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THE EFFECT OF CONCOMITANT STIMULATION WITH CHOLECYSTOKININ AND EPIDERMAL GROWTH FACTOR ON EXTRACELLULAR SIGNAL-REGULATED KINASE (ERK) ACTIVITY IN PANCREATIC ACINAR CELLS.

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The transmission of extracellular proliferation and differentiation signals into their intracellular targets is mediated by a signaling cascade culminating in mitogen-activated protein kinase (MAPK) also known as ERK. In pancreatic acinar cells both cholecystokinin (CCK) and epidermal growth factor (EGF) are known to stimulate ERK. Regulatory interactions among individual receptor-coupled signaling cascades are critically important for establishing cellular responses in the face of multiple stimuli. The aim of our study was to evaluate the effect of concomitant stimulation of G protein-coupled receptors (GPCR) and EGF receptors on ERK activity in isolated pancreatic acinar cells. ERK activity was determined by means of Western-blotting, with the use of the antibody which recognizes active, tyrosine-phosphorylated kinase (pY-ERK). pY-ERK level was strongly elevated by 10 nM CCK-8, 100 μM carbachol (CAR), or 100 nM EGF. The addition of EGF to 60 min-lasting incubations of acini with CCK-8 or CAR caused abrupt decrease of pY-ERK level to 56 and 59% of control, respectively. Similar phenomenon was observed when short stimulation with CCK-8 or CAR was superimposed on the effect of EGF. After the addition of EGF to acini incubated previously with phorbol ester TPA, strong decrease in pY-ERK level was also observed. In conclusion, in pancreatic acinar cells, concomitant stimulation with CCK or CAR and EGF has strong inhibitory effect on ERK cascade. This inhibitory cross-talk may be mediated, at least partially, by protein kinase C (PKC). These mutual inhibitory interactions demonstrate novel mechanism for integration of multiple signals generated by activation of G-protein-coupled and growth factor receptors in pancreatic acinar cells.

Key words: *cholecystokinin, epidermal growth factor, extracellular signal-regulated kinase, mitogen-activated protein kinases, pancreatic acinar cells*

INTRODUCTION

A great progress in the understanding mechanisms of transmission of intracellular signals after stimulation of receptors at the cell surface has been done during last years. It was found that agonists which stimulate enzyme and pancreatic juice secretion also influence cellular growth, differentiation and regeneration (1 - 3). The transmission of this signal is mediated by a signaling cascade, which consists of several interacting proteins. There are several parallel pathways to transmit information in the cell. In some circumstances this pathways can interact with each other. One of the most important intracellular signaling cascade is MAPK cascade (3 - 10). MAPK represents a serine-threonine protein kinase which is culminating point of different intracellular pathways. It regulates many cellular processes like growth, differentiation, survival or apoptosis as well as cytokine production (11 - 14). Nowadays we know five MAPK cascades. Three of them, culminating in ERKs, p38MAPK or c-jun N-terminal kinase (JNK) also known as stress activated protein kinase (SAPK) were identified in pancreatic acinar cells (3 - 6, 15, 16). Two isoforms of ERK: ERK 1 (44 kDa) and ERK 2 (42 kDa) are known. ERK is very rapidly activated by a variety of cell surface receptors and after translocation to the nucleus active ERK regulates expression of genes which are responsible for growth and differentiation processes. Interestingly, in the same cell, activation of ERK may be mediated by a multiple agents. About 160 substrates have already been discovered for ERK. Many of these substrates are localized in the nucleus, and participate in regulation of transcription upon stimulation. Other substrates are found in cytosol and cellular organelles (17).

