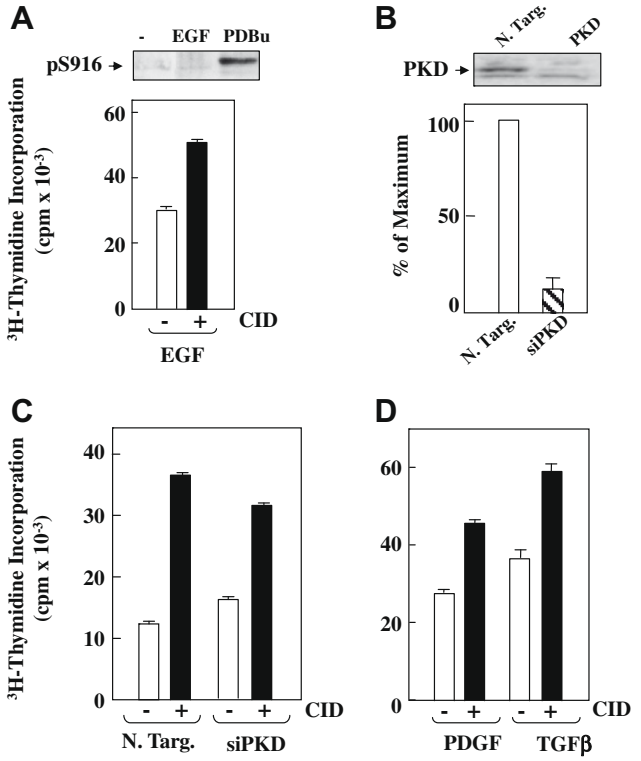

Fig. 3. CID755673 potentiates DNA synthesis in response to EGF. (A, upper) Confluent Swiss 3T3 cells were washed twice with DMEM and incubated for 10 min with either 5 ng/ml of EGF or 100 nM PDBu (for comparison). Cell lysates were analyzed by SDS–PAGE and immunoblotting with a pS916 antibody. (Lower) Confluent Swiss 3T3 cells were washed and incubated in DMEM/Waymouth's medium containing [^{3}H]thymidine and 25 μM of CID755673 for 1 h prior to stimulation with 5 ng/ml EGF. After 40 h, DNA synthesis was assessed by measuring the [^{3}H]thymidine incorporated into acid-precipitable material. Results are expressed as cpm/culture × 10^{-3}, mean ± SE (n = 3); maximal stimulation, obtained with 10% FBS, was 88 × 10^{-3} cpm/culture. (B) Swiss 3T3 cells were transfected with either non-targeting negative control (N. Targ.) or 75 nM PKD siRNA (siPKD), as indicated. After 6 days, the cells were lysed and PKD1 protein expression was assessed by Western blotting using the anti-PKD-C20 antibody. Shown here is a representative autoluminogram; similar results were obtained in four independent experiments. Autoluminograms were quantified by densitometric scanning. The results shown are the mean ± SEM (n = 4) and are expressed as percentage of the maximum level of PKD1 in non-targeting negative control cells (open bars). (C) Swiss 3T3 cells were transfected with either non-targeting negative control (Non. Targ.) or 75 nM PKD siRNA (siPKD). After 6 days, the cultures were incubated in DMEM/Waymouth's medium containing [^{3}H]-thymidine and 5 ng/ml EGF either in the absence (open bars) or presence of 25 μM CID755673. After 40 h, DNA synthesis was assessed by [^{3}H]thymidine incorporation. Results are expressed as cpm/culture × 10^{-3}; maximal stimulation, obtained with 10% FBS, was 94 × 10^{-3} cpm/culture. (D) Confluent Swiss 3T3 cells were washed and incubated in DMEM/Waymouth's medium containing [^{3}H]thymidine without (open bars) or with 25 μM CID755673 for 1 h prior to stimulation with 2.5 ng/ml PDGF or 2 ng/ml TGFβ. After 40 h, DNA synthesis was assessed by [^{3}H]thymidine incorporation. Results are expressed as cpm/culture × 10^{-3}, mean ± SE (n = 3); maximal stimulation, obtained with 10% FBS, was 105 × 10^{-3} cpm/culture.

CID755673 enhances cell cycle progression induced by bombesin, EGF or PDBu

Since the finding that cell treatment with CID755673 strikingly enhanced [^{3}H]thymidine incorporation in response to multiple agonists was unexpected, we determined whether the stimulatory effect of this compound reflects an increase in DNA replication through the S phase of the cell cycle rather than an increase in the transport and/or phosphorylation of [^{3}H]thymidine. Consequently, we used flow cytometric analysis to determine the proportion of cells in the various phases of the cell cycle. As shown in Fig. 4, addition of 25 μM CID755673 strikingly increased the movement from G_1 to S and G_2 plus M induced by bombesin, EGF or PDBu in Swiss 3T3 cells. Thus, CID755673 markedly stimulated progression through the cell cycle induced by growth-promoting stimuli that act either through PKD1-dependent (e.g. bombesin, PDBu) or PKD1-independent (e.g. EGF) signaling pathways in Swiss 3T3 cells.

