Calcium mobilization and protein kinase C activation are required for cholecystokinin stimulation of pancreatic cholesterol esterase secretion

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The bile salt-stimulated cholesterol esterase is a digestive enzyme synthesized by the acinar cells of the pancreas. Previous results have shown that cholesterol esterase biosynthesis and secretion in the AR42J pancreatoma cells could be increased 3–5-fold by intestinal hormones such as cholecystokinin (CCK). The purpose of the current study is to explore the signalling mechanism by which CCK stimulation of AR42J cells results in increased biosynthesis and secretion of the cholesterol esterase. The results showed that the CCK-induced cholesterol esterase secretion could be mimicked by addition of the Ca^{2+} ionophore A23187 or by transient incubation of AR42J cells with the protein kinase C

activator phorbol 12-myristate 13-acetate (PMA). Cholesterol esterase stimulation by CCK, A23187 and PMA could be abolished by the calcium chelator BAPTA or by specific protein kinase C inhibitors such as chelerythrine. Additionally, prolonged incubation of AR42J cells with PMA to reduce the protein kinase C level, also reduced CCK-stimulated cholesterol esterase secretion to a level similar to that observed in control cells. Taken together, these data suggested that CCK activation of cholesterol esterase secretion may be mediated by a Ca^{2+} -dependent protein kinase C pathway, requiring increases in calcium mobilization and activation of protein kinase C.

INTRODUCTION

Intestinal hormones are produced *in vivo* in response to different nutrients present in the gut [1]. For example, secretin is produced in response to fat consumption while cholecystokinin (CCK) is released from intestinal cells in response to increased protein influx. The effects of these hormones in the regulation of digestive enzyme biosynthesis can be studied using the rat pancreatoma cell line AR42J. This cell line is a viable model for studying the acute response of pancreatic acinar cells to hormonal stimulation due to its production of amylase, chymotrypsin, lipase and trypsinogen [2]. Moreover, the AR42J cells have been shown to possess hormone receptors similar to those found in normal rat pancreatic acini [3].

Previously, CCK was reported to stimulate amylase and chymotrypsinogen biosynthesis in AR42J cells [4,5]. The amylase and chymotrypsin mRNA isolated from hormone-stimulated AR42J cells were translated more efficiently than those obtained from unstimulated cells [4], thus suggesting a post-transcriptional regulatory mechanism. Using the AR42J cells as a model to study hormonal regulation of cholesterol esterase biosynthesis, our laboratory has reported that cholesterol esterase biosynthesis could be increased by either secretin, CCK or bombesin stimulation of the AR42J cells [6,7]. The hormone-induced cholesterol esterase biosynthesis was shown to be mediated by activation of a post-transcriptional event, probably the increased translation of cholesterol esterase mRNA [6].

The exact mechanism by which CCK and secretin activate cholesterol esterase biosynthesis remains unknown. However, the initial event for hormone-induced pancreatic functions is the binding of the hormone to receptors on the cell membrane [8,9]. Hormone–receptor interactions trigger a series of signal trans-

duction pathways leading to the production of physiological responses. For example, secretin binding to the secretin receptor results in adenylate cyclase stimulation and an increased level of cyclic AMP [10]. The elevated level of cyclic AMP activates protein kinase A, which then phosphorylates specific proteins that may be involved with digestive enzyme synthesis and secretion [11]. In contrast, the binding of CCK, a cholinergic agent, to its receptor on pancreatic cell surfaces results in the activation of signal-transduction mediated by G-proteins [12,13]. The G-proteins activate phospholipase C-catalysed hydrolysis of phosphatidylinositol-4,5-bisphosphate to *sn*-1,2-diacylglycerol (1,2-DAG) and D-inositol 1,4,5-trisphosphate. These products in turn act on other intracellular targets, leading ultimately to increased protein biosynthesis. The 1,2-DAG activates the calcium- and phospholipid-dependent protein kinase C, while the inositol trisphosphate stimulates the release of calcium from an intracellular pool (reviewed in [14]). The increase in calcium flux may act either synergistically with protein kinase C activation [15,16] or independent of protein kinase C [17] in the regulation of gene expression.

The purpose of this investigation was to determine the signalling mechanism for CCK-stimulation of cholesterol esterase biosynthesis and secretion by AR42J cells. The results show that both increased calcium flux and protein kinase C activation are required for CCK-induction of cholesterol esterase.

EXPERIMENTAL PROCEDURES

Materials

The rat pancreatic AR42J cell line was obtained from the American Tissue Culture Collection (Rockville, MD, U.S.A.). The calcium chelator BAPTA was purchased from Molecular

Abbreviations used: CCK, cholecystokinin; PMA, phorbol 12-myristate 13-acetate; DMEM, Dulbecco's modified Eagle's medium; DAG, 1,2-diacylglycerol.

