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ORIGINAL ARTICLE

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Cholecystokinin-stimulated enzyme secretion from dispersed rabbit pancreatic acinar cells: phosphorylation-dependent changes in potency and efficacy

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Abstract In order to establish a regulatory role for phosphoproteins in receptor-stimulated enzyme secretion, dispersed rabbit pancreatic acinar cells were stimulated with the COOH-terminal octapeptide of cholecystokinin (CCK_8) in the absence and presence of staurosporine and/or 12-*O*-tetradecanoylphorbol 13-acetate (TPA) or forskolin. The dose/response curve for the stimulatory effect of CCK_8 on amylase secretion was biphasic, with a mean half-maximal concentration (EC_{50}) of 21 pM. Staurosporine (1 μM) did not affect secretion elicited by CCK_8 concentrations below 0.1 nM, but reduced the response to CCK_8 concentrations above 0.1 nM. As a result, the mean EC_{50} for CCK_8 decreased to 8 pM and its efficacy to 70%. The phorbol ester TPA (0.1 μM) attenuated secretion evoked by CCK_8 concentrations below 0.1 nM and potentiated the response to CCK_8 concentrations above 0.1 nM. As a result, the mean EC_{50} for CCK_8 increased to 0.14 nM and its efficacy to 300%. Staurosporine abolished both the inhibitory and the potentiating effect of TPA, thereby turning the inhibitory effect into a strong potentiating effect. As a result, the mean EC_{50} for CCK_8 decreased to 3 pM, whereas its efficacy increased to 190%. Forskolin (30 μM) potentiated the response to both the lower and the higher CCK_8 concentrations. As a result, the mean EC_{50} for CCK_8 increased to 28 pM and its efficacy to 300%. Staurosporine enhanced the potentiating effect of forskolin at CCK_8 concentrations below 0.1 nM, but abolished potentiation at CCK_8 concentrations above 0.1 nM. As a result, the mean EC_{50} for CCK_8 decreased to 1.4 pM, whereas its efficacy increased to 260%. The data presented demonstrate that the apparent sensitivity of dispersed pancreatic acinar cells to stimulation of the process of enzyme secretion by CCK_8 decreases when kinases are activated and increases when kinases are inactivated. Moreover, they show that the efficacy of CCK_8 increases by the action of kinases, both sensitive and insensitive to staurosporine.

Key words Pancreatic acinar cell · Cholecystokinin-octapeptide · TPA · Forskolin · Thapsigargin · Staurosporine · Secretion

Introduction

Stimulation of pancreatic digestive enzyme secretion by the hormones cholecystokinin (CCK) and secretin and the neurotransmitters acetylcholine (Ach) and vasoactive intestinal polypeptide (VIP), which interact with specific receptors located on the surface of the acinar cell, is paralleled by either the production of cyclic adenosine 5′-monophosphate (cAMP) [10], activating protein kinase A (PKA), or the simultaneous formation of 1,2-diacylglycerol [37], activating protein kinase C (PKC) [23], and inositol 1,4,5-trisphosphate [37], releasing Ca^{2+} from intracellular stores [3].

In all species tested, the Ca^{2+}-mobilizing secretagogues CCK and Ach readily stimulate the process of enzyme secretion [10]. On the other hand, the cAMP-generating stimulants secretin and VIP are secretory active in the guinea-pig and in the rat but not in the rabbit[1]. Concerning the physiological significance of cAMP in pancreatic digestive enzyme secretion, most studies, including those using permeabilized acinar cells [24], tend towards a modulatory rather than a stimulatory role, since in all species tested the secretory response to Ca^{2+}-mobilizing secretagogues is markedly potentiated in the presence of elevated acinar cell cAMP levels [10, 37].

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The Ca^{2+}-mobilizing secretagogues CCK and Ach activate a bifurcating signal transduction pathway resulting in the simultaneous activation of PKC and modulation of Ca^{2+}-dependent processes [3, 23]. Both routes can be activated separately, in that phorbol esters mimic the stimulatory effect of 1,2-diacylglycerol on PKC and Ca^{2+} ionophores increase the free cytosolic Ca^{2+} concentration. Many studies have demonstrated that artificial activation of each one of these two routes only moderately stimulates secretion [2, 4–8, 11, 14, 15, 19–21, 25, 29, 30], whereas simultaneous activation of both routes mimics the stimulatory effect of the Ca^{2+}-mobilizing secretagogues [5, 19, 25].

Putative inhibitors of PKC activity, such as H-7 and staurosporine, have been used in an attempt to ascertain the relative importance of both routes in the mechanism of action of the Ca^{2+}-mobilizing secretagogues [7, 21, 25, 29]. Staurosporine was found to completely inhibit the secretory response to the phorbol ester 12-*O*-tetradecanoylphorbol 13-acetate (TPA) [7, 21, 29], whereas the drug only partly reduced the secretory response to carbachol [21, 29], CCK [7, 29] and caerulein [21]. On the other hand, H-7 was found to potentiate rather than inhibit the secretory response to TPA [25], CCK [7, 25] and carbachol [25]. Similarly, TPA-evoked down-regulation of PKC was demonstrated to decrease the efficacy of both CCK and carbachol [27].

Pretreatment of pancreatic acinar cells with a phorbol ester has been demonstrated to result in inhibition of the secretory response to Ca^{2+}-mobilizing secretagogues [2, 4, 14, 15, 21, 33]. Virtually complete inhibition was observed with submaximal stimulatory secretagogue concentrations [14, 21, 33]. In one study, this inhibitory effect of the phorbol ester was paralleled by a significant reduction in efficacy of CCK [14]. In contrast, in a second study the efficacy of the secretagogue was found to remain unaffected (caerulein) or to become slightly increased (carbamylcholine) by TPA treatment [21]. Finally, in a third study, the phorbol ester was demonstrated to markedly potentiate the secretory response to an optimal concentration of CCK [6]. So far, the reason for these discrepancies is unclear and forms the object of the present study.