Conclusions and implications

PKD signaling is increasingly implicated in the regulation of multiple cellular activities and in the mechanism of action of multiple stimuli (see Introduction for Refs.). Therefore, the identification of specific PKD inhibitors would be extremely useful in helping to define the molecular substrates and physiological functions of the members of the PKD family and may open up new avenues for the development of novel therapeutic approaches in a variety of conditions, including disorders of cell growth.

Recently, CID755673 has been reported to act as a potent and highly selective inhibitor of the catalytic activity of members of the PKD family [27]. However, the salient and unexpected feature of the results shown here is that CID755673 induced a potent enhancement of cell cycle progression in bombesin-stimulated Swiss 3T3 cells, as judged by [^{3}H]thymidine incorporation assays or by flow cytometric analysis. The effects were obtained at CID755673 concentrations lower than those required to produce substantial inhibition of PKD1 activation in intact cells, were not affected by the level of PKD1 expression and were corroborated with CID755673 obtained from different sources. Furthermore, CID755673 strikingly enhanced DNA synthesis induced by EGF, PDGF or TGFβ that induce DNA synthesis in Swiss 3T3 cells via PKD1-independent pathways [21]. Indeed, siRNA-mediated knockdown of PKD1 protein did not prevent the increase in DNA synthesis induced by CID755673 in Swiss 3T3 cells. Collectively, these results imply that the stimulatory effect of CID755673 on cell cycle progression of 3T3 cells is not mediated by PKD1.

We conclude that CID755673 induces cellular responses through molecular targets other than PKD1. While the identification of the putative target(s) of CID755673 that mediates its growth-stimulatory effects is of interest, the major point raised

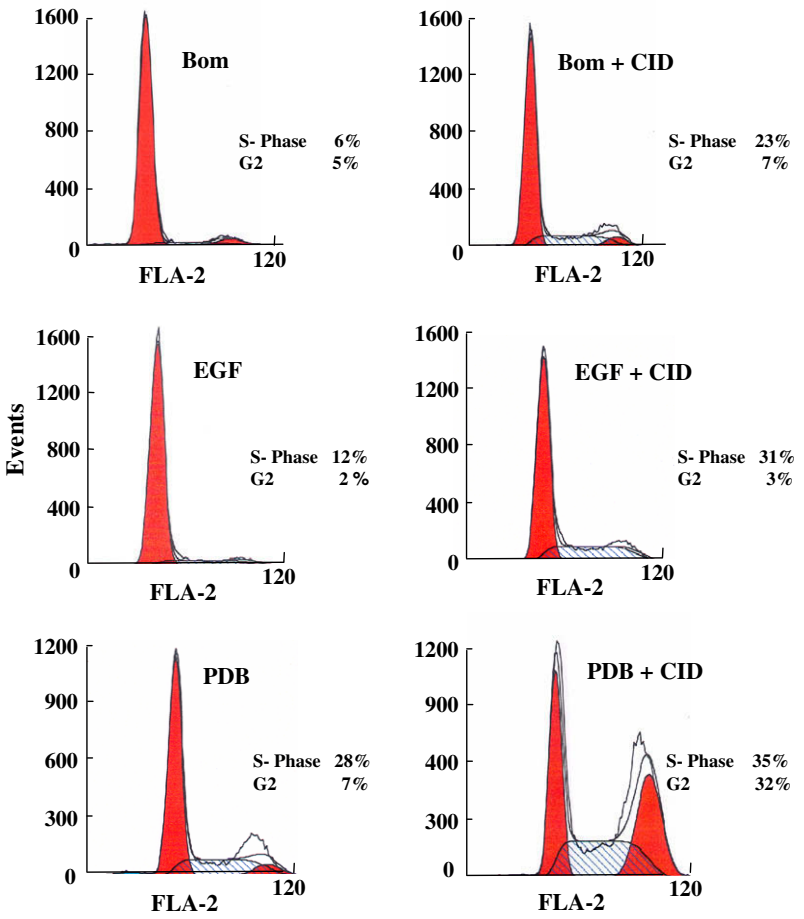

Fig. 4. CID755673 stimulates cell cycle progression induced by bombesin, EGF or PDBu. Confluent and quiescent Swiss 3T3 cells grown on 100 mm dishes were washed and incubated at 37 °C in 10 ml DMEM/Waymouth's medium containing 10 nM bombesin, 5 ng/ml EGF or 100 nM PDBu. Flow cytometric analysis was performed as described in Materials and methods. Similar results were obtained in two independent experiments.

by our study is that CID755673 can not be considered a specific inhibitor of PKD and it should be used with great caution in experiments attempting to elucidate the role of PKD family members in cellular process, particularly cell cycle progression from G_1/G_o to S phase.

Acknowledgments

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