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Probes (Eugene, OR, U.S.A.) and the calcium ionophore A23187 was purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Protein kinase inhibitors chelerythrine and staurosporine were obtained from LC Services (Woburn, MA, U.S.A.). Phorbol 12-myristate 13-acetate (PMA) was the product of Calbiochem (La Jolla, CA, U.S.A.) and cholesteryl [^{14}C]oleate was obtained from DuPont New England Nuclear (Boston, MA, U.S.A.).

Cell culture

The AR42J cells were cultured at 37 °C with 5% CO_2 in Dulbecco's modified Eagles medium (DMEM) containing 10% fetal bovine serum, 2 mM glutamine and 4.5 g/l of glucose. The stock cultures were maintained in T75 flasks and the media were changed every 2 days. When the culture reached 80% confluence, cells were dissociated with 0.25% trypsin/0.05% EDTA and replated into new flasks at a split ratio of 1:3. Experiments were performed with cells at the 30th–50th passages. The AR42J cells were seeded into six-well plates at $(2\text{--}3) \times 10^4/\text{cm}^2$ and were grown to 80% confluency.

On the day of the experiments, the AR42J cells were rinsed in DMEM media without fetal bovine serum and experiments were carried out in DMEM media without serum supplementation. The test reagents, including 10 μM BAPTA, 1 μM chelerythrine and 5 μM staurosporine, were added to the cells 5 min before stimulation. The AR42J cells were stimulated with agonists such as 4 nM CCK, 10 μM PMA or 50 μM A23187 for 15 min at 37 °C. At the end of the incubation period, the media were collected and centrifuged in Eppendorf tubes at $5000 \times g$ for 5 min to remove cell debris.

Cholesterol esterase assay

Samples of media or purified rat pancreatic enzyme [18] were tested for activity by measuring bile salt-dependent hydrolysis of cholesteryl [^{14}C]oleate. The assay mixture contained 50 mM Tris/HCl (pH 7.5), 33 mM cholate and 1.96 nmol of cholesteryl [^{14}C]oleate (DuPont New England Nuclear, Boston, MA, U.S.A.) mixed with 5 μl of media and water to give a final volume of 200 μl in each assay tube. The tubes were incubated at 37 °C for 1 h. The reactions were terminated by the addition of 3.25 ml of methanol/chloroform/heptane (1.41:1.25:1.0, by volume) and 1.05 ml of 50 mM sodium carbonate with 50 mM sodium borate (pH 10). Samples were vortexed and then centrifuged at $1500 \times g$ for 38 min to separate the phases. The amount of the product, [^{14}C]oleate, extracted into the aqueous phase was determined by mixing 0.5 ml aliquots of each sample with 10 ml of scintillation fluid for counting in a Packard 1900 TR scintillation analyser. The extraction efficiency was determined empirically to be 70%.

Initial experiments performed with purified rat pancreatic cholesterol esterase revealed a linear range of substrate hydrolysis ($r = 0.984$) with 2–8 ng of purified protein. Under the same assay conditions, 5 μl of AR42J-conditioned medium also resulted in cholesteryl oleate hydrolysis in the same range. Therefore, this volume of cultured medium was consistently used in subsequent experiments to measure cholesterol esterase produced by the pancreatoma cells.

RESULTS

Previous studies from our laboratory have indicated that incubating AR42J cells with 4 nM CCK resulted in a 2–5-fold increase in cholesterol esterase biosynthesis [6]. When cholesterol esterase activity in the AR42J cell medium was determined, an approximately 2-fold higher enzyme activity was observed in the

Table 1 Inhibition of CCK-stimulated cholesterol esterase synthesis and secretion

The AR42J cells were pre-incubated at 37 °C for 5 min in DMEM media in the presence or absence of 10 μM BAPTA and/or 1 μM chelerythrine before addition of CCK to a final concentration of 4 nM. The incubations were continued for 15 min at 37 °C before collection of the incubation media. The amount of cholesterol esterase synthesized and secreted into the media was determined by the hydrolysis of cholesteryl [^{14}C]oleate with 5 μl of the media. The effects of CCK and the various inhibitors were compared with basal cholesterol esterase secretion in the absence of these reagents (control). The results represent the average of four separate experiments $\pm$ S.D. ** $P < 0.05$.