In a previous study, using dispersed rabbit pancreatic acinar cells, it was observed that the dose/response curve for the stimulatory effect of CCK_8 on amylase secretion was relatively broad, in that it ranged from a minimally effective concentration of 1 pM to a maximally effective concentration of 10 nM [34]. In addition, the efficacy of CCK_8 was found to be rather low as compared to pancreatic acini. In contrast, the dose/response curve for the recruitment of dispersed acinar cells in terms of CCK_8-evoked Ca^{2+} mobilization was found to be less broad, in that it ranged from a minimally effective concentration of 1 pM to a maximally effective concentration of 0.1 nM. Preliminary experiments with dispersed acinar cells revealed that TPA not only decreased the maximally effective CCK_8 concentration for the stimulation of amylase secretion but, in addition, markedly potentiated the secretory response to this particular secretagogue concentration. Thus, TPA caused both a narrowing of the dose/response curve for CCK_8 and an increase in the efficacy of the secretagogue. These observations urged us to investigate the effects of kinase activation and inactivation on the shape of the dose/response curve for the stimulatory effect of CCK_8 on amylase secretion from dispersed pancreatic acinar cells.

Materials and methods

Pancreatic acinar cells

Rabbit pancreatic acinar cells were prepared as described previously [34, 35].

Amylase secretion experiments

For secretion studies, dispersed acinar cells were resuspended in a HEPES/Tris (where HEPES denotes 4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid) medium (pH 7.4) containing 133 mM NaCl, 4.2 mM-KCl, 1.0 mM-$CaCl_2$, 1.0 mM-$MgCl_2$, 5.8 mM glucose, 0.2 mg/ml soybean trypsin inhibitor and 10 mM HEPES, adjusted with Tris to pH 7.4; the solution was gassed thoroughly with 100% O_2. After preincubation for 10 min at 37°C, 200 µl aliquots of the suspension were transferred to Eppendorf microtubes containing the indicated concentrations of CCK_8, and incubated for another 20 min at 37°C. The incubations were stopped by rapid centrifugation at 10000 *g* for 10 s (Eppendorf minifuge) and the supernatant was used for determination of medium amylase activity. At the beginning of the experiment, an aliquot of the suspension was removed and the acinar cells were homogenized for determination of total amylase activity. Amylase activity was measured by means of the Phadebas test and medium amylase activity was expressed as a percentage of total amylase present in the acini at the beginning of incubation. Staurosporine (1 µM) was present from the beginning of the preincubation period, forskolin (30 µM) was added after 5 min and TPA was added immediately before transferring the 200-µl aliquots to the microtubes containing CCK_8. Thapsigargin was present in the microtubes. In the case of testing various TPA concentrations, the phorbol ester was present in the microtubes.

cAMP measurements

The cellular cAMP content was determined as described previously [31, 32]. Staurosporine (1 µM) and 3-isobutyl-1-methylxanthine (IBMX, 0.1 mM) were present from the beginning of preincubation. TPA was added immediately before transferring the 200 µl aliquots to the microtubes. CCK_8 (10 nM) and forskolin (30 µM) were present in the microtubes. The reaction was quenched after 10 min and the cellular cAMP content was expressed in pmoles cAMP per milligram protein. Protein was determined with a commercial Coomassie blue kit (Bio-Rad, Richmond, Calif., USA) using bovine serum albumin (BSA, Hoechst-Behring, Marburg, Germany) as a standard.

Analysis of the data

Half-maximally stimulatory concentrations and maximal release values were calculated by means of the nonlinear regression computer

programme InPlot (Graphpad Software for Science, San Diego, Calif., USA). Differences between the mean values were determined by analysis of variance [26]; $P < 0.5$ was considered statistically significant.

Materials

Collagenase was purchased from Cooper Biomedical, Malvern, Pa., USA; BSA, soybean trypsin inhibitor, Trypan blue, TPA and CCK_8 from Sigma, St. Louis, Mo., USA. Hyaluronidase, cAMP and staurosporine were obtained from Boehringer, Mannheim, Germany; IBMX from Aldrich, Milwaukee, Wis., USA and [^{3}H]cAMP (666 GBq/mmol) from Amersham, UK. Forskolin was purchased from Calbiochem, La Jolla, Calif., USA and thapsigargin from LC Services, Woburn, Mass., USA. The Phadebas test kit was obtained from Pharmacia, Uppsala, Sweden and activated charcoal (Norit, SX-1) from Norit, Amersfoort, The Netherlands. The cAMP binding protein was isolated from bovine adrenal cortex. All other chemicals were of reagent grade.

Results

Time course for the effect of CCK_8 on amylase release from isolated pancreatic acini and dispersed pancreatic acinar cells

Dispersed pancreatic acinar cells, prepared according to the method of Amsterdam and Jamieson [1] as described previously [34, 35], released amylase at a considerably slower rate than isolated acini when stimulated with a maximally effective concentration of 10 nM CCK_8 (Fig. 1). Pancreatic acini were prepared by omitting the divalent cation chelating step from the protocol for the isolation of dispersed acinar cells as described previously [31–33].