In pancreatic acinar cells, ERK cascade is activated by two major classes of cell surface receptors, tyrosine growth factor receptors – represented by EGF receptor (EGFR), and GPCRs, like CCK or muscarinic. EGFR does it through Ras-dependent pathway, while CCK-receptor seems to activate ERKs predominantly through a Ras-independent pathway involving PKC (4). The mechanism of EGFR-stimulated mitogenic signaling involves formation of complexes of the guanine nucleotide exchange protein SOS, and the SH2 and SH3 domain-containing adaptor protein Grb2 with either autophosphorylated growth factor receptors or another Tyr phosphorylated adapter protein Shc. These interactions result in the translocation of SOS from cytosol to the plasma membrane and activation of Ras. Different mechanisms are involved in stimulation of pancreatic acini with CCK or carbachol. Activation of GPCRs cause stimulation of phospholipase C which hydrolizes phosphatidyloinositol 4,5-bisphoshpate generating inositol-1,4,5-triphosphate (IP3) and diacylglycerol. The latter in turn activates PKC.

Both, CCK and acetylcholine are major physiologic stimuli activating pancreatic acinar cells for enzyme secretion (18). EGF is normally present in pancreatic juice, however, its effect on pancreatic secretion is controversial. Some investigators showed no significant effect (19, 20), while the others reported

stimulatory role of EGF on pancreatic secretion, both *in vivo* and *in vitro* (21, 22). Multiple signaling pathways are integrated into intracellular protein networks. Within these networks, cross-talk between the individual pathways may result in signal amplification or attenuation. Regulatory interactions among individual receptor-coupled signaling cascades are critically important for establishing cellular responses in the face of multiple stimuli. Therefore, the aim of our study was to evaluate the effect of concomitant stimulation of G-protein-coupled and EGF receptors on activation of ERK in pancreatic acinar cells.

MATERIAL AND METHODS

Materials

Mouse natural EGF was purchased from ICN Biomedicals Inc. CCK octapeptide was from Squibb Research Institute and 12-*O*-tetradecanoylphorbol-13-acetate (TPA) was from LC Laboratories (Woburn, MA). Prestained molecular weight standards were from Bio-Rad (Hercules, CA), nitrocellulose membranes were from Schleicher&Schuell (Keene, NH) Chromatographically purified collagenase was from Worthington Biochemicals (Freehold, NJ). The enhanced chemiluminescence (ECL) detection system and X-ray film were from Amersham (Arlington Heights, IL) The antibody specific for the thyrosine phosphorylated form of ERKs was from Santa Cruz Biotechnology (Santa Cruz, CA). All other reagents were obtained from Sigma (St. Luis, MO).

Preparation of acini and cell-free extract

The preparation of pancreatic acini has been done by method described by Williams et al. (6, 7, 15). Pancreases from male Wistar rats were digested with purified collagenase, mechanically dispersed and passed through a 150-µm mesh nylon cloth. Acini were than purified by centrifugation at 50 g for 3 min in a solution containing 4% bovine serum albumin (BSA) and were resuspended in incubation buffer that consists of an *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES)-buffered Ringer solution supplemented with 11.1 mM glucose, Eagle's minimal essential amino acids, 0.1mg/ml soybean trypsin inhibitor, and 1% BSA. Acini were preincubated at 37°C with minimal shaking for 180 min and then stimulated with different agonists in 1-ml aliquots in polystyrene vials for indicated times. As a control we used isolated pancreatic acinar cells incubated without stimulation. Acini were then pelleted in a microcentrifugate, washed once with 1 ml of ice-cold phosphate-buffered saline containing 1 mM Na_3VO_4 (pH 7.4) and sonicated for 5 s in 0.5 ml of ice-cold lysis buffer [50 nM β-glycerolphosphate, 1.5 mM ethylene glycol-bis(β-amino-ethyl ether)-*N,N,N',N'*-tetraacetic acid, 1nM phenylmethyl-sulfonyl fluoride, 1nM Na_3VO_4, 1 nM dithiothreitol, 10 µg/ml leupeptin, and 10 µg/ml aprotinin (pH 7.4). The lysates were than centrifugated in a microcentrifuge at 4° C for 15 min, and the supernatant was boiled with stop buffer for polyacrylamide gel electrophoresis (SDS-PAGE). The amount of protein in cell extracts was assayed by the Bio-Rad protein assay reagent.

Western blotting

Pancreatic acini extracts were subjected to SDS-PAGE, followed by Western blot analysis with the indicated antibody using an ECL detection system. Probing for active ERKs was done with the use of antibodies specifically recognizing active, tyrosine-phosphorylated ERK (pY-ERK).