Incubation conditions	Cholesteryl [^{14}C]oleate hydrolysis (d.p.m./h)	Control (%)
Control	1229 $\pm$ 97	100
+ CCK	2381 $\pm$ 59**	194**
+ CCK + BAPTA	1073 $\pm$ 145	87
+ CCK + chelerythrine	864 $\pm$ 121	70.3
+ CCK + BAPTA + chelerythrine	585 $\pm$ 13**	47.6**

Table 2 Effects of calcium ionophore on cholesterol esterase secretion by the AR42J cells

The AR42J cells were pre-incubated at 37 °C for 5 min in DMEM media in the presence or absence of 10 μM BAPTA and/or 1 μM chelerythrine, or with 5 μM staurosporine, before adding 5 μM of the calcium ionophore A23187. The incubations were continued for 15 min at 37 °C before collection of the incubation media. The amount of cholesterol esterase synthesized and secreted into the media was determined by the hydrolysis of cholesteryl [^{14}C]oleate with 5 μl of the media. The effects of A23187 and the various inhibitors were compared with basal cholesterol esterase secretion in the absence of these reagents (control). The results represent the average of four separate experiments $\pm$ S.D. ** $P < 0.05$.

Incubation conditions	Cholesteryl [^{14}C]oleate hydrolysis (d.p.m./h)	Control (%)
Control	1229 $\pm$ 97	100
+ A23187	2215 $\pm$ 280**	180**
+ A23187 + BAPTA	1321 $\pm$ 508	107
+ A23187 + chelerythrine	1026 $\pm$ 166	84
+ A23187 + staurosporine	807 $\pm$ 222	66

medium of CCK-stimulated cells in comparison with that of control cells (Table 1). However, if the AR42J cells were preincubated with the calcium chelator BAPTA before CCK stimulation, the amount of cholesterol esterase secreted by the AR42J cells was similar to that observed in unstimulated control cells (Table 1). Preincubation of the AR42J cells with the protein kinase C inhibitor chelerythrine also abolished the CCK-induced cholesterol esterase biosynthesis and secretion (Table 1). The effects of BAPTA and chelerythrine appeared to be additive, as the addition of both reagents before CCK stimulation reduced the amount of cholesterol esterase secretion to a level below that observed in control cells (Table 1).

The results of the above experiments suggest that CCK-stimulated cholesterol esterase secretion may be related to CCK-induced calcium mobilization and/or protein kinase C activation. This hypothesis was examined directly by testing the effects of the calcium ionophore A23187 on cholesterol esterase secretion by AR42J cells. Results showed that the preincubation of AR42J with the ionophore increased cholesterol esterase secretion approximately 2-fold (Table 2). Preincubation of the AR42J cells

Table 3 Effects of phorbol esters on cholesterol esterase secretion by the AR42J cells

The AR42J cells were pre-incubated at 37 °C for 5 min in DMEM media in the presence or absence of 10 μ M BAPTA and/or 1 μ M chelerythrine, or with 5 μ M staurosporine, before addition of the DAG analogue PMA to a final concentration of 10 μ M. The incubations were continued for 15 min at 37 °C before collection of the incubation media. The amount of cholesterol esterase synthesized and secreted into the media was determined by the hydrolysis of cholesteryl [14 C]oleate with 5 μ l of the media. The effects of PMA and the various inhibitors were compared with basal cholesterol esterase secretion in the absence of these reagents (control). The results represent the average of 4 separate experiments $\pm$ S.D. ** P < 0.05.

Incubation conditions	Cholesteryl [14 C]oleate hydrolysis (d.p.m./h)	Control (%)
Control	1229 $\pm$ 97	100
+ PMA	3142 $\pm$ 280**	256**
+ PMA + BAPTA	1620 $\pm$ 87	132
+ PMA + chelerythrine	1298 $\pm$ 86	106
+ PMA + staurosporine	1065 $\pm$ 66	87

Table 4 Effects of protein kinase C down-regulation on cholesterol esterase secretion by AR42J

The AR42J cells were pre-incubated with PMA at the indicated concentration for 24 h at 37 °C in DMEM media. At the end of the pre-incubation period, the cells were washed extensively and then incubated with or without CCK. The amount of cholesterol esterase synthesized and secreted into the media was determined by the hydrolysis of cholesteryl [14 C]oleate with 5 μ l of the media. The effects of PMA and CCK were compared with basal cholesterol esterase secretion in the absence of these reagents (control). The results represent the average of four separate experiments $\pm$ S.D. ** P < 0.05.

PMA (μ M)	CCK (nM)	Cholesteryl [14 C]oleate hydrolysis (d.p.m./h)	Control (%)
0	0	1055 $\pm$ 780	100
0	4	3666 $\pm$ 940**	347**
0.05	0	1290 $\pm$ 390	122
0.05	4	1298 $\pm$ 1110	123

with the calcium chelator BAPTA abolished the ionophore-induced cholesterol esterase secretion (Table 2). Interestingly, the protein kinase C inhibitor chelerythrine, as well as the non-specific protein kinase inhibitor staurosporine, also inhibited the A23187-induced cholesterol esterase secretion by AR42J cells (Table 2).