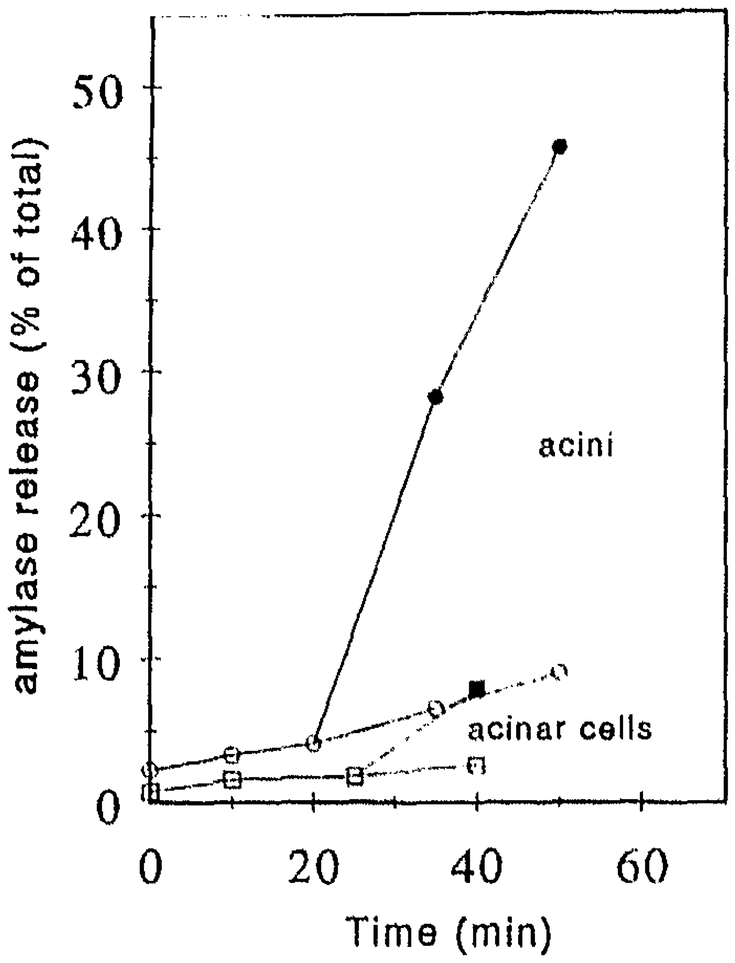
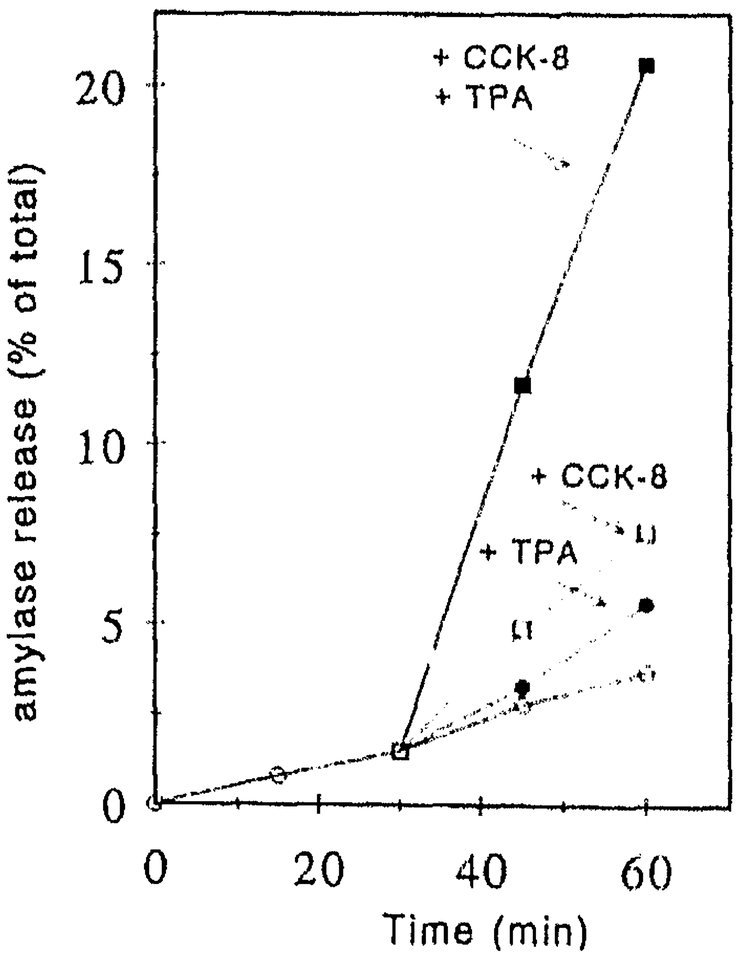


Fig. 1 Time course for the effect of the COOH-terminal octapeptide of cholecystokinin (CCK_8) on amylase release from isolated pancreatic acini and dispersed pancreatic acinar cells. Rabbit pancreatic acini (*circles*) and dispersed pancreatic acinar cells (*squares*) were resuspended in a HEPES/Tris medium and incubated at 37°C. CCK_8 (*closed symbols*) was added at a concentration of 10 nM and samples for measurement of medium amylase activity were removed at the indicated times. Amylase activity is expressed as a percentage of the initial cellular amylase content

Time course for the effects of CCK_8 and TPA, alone and in combination, on amylase release from dispersed pancreatic acinar cells

Stimulation of dispersed pancreatic acinar cells with 10 nM CCK_8 evoked a nearly linear increase in amylase release with time during the first 30 min (Fig. 2). At this concentration, which was a maximally effective concentration with this acinar preparation (Fig. 3), CCK_8 released an average of 4.1% (SEM ± 0.5, $n = 17$) of the total amylase originally present in the acinar cells during the first 20 min of stimulation. Compared to the effect of CCK_8, the phorbol ester, TPA, added at a maximally effective concentration of 0.3 µM (Fig. 4), only slightly enhanced the rate of amylase release. Surprisingly, however, TPA markedly potentiated the stimulatory effect of CCK_8. The increase in amylase release, evoked by the combination of secretagogue and phorbol ester, was virtually linear with time during the first 30 min.