Integrated density of bands corresponding to pY-ERK was quantitatived by the use of Gel-Pro Analyzer program and expressed as a percentage of the average control value.

Statistical analysis

Where appropriate, the data have been analyzed by Student's *t*-test.

RESULTS

The effect of EGF, CCK and carbachol on the pY-ERK level in rat pancreatic acinar cells

Figure 1 presents the time course of EGF-, CCK- and carbachol-induced activation of ERKs. The densities of both, ERK1 and ERK2 bands were calculated together and shown in the lower panel of *Fig. 1*. Incubation of pancreatic acinar cells with 100 nM EGF caused abrupt increase of pY-ERK level, compared to control (*Fig. 1A*). EGF rapidly increased activity of both ERKs which reached a maximum at 2 and 5 min, 236% and 257% of control, respectively. At 60 min, pY-ERK level was still 2-fold higher than control. Strong elevation of pY-ERK level was observed after stimulation of pancreatic acini with 10 nM CCK (*Fig. 1B*). However, in this case the increase was not so rapid, and at 2 min, pY-ERK was at the level of 139% of control. The difference in the effect of both agonists on the pY-ERK level at 2 min was statistically significant ($p<0.017$). Both, CCK and EGF caused sustained ERK activation and after 60 min pY-ERK level was still 2-fold higher than control. Similarly to CCK, carbachol, which is M3 muscarinic receptor agonist caused strong and rapid increase of pY-ERK level, reaching 225% of control at 2 min (*Fig. 1C*). The pY-ERK level at 60 min was almost 5-fold higher than control and this effect was much stronger than caused by EGF ($p<0.01$) or CCK ($p<0.05$).

The effect of concomitant stimulation of pancreatic acinar cells with the mixture of EGF with CCK or CAR on pY-ERK level

Figure 2 shows the time course of the concomitant stimulation of pancreatic acini with the mixture of 100 nM EGF and 10 nM CCK on pY-ERK level. Surprisingly, after stimulation of acini with two agonists interacting with different receptors, instead of potentialization, we observed significant inhibition of ERK activity during the whole incubation period ($p<0.01$). This inhibitory interaction was especially strong in the first 2, 5 and 10 min, when pY-ERK level dropped even below the control, reaching 56 ± 17%, 54 ± 11% and 86 ± 36% respectively. This kind of inhibition was also observed after stimulation with the mixture of 100 nM EGF and 100 μM CAR (*Fig. 3*). However, in this case, it was restricted to the period of the initial 10 min. Compared to the stimulation with the individual agents at the first 2, 5 and 10 min of concomitant incubation, pY-ERK level dropped to 59 ± 13%, 76 ± 39% and 131 ± 16% of control, respectively ($p<0.01$). Interestingly, at