The role of protein kinase C activation in cholesterol esterase secretion was examined by taking advantage of the availability of the diacylglycerol analogue (PMA), which has been shown previously to modulate the activity of protein kinase C [19]. The transient preincubation of AR42J cells with PMA resulted in a 2.2-fold increase in cholesterol esterase secretion (Table 3). This stimulation could be inhibited by preincubating the cells with either the protein kinase C-specific inhibitor chelerythrine, the non-specific kinase inhibitor staurosporine, or the calcium chelator BAPTA (Table 3).

The role of protein kinase C in mediating CCK stimulation of cholesterol esterase secretion was explored directly by measuring the CCK stimulation of cholesterol esterase secretion after an overnight incubation of the AR42J cells with PMA. The prolonged incubation of AR42J with PMA, which reduces the

protein kinase C level in tissue culture cells [14], resulted in inhibition of CCK-activated cholesterol esterase secretion. The amount of cholesterol esterase found in the medium of CCK-treated cells after prolonged incubation with PMA was similar to that observed in the medium of unstimulated control AR42J cells (Table 4).

DISCUSSION

The results of this study showed that CCK stimulation of cholesterol esterase secretion could be abolished by calcium chelators or agents that inhibit protein kinase C activation. Additionally, increasing calcium flux with the ionophore A23187 or stimulation of protein kinase C with phorbol esters was also effective in activating cholesterol esterase biosynthesis and secretion from AR42J cells. At first glance, these results seemed to suggest that calcium mobilization and protein kinase C activation act independently, and that stimulation of one such pathway is sufficient to induce cholesterol esterase secretion. However, intensive scrutiny of the data revealed that increased calcium flux alone is not sufficient to induce cholesterol esterase secretion by AR42J cells. Activation of the protein kinase C pathway is necessary for increased cholesterol esterase secretion.

The requirement of protein kinase C activation in the stimulation of cholesterol esterase secretion was supported by two separate experiments in the current study. First, the protein kinase C inhibitor chelerythrine was effective in abolishing both CCK- and A23187-induced cholesterol esterase secretion by AR42J cells (Table 2). Since previous studies have shown that this reagent is specific for protein kinase C inhibition and has no effect on calcium flux [20], these results indicate that increased calcium flux without protein kinase C activation is not sufficient to activate cholesterol esterase secretion in AR42J cells. Secondly, the down-regulation of protein kinase C level, by an overnight incubation with PMA, also abolished the effects of CCK on cholesterol esterase secretion by the AR42J cells (Table 4). Taken together, these data document that protein kinase C activation is necessary for induction of cholesterol esterase biosynthesis/secretion by AR42J. Thus, the ability of the calcium chelator BAPTA to inhibit CCK and PMA stimulation of cholesterol esterase production is most likely to be mediated by inhibition of a Ca^{2+} -dependent protein kinase C [14].

The results of the current experiments also show that the induction of calcium influx with the ionophore A23187 also results in increased cholesterol esterase secretion by the AR42J cells. Although the experiments did not include any exogenously added activator of protein kinase C, it is possible that the A23187-induced cellular calcium may trigger protein kinase C activation. In support of this hypothesis is the reported inhibition of CTP:phosphocholine cytidyltransferase activity in rat pancreas by the calcium ionophore A23187 [21]. This enzyme catalyses the formation of CDP-choline from phosphocholine in the phosphatidylcholine synthetic pathway. Thus, inhibition of this enzyme will result in cellular accumulation of DAG, a physiological activator of protein kinase C. Interestingly, CCK-treated pancreatic acini were also shown to have reduced CTP:phosphocholine cytidyltransferase activity and prolonged accumulation of DAG [21]. Thus, it is possible that A23187-induced cholesterol esterase secretion was related to a calcium-dependent accumulation of DAG and the resulting activation of protein kinase C. The observed inhibition of A23187-induced cholesterol esterase biosynthesis by chelerythrine was supportive of this conclusion.

In summary, the results of this study show that CCK-induced cholesterol esterase biosynthesis is mediated by increasing cal-

cium flux and activation of protein kinase C-dependent pathways. In view of the putative role of cholesterol esterase in mediating dietary cholesterol absorption through the intestine [22], these studies suggest that therapeutic modulation of pancreatic signal transduction pathways may be a potentially useful strategy in reducing cholesterol absorption efficiency, thus lowering plasma cholesterol level and reducing the risk of cardiovascular disease.

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