Fig. 2 Time course for the potentiating effect of 12-*O*-tetradecanoylphorbol 13-acetate (TPA) on CCK_8-induced amylase secretion from dispersed pancreatic acinar cells. Dispersed rabbit pancreatic acinar cells were resuspended in a HEPES/Tris medium and incubated at 37°C. TPA (0.3 µM, *closed symbols*) and CCK_8 (10 nM, *squares*) were added at 30 min and samples for determination of medium amylase activity were removed at the indicated times. Amylase activity is expressed as a percentage of the initial cellular amylase content



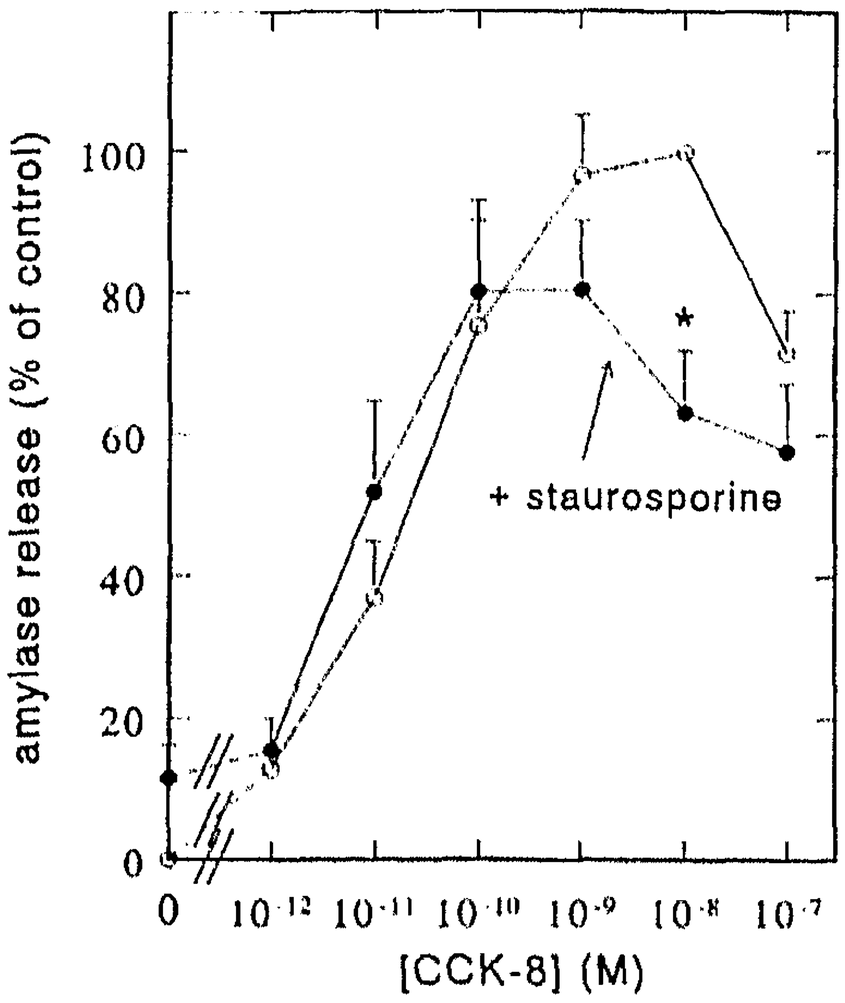


Fig. 3 Effect of staurosporine on the dose/response curve for CCK_8-induced amylase secretion from dispersed pancreatic acinar cells. Dispersed rabbit pancreatic acinar cells were resuspended in a HEPES/Tris medium and preincubated in the presence of either dimethylsulphoxide (*open circles*) or 1 μM staurosporine (*closed circles*) for 10 min at 37°C. Aliquots of the suspension were transferred to Eppendorf microtubes containing the indicated concentrations of CCK_8. Medium amylase activity was determined at 20 min following the onset of stimulation and expressed as a percentage of the initial cellular amylase content. After correction for basal amylase release, the release value obtained with 10 nM CCK_8 is set at 100%, to which all other values are related. The data presented are the mean ± SEM of 8 experiments. *Significantly different ($P < 0.05$) from corresponding value for CCK_8-stimulated control cells