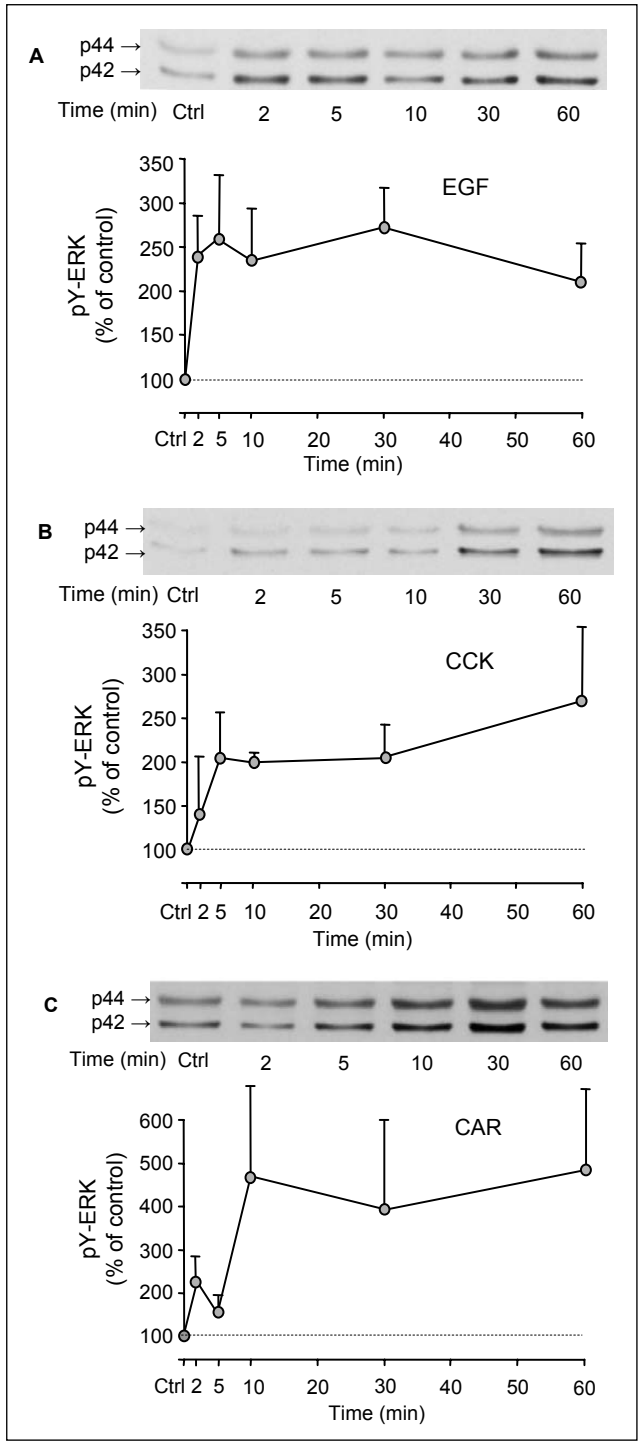

Fig. 1 (A,B,C). The effect of EGF (A), CCK (B) and CAR (C) on the pY-ERK level in rat pancreatic acini. Acini were stimulated for different time periods with 100 nM EGF, 10 nM CCK or 100 μM carbachol. The pY-ERK level was determined by means of Western-blotting, with the use of the antibody which recognizes active, tyrosine-phosphorylated kinase. Each point of the diagram represents mean ± SD of 3-8 independent experiments, each performed in duplicate. Upper panel of the figure shows a sample of representative Western blotting with bands corresponding to two forms of ERK. Ctrl - acinar cells incubated 60 minutes without stimulation. Ctrl level marked as dotted line.

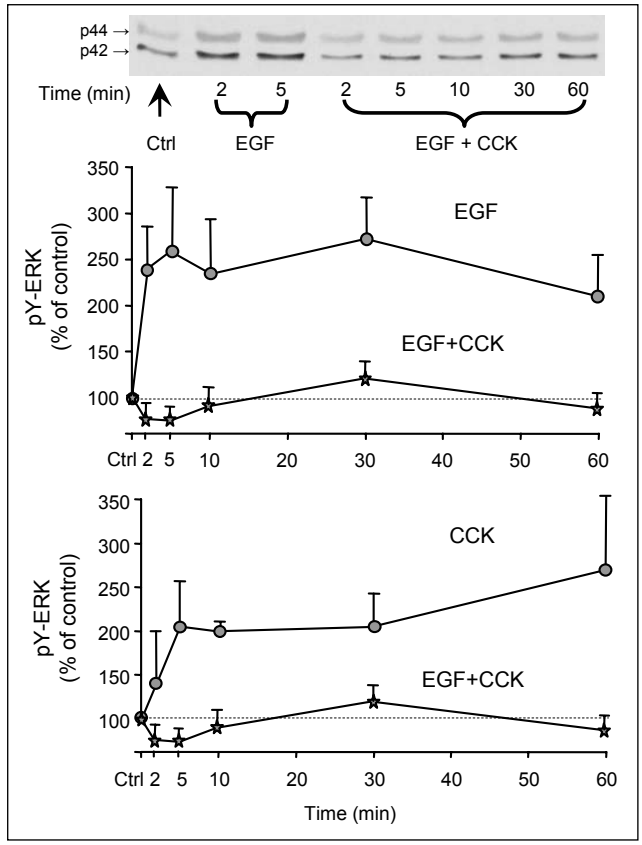

Fig. 2. The effect of CCK and EGF on the pY-ERK level in rat pancreatic acini. Acini were stimulated for different time periods with 10 nM CCK or 100 nM EGF applied alone or as a mixture. All measurements performed as described in *Fig. 1.*

30 and 60 min of acini incubation with the mixture of EGF and CAR, pY-ERK level increased to 285 ± 114% and 475 ± 92% of control levels, respectively.