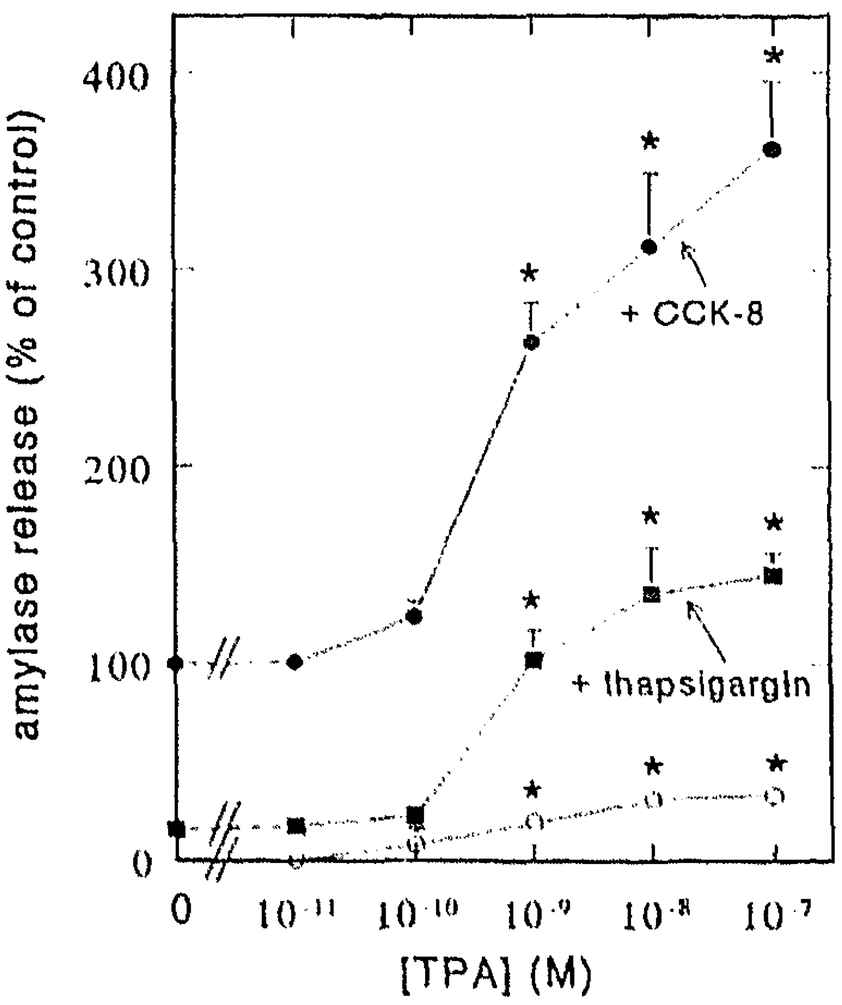


Fig. 4 Dose/response curve for the effect of TPA alone and in combination with either thapsigargin or CCK_8 on amylase release from dispersed pancreatic acinar cells. Dispersed rabbit pancreatic acinar cells were resuspended in a HEPES/Tris medium and preincubated for 10 min at 37°C. Aliquots of the suspension were transferred to Eppendorf microtubes containing the indicated concentrations of TPA alone (*open circles*) or in combination with either 1 μM thapsigargin (*closed squares*) or 10 nM CCK_8 (*closed circles*). Medium amylase activity was determined at 20 min following the onset of stimulation and expressed as a percentage of the initial cellular amylase content. After correction for basal amylase release, the release value obtained with 10 nM CCK_8 is set at 100%, to which all other values are related. The data presented are the mean ± SEM of 4 experiments. *Significantly different ($P < 0.05$) from corresponding control cells

Dose/response curve for the effect of TPA on thapsigargin- and CCK_8-stimulated amylase secretion from dispersed pancreatic acinar cells

TPA stimulated amylase secretion dose dependently from dispersed acinar cells (Fig. 4). The lowest concentration of the phorbol ester producing a significant effect was 1 nM, whereas the maximal effect was reached with 10 nM TPA. At this concentration, the stimulatory effect was not significantly different from that obtained with 0.1 μM TPA. This observation suggests that supramaximal stimulation with TPA does not lead to inhibition of the secretory response. The mean half-maximal TPA concentration was calculated to be 0.66 nM (Fig. 5). The maximal effect of TPA was only 35% of that obtained with CCK_8 alone. TPA potentiated the stimulatory effect of 1 μM thapsigargin dose dependently (Fig. 4). The lowest concentration of the phorbol ester producing a significant potentiating effect was 1 nM, whereas the maximal effect was reached with 0.1 μM TPA. At this concentration, the potentiating effect of TPA was significantly higher than that obtained with 1 nM TPA, whereas it was not significantly different from that obtained with 10 nM TPA. The mean half-maximal TPA concentration was calculated to be 0.58 nM (Fig. 5); no supramaximal inhibition was observed. The maximal effect reached with the combination of TPA (0.1 μM) and thapsigargin (1 μM) was 1.4 times higher than that obtained with CCK_8 alone. TPA markedly potentiated CCK_8-stimulated amylase secretion. The effect of the phorbol ester was dose dependent. Again, the minimally effective concentration of TPA was 1 nM. The mean half-maximally effective phorbol ester concentration was calculated to be 0.58 nM (Fig. 5). In the presence of 0.1 μM TPA, the stimulatory effect of 10 nM CCK_8 was increased by a factor of 3.5.

Dose/response curve for the effect of thapsigargin on TPA- and CCK_8-stimulated amylase secretion from dispersed pancreatic acinar cells

Thapsigargin stimulated amylase secretion dose dependently from dispersed acinar cells (Fig. 6). The lowest concentration producing a significant effect was 10 nM. The release values obtained with 10 nM, 0.1 μM and 1 μM thapsigargin were not significantly different and the maximal effect of thapsigargin was 15% of that obtained with CCK_8 alone. No supramaximal