Stimulation of pancreatic acini with the mixture of GPCRs agonists, namely CCK and CAR, had no inhibitory effect on ERK activity (*Fig. 4*). After simultaneous incubation with 10 nM CCK and 100 μM CAR, at 5 and 60 min, pY-ERK level reached 203 ± 86% and 356 ± 103% of control, respectively, and generally remained at the level corresponding to the effect of CAR or CCK alone.

These regulatory interactions have been also found when short stimulation (2 or 5 min) with CAR or CCK was superimposed on 60 min-lasting incubation of acini with EGF or the opposite. The addition of EGF, for 2 min, to 60 min-lasting incubations of acini with CCK or CAR caused abrupt decrease of ERK activity to 81 or 82% of control level ($p<0.002$) (*Fig. 5*). Similar phenomenon was observed when short stimulation with CCK or CAR was superimposed on 60 min-lasting incubation of acini with EGF (127 ± 64% and 117 ± 46% respectively, $p<0.05$). Interestingly, these inhibitory effects were not found when CCK stimulation was superimposed on short stimulation with CAR or the opposite.

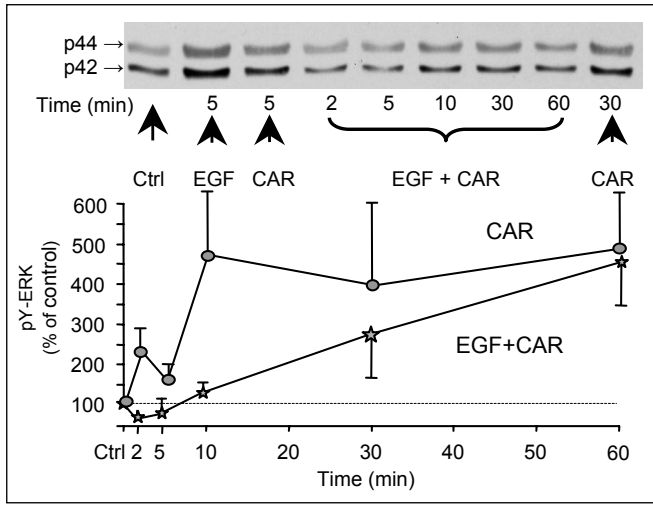

Fig. 3. The effect of EGF and CAR on the pY-ERK level in rat pancreatic acini. Acini were stimulated for different time periods with 100 nM EGF or 100 μM CAR applied alone or as a mixture. All measurements performed as described in *Fig. 1.*

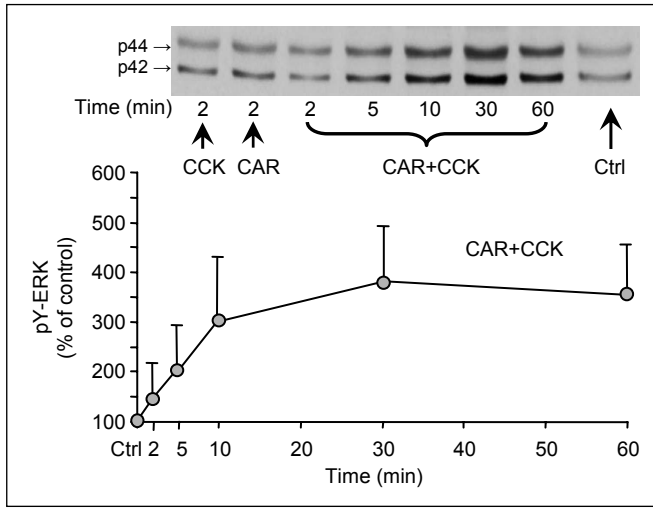

Fig. 4. The effect of CCK and CAR on the pY-ERK level in rat pancreatic acini. Acini were stimulated for different time periods with the mixture of 10 nM CCK and 100 μM CAR. All measurements performed as described in *Fig. 1.*

The effect of the concomitant stimulation of pancreatic acinar cells with EGF and TPA on pY-ERK level

Incubation of pancreatic acini with 1 μM TPA directly stimulating PKC, caused very strong, more than 5-fold, activation of ERK at 60 min (*Fig. 6*). The addition of EGF, for 2 min, to 60 min-lasting incubation of acini with TPA caused statistically significant decrease of ERK activity to 170 ± 37% of control level ($p<0.005$). Similar effect was observed when short stimulation with TPA was superimposed on 60 min-lasting incubation of acini with EGF (152 ± 105%).

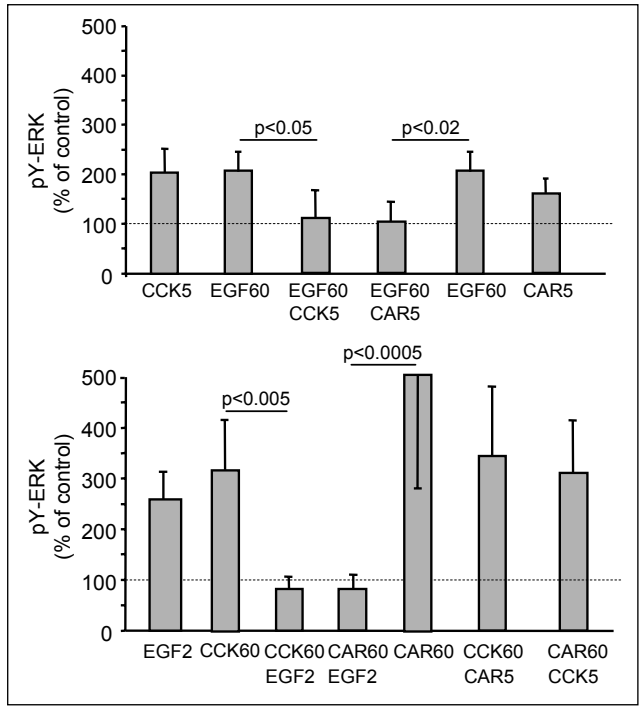

Fig. 5. The pY-ERK level in pancreatic acinar cells stimulated with EGF, CCK or CAR for different time periods. Each bar represents mean ± SD of 3-8 independent experiments, each performed in duplicate. The double labels below the individual bars mean, for example: "EGF60/CCK5" - the addition of CCK for 5 min to 60 min-lasting stimulation of acini with EGF.

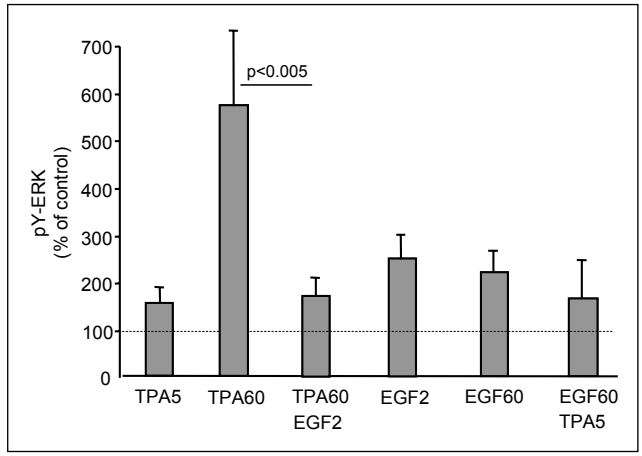

Fig. 6. The pY-ERK level in pancreatic acinar cells stimulated with EGF or TPA in different time periods. Each bar represents mean ± SD of 3-8 independent experiments, each performed in duplicate. The double labels below the individual bars mean, for example: "EGF60/TPA5" - the addition of TPA for 5 min to 60 min-lasting stimulation of acini with EGF.