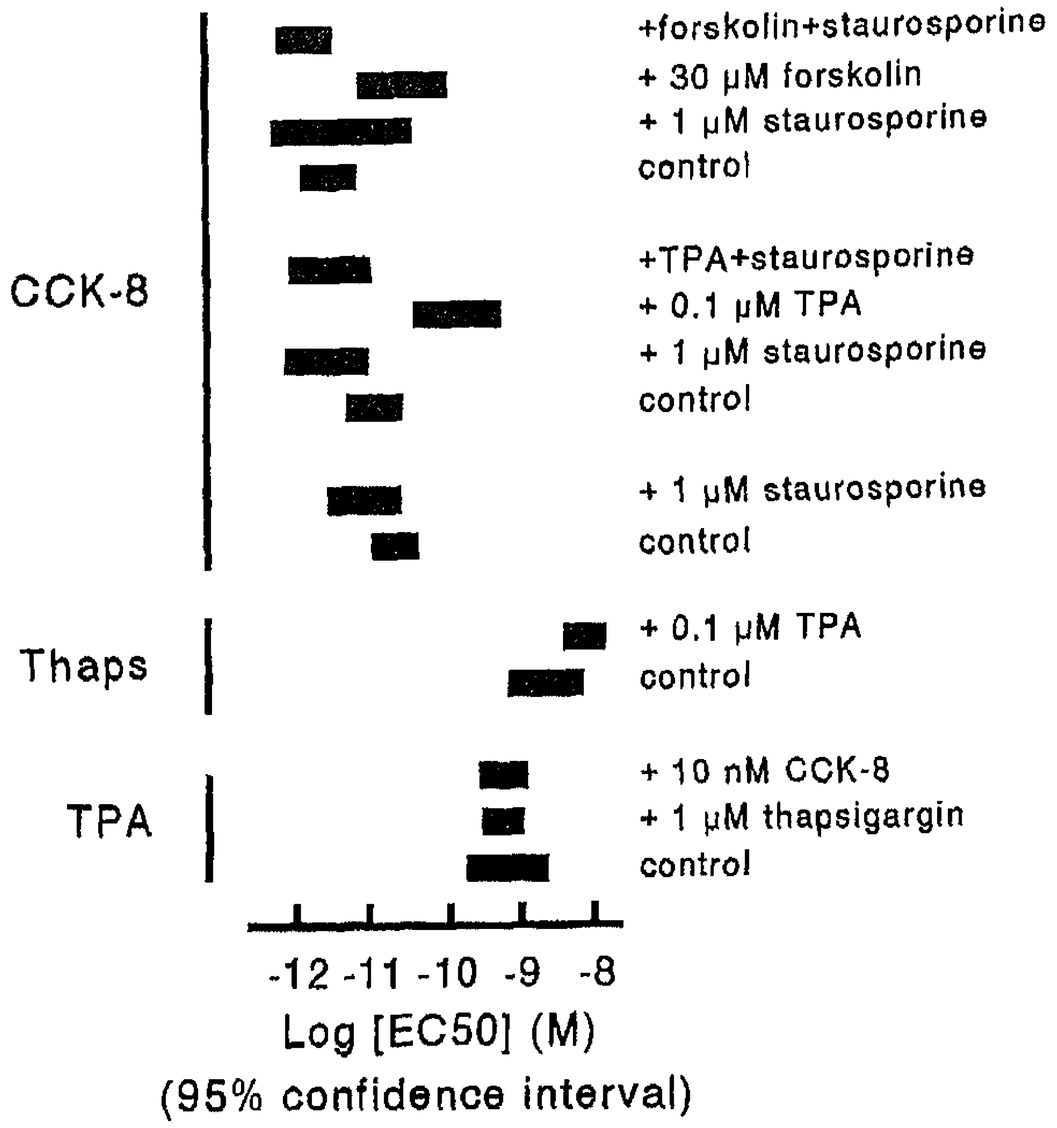


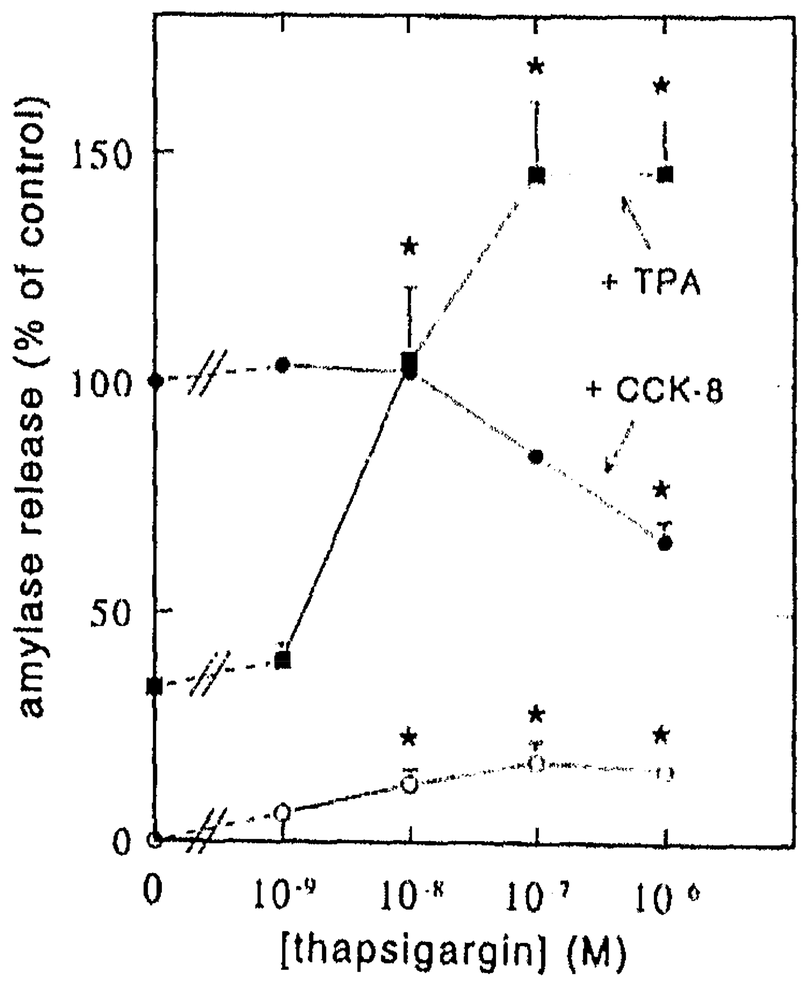
Fig. 5 Effect of various treatments on the half-maximally stimulatory concentrations ([EC_{50}]) of CCK_8, TPA and thapsigargin. The half-maximally stimulatory concentrations for CCK_8, TPA and thapsigargin were calculated from the data presented in the Figs. 3, 4, 6–8 by means of the nonlinear regression computer programme InPlot (Graphpad Software for Science, San Diego, Calif., USA). Each *bar* represents the 95% confidence interval

inhibition of amylase secretion was observed with thapsigargin. The mean half-maximal concentration was calculated to be 2.2 nM (Fig. 5). Thapsigargin markedly potentiated the stimulatory effect of 0.1 µM TPA. The minimally effective concentration was 10 nM, whereas maximal potentiation was reached with 0.1 µM thapsigargin. The potentiating effect obtained with 0.1 µM thapsigargin was not significantly different from that obtained with 1 µM thapsigargin. The mean half-maximal concentration was calculated to be 7.0 nM (Fig. 5). Again, no supramaximal inhibition was observed. The maximal effect reached with thapsigargin (either 0.1 µM or 1 µM) in combination with TPA (0.1 µM) was 1.5 times higher than that obtained with CCK_8 alone. In contrast to TPA, thapsigargin inhibited the secretory response to 10 nM CCK_8 dose dependently. The minimally inhibitory concentration of thapsigargin was 0.1 µM. At a concentration of 1 µM, thapsigargin inhibited the response to 10 nM CCK_8 by approximately 35%.

Effect of staurosporine on the dose/response curve for the stimulatory effect of CCK_8 on amylase secretion from dispersed pancreatic acinar cells

CCK_8 stimulated amylase secretion dose dependently from dispersed pancreatic acinar cells (Fig. 3). The dose/response curve did not show a clear maximum in that the release values obtained with 0.1 nM and 1 nM CCK_8 were not significantly different from that obtained with 10 nM CCK_8. The effect of 0.1 µM CCK_8 was significantly lower than that obtained with 10 nM CCK_8, demonstrating the occurrence of supramaximal inhibition. The mean half-maximal concentration was calculated to be 21 pM (Fig. 5). Staurosporine (1 µM) did not affect the stimulatory effect of CCK_8 concentrations below 0.1 nM, but tended to reduce the secretory response to secretagogue concentrations above 0.1 nM. Statistical significance was reached with 10 nM CCK_8. Staurosporine alone tended to increase the basal rate of amylase secretion. However, this effect was not statistically significant. The release values obtained with 10 pM, 10 nM and 0.1 µM CCK_8 were significantly lower than the value reached with 1 nM CCK_8, whereas the release value obtained with 0.1 nM CCK_8 did not differ significantly from the latter value. In the presence of staurosporine, the mean half-maximal concentration of CCK_8 was calculated to be 8 pM (Fig. 5). The maximal effect of CCK_8, after correction for the stimulatory effect of staurosporine alone (12%), was reduced by 30%.

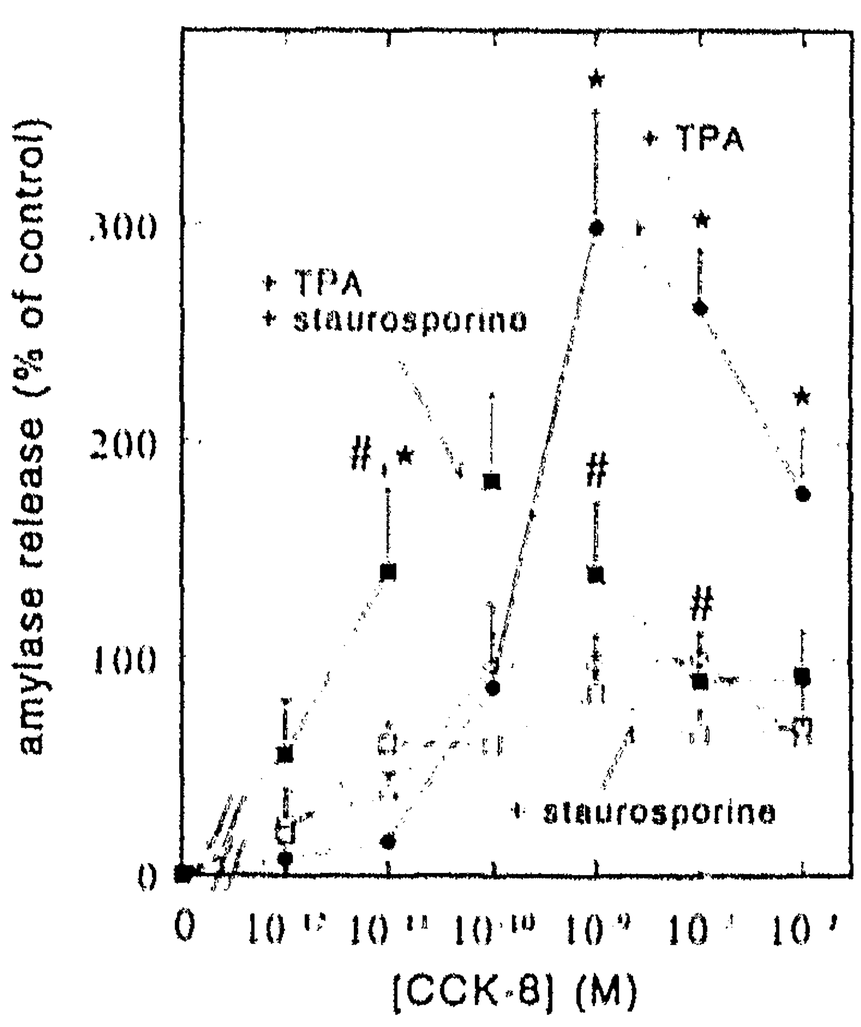
Fig. 6 Dose/response curve for the effect of thapsigargin alone and in combination with either TPA and CCK_8 on amylase release from dispersed pancreatic acinar cells. Dispersed rabbit pancreatic acinar cells were resuspended in a HEPES/Tris medium and preincubated for 10 min at 37°C. Aliquots of the suspension were transferred to Eppendorf microtubes containing the indicated concentrations of thapsigargin alone (*open circles*) or in combination with either 0.1 µM TPA (*closed squares*) or 10 nM CCK_8 (*closed circles*). Medium amylase activity was determined at 20 min following the onset of stimulation and expressed as a percentage of the initial cellular amylase content. After correction for basal amylase release, the release value obtained with 10 nM CCK_8 is set at 100%, to which all other values are related. The data presented are the mean ± SEM of 4 experiments. *Significantly different ($P < 0.05$) from corresponding control cells