DISCUSSION

In mammals, MAPK superfamily is composed of several subfamilies including ERK, JNK/SAPK, p38 and ERK5 (23). Each MAPK has its own activators, substrates, and inactivators. ERK is activated mainly by mitogenic stimuli and many authors compared it to the “information highway“ used by growth factors

and hormones that trigger proliferation and cell differentiation. In different biological systems, ERK activation is known to be correlated with more than one physiological response to a specific stimulus, and this raises the question of how the same ERK cascade can affect different physiological responses (4). In the last years, it has become increasingly clear that signaling pathways are not organized in a simply linear fashion but are connected to complex signaling networks (24). In normal and pathological conditions not one, but many agonists with the potential to activate ERK interact with pancreatic acinar cells at the same time. Pancreatic acinar cells contain a large number of cell surface receptors associated with an individualized signal transduction system to generate intracellular responses to receptor activation. However, it has been found that the multitude of signals generated from multiple receptors must integrate at some point of the signaling cascade. Many mechanisms have been identified by which this can occur, for example cross-regulation and cross-activation of receptors or activation of specific phosphatases. GPCRs may recruit multiple and redundant mechanisms to stimulate ERK activity. Gi- and Gq-coupled receptors mainly promote transactivation of EGF receptor by activation of matrix metalloproteinases leading to the release of EGF-like ligands which subsequently activate the receptor tyrosine kinase (25-27). Alternatively, the non-receptor tyrosine kinase Src may be activated by GPCRs via multiple mechanisms resulting in Ras activation mediated by the adaptor protein Shc. Finally, Gq protein-coupled receptors may stimulate ERK activity independent of Ras but involving PKC (6, 7, 15).

Although the individual signaling cascades coupled to EGF, CCK or muscarinic receptors have been identified in some detail, there is little information about modulatory interactions between signaling cascades activated by these types of receptors. It is known that in pancreatic acinar cells EGF and CCK or carbachol activate both ERK's isoforms of $p44^{MAPK}$ and $p42^{MAPK}$ known as ERK 1 and ERK 2 (5, 7, 15). In the present study we found that stimulation of pancreatic acinar cells with individual agonist, namely EGF or CCK alone, caused strong increase of pY-ERK level. Incubation of pancreatic acini with carbachol also caused rapid and sustained increase of pY-ERK level. Therefore, our results are in line with other studies (4, 28).

Currently, we evaluated partially activated kinase with the use of specific antibody which recognizes tyrosine-phosphorylated kinase (pY-ERK). As we know, to reach the full activation of ERK, double phosphorylation on tyrosine and threonine residues is required (pTpY-ERK) (29). On the other hand, pY-ERK exerts a lot of very important biological functions in the cell. pY-ERK has been found constitutively present in the nucleus, and localized to the Golgi complex of cells that are in the late G2 or early mitosis of the cell cycle. Increased ERK phosphorylation causes punctate distribution of several Golgi proteins, indicating disruption of the Golgi structure. It has been recently suggested that pY-ERK and not ERK kinase activity regulates Golgi structure and may be involved in mitotic Golgi fragmentation and reformation (30).

Until now, there was no information about the effect of concomitant stimulation of pancreatic acinar cells with two different stimuli able to activate ERKs. We have shown, for the first time, that in isolated pancreatic acinar cells concomitant activation of growth factor receptor and G-protein coupled receptor caused not augmentation, but abrupt and strong inhibition of ERK activity. In concomitant stimulation with EGF and CCK, pY-ERK level dropped even below control, which indicates some powerful inhibitory mechanisms. Similar phenomenon was observed when short stimulation with one agonist was superimposed on long-lasting incubation of acini with the other agonist. Interestingly, it was not found after the concomitant incubation of acini with CCK and CAR, stimulating the same type of cellular receptors. Therefore, this inhibitory interaction may represent the novel mechanism of ERK activity regulation.

One of the possible explanations of this inhibitory cross-talk is the activation of distinct intracellular pools of ERK by EGF and CCK. Because ERK has targets in different compartments of the cell, it is possible that EGF and CCK may activate distinct pools of ERK in pancreatic acinar cells. Compartmentalization of the ERK cascade was also suggested in the study showing that insulin and EGF regulate distinct pools of Grb2-SOS in the control of Ras activation (31). However, the exact mechanism of the inhibitory cross-talk between different pathway remains unknown.