Effect of TPA alone and in combination with staurosporine on the dose/response curve for CCK_8-stimulated amylase secretion from dispersed pancreatic acinar cells

TPA, added at a maximally effective concentration of 0.1 μM (Fig. 4), tended to decrease the secretory response to CCK_8 concentrations below 0.1 nM and markedly potentiated secretion evoked by secretagogue concentrations above 0.1 nM (Fig. 7). The release values obtained with 1 nM, 10 nM and 0.1 μM CCK_8 were significantly higher in phorbol-ester-treated cells than in control cells. In the presence of TPA, the dose/response curve for the stimulatory effect of CCK_8 tended to adopt its characteristic biphasic shape. However, the release value obtained with 10 nM CCK_8 was not significantly different from that obtained with 1 nM CCK_8. In the presence of TPA, the mean half-maximal concentration of CCK_8 was calculated to be 0.14 nM (Fig. 5). Staurosporine (1 μM) not only counteracted the inhibitory effect of TPA on the stimulatory effect of the lower concentrations of CCK_8, but even turned it into a potentiating effect. This potentiating effect reached statistical significance with 10 pM CCK_8. Note that staurosporine alone did not markedly affect the secretory response to these lower CCK_8 concentrations (see also, Fig. 3). On the other hand, staurosporine markedly inhibited the potentiating effect of TPA on the secretory response to CCK_8 concentrations above 0.1 nM. The effect was statistically significant with 1 nM and 10 nM CCK_8. In the combined presence of TPA and staurosporine, the dose/response curve for CCK_8-stimulated amylase secretion was shifted to the left and the mean half-maximal CCK_8 concentration was calculated to be 3 pM (Fig. 5). Moreover, in the combined presence of TPA and staurosporine, the dose/response curve for CCK_8-stimulated amylase secretion tended to adopt its characteristic biphasic shape. However, the release values obtained with 10 pM and 1 nM CCK_8 were not significantly different from that obtained with 0.1 nM CCK_8.

Fig. 7 Effect of TPA alone and in combination with staurosporine on the dose/response curve for CCK_8-induced amylase secretion from dispersed pancreatic acinar cells. Dispersed rabbit pancreatic acinar cells were resuspended in a HEPES/Tris medium and preincubated in the presence of either dimethylsulphoxide (*circles*) or 1 μM staurosporine (*squares*) for 10 min at 37°C. Aliquots of the suspension were transferred to Eppendorf microtubes containing the indicated concentrations of CCK_8 either alone (*open symbols*) or in combination with 0.1 μM TPA (*closed symbols*). Medium amylase activity was determined at 20 min following the onset of stimulation and expressed as a percentage of the initial cellular amylase content. After correction for basal amylase release, the release value obtained with 10 nM CCK_8 alone is set at 100%, to which all other values are related. The data presented are the mean ± SEM of 3 experiments. * Significantly different ($P < 0.05$) from corresponding value for CCK_8-stimulated control cells. # Significantly different ($P < 0.05$) from corresponding value for CCK_8-stimulated TPA-treated cells



Effect of forskolin alone and in combination with staurosporine on the dose/response curve for CCK_8-stimulated amylase secretion from dispersed pancreatic acinar cells

Stimulation of adenylyl cyclase activity by means of forskolin (30 μM), resulted in a marked potentiation of the secretory response to CCK_8 (Fig. 8). This potentiating effect reached statistical significance with 1 nM, 10 nM and 0.1 μM CCK_8. In the presence of forskolin, the mean half-maximal CCK_8 concentration was calculated to be 28 pM (Fig. 5). Although the dose/response curve for the stimulatory effect of CCK_8 tended to adopt its characteristic biphasic shape in forskolin-treated cells, the release values obtained with 0.1 nM, 10 nM and 0.1 μM CCK_8 were not significantly different from that obtained with 1 nM CCK_8. Forskolin alone did not change the basal rate of amylase secretion (see also, [32]). In the combined presence of forskolin and staurosporine (1 μM), the dose/response curve was shifted to the left and the mean half-maximal CCK_8 concentration was calculated to be 1.4 pM (Fig. 5). Compared to control cells, the secretory response to 1 pM, 10 pM and 0.1 nM CCK_8 was significantly increased in cells pretreated with the combination of forskolin and staurosporine. Staurosporine did not significantly affect the efficacy of CCK_8 in forskolin-treated cells. However, staurosporine markedly reduced the potentiating effect of forskolin on secretion evoked by CCK_8 concentrations above 0.1 nM. This inhibitory effect reached statistical significance with 10 nM and 0.1 μM CCK_8. In the combined presence of forskolin and staurosporine the dose/response curve for CCK_8-stimulated amylase secretion tended to adopt its characteristic biphasic shape. However, the release values obtained with 10 pM and 1 nM were not significantly different from that obtained with 0.1 nM CCK_8.

Table 1 Effect on the cAMP content in rabbit pancreatic acinar cells. Rabbit pancreatic acinar cells were resuspended in a HEPES/Tris medium. The acinar cells were preincubated in the absence and presence of either 1 μM staurosporine and/or 0.1 mM 3-isobutyl-1-methylxanthine (IBMX) for 10 min at 37°C. 12-*O*-tetradecanoylphorbol 13-acetate (TPA, 1 μM) was added immediately before transferring 200-μl aliquots of each suspension to microtubes. COOH-terminal octapeptide of cholecystokinin (CCK_8, 10 nM) and forskolin (30 μM) were present in the microtubes. The reaction was