A currently accepted model maintains that ERK1 and ERK2 are regulated similarly and contribute to intracellular signaling by phosphorylating a largely common subset of substrates in the cytosol and nucleus. Recent evidence suggests that there are quantitative differences in ERK1 and ERK2 dynamics and that these could have significant role in their regulation. It is possible that although ERK2 is essential for transduction of signals, ERK1 could instead have an accessory role, possible enabling a fine tuning of ERK2 activity. There are some evidences that ERK1 acts by attenuating ERK2 activity (32, 33). In a very recent paper the authors reveal an unexpected interplay between ERK1 and ERK2 in transducing Ras-dependent cell signaling and proliferation in mouse embryo fibroblasts and NIH 3T3 cells (34). Whereas ERK2 seems to have a positive role in controlling normal and Ras-dependent cell proliferation, ERK1 probably affects the overall signaling output of the cell by antagonizing ERK2 activity. Perhaps this kind of interaction, between ERK1 and ERK2, may participate in decreasing of pY-ERK level after concomitant stimulation of pancreatic acini with EGF and CCK or carbachol.

In our opinion, the best possible explanation of strong ERK inhibition, after simultaneous stimulation with EGF and CCK or CAR is a quick activation of protein tyrosine phosphatases (MAPK phosphatases) which downregulate ERK (35).

Different kinds of receptor interactions have been recently reported (36-38). Repeated agonist stimulation triggers a negative feedback regulatory mechanism that attenuates GPCR-mediated signal transduction and is known as desensitization. The initial event of desensitization is the phosphorylation of GPCR catalyzed by G-protein-coupled receptor kinases (GRKs) (36). GRKs are a key modulator of GPCR signaling. A recent study has shown that in HEK293

cells overexpression of GRK2 enhances EGF-induced ERK activation, suggesting that GRK2 may exert a positive regulation on EGF-induced ERK phosphorylation (37). Simultaneously GRK-catalyzed GPCR phosphorylation leads to GPCR desensitization and subsequent internalization and degradation. In the COS-7 signaling network high levels of cAMP produced, for example, by co-stimulation of β2-adrenergic receptor (β2-AR) and bradykinin B2 receptor (BKR) may affect EGF receptor-mediated activation of ERK (38).

Regulatory interactions among the signals generated by muscarinic M3 and EGF receptors were examined in human neuroblastoma SH-SY5Y cells (28). This study revealed that there are mutual inhibitory interactions in the signaling cascades activated by each of these receptors, with PKC mediating inhibitory effects of stimulated muscarinic M3 receptors on EGF receptor-linked signaling, and reactive oxygen species mediating inhibitory effects of stimulated EGF receptors on muscarinic M3 receptor-linked signaling. It is possible that neurons likely employ these inhibitory cross-talk interactions to integrate signals emanating from G-protein coupled and growth factor receptor. Although ERK activity was not assessed directly in this study, these data are in accordance with our findings. In our study, we used phorbol ester TPA instead of CCK and CAR to determine potential sites of these mutual inhibitory interactions. In pancreatic acini, activation of ERK by CCK and CAR is almost entirely PKC-dependent. TPA stimulates ERK directly through the activation of PKC, without stimulation of GPCRs. The decrease of pY-ERK level after stimulation of acini with the mixture of EGF and TPA suggest that PKC plays an important role in the inhibitory cross-talk phenomenon we have found.

In conclusion, we have made the observation of novel regulatory interactions in transmission of intracellular signals after activation with the multiple stimuli in freshly isolated pancreatic acinars cells. We believe that these kind of inhibitory interactions play a crucial role in the integration of multiple signals generated by activation of G-protein-coupled (i.e. CCK or muscarinic) and growth factor (i.e. EGF) receptors in pancreatic acinar cells. Further work is necessary for full evaluation of potential sites and mechanisms of mutual inhibitory interactions in the course of ERK cascade activation in pancreatic acinar cells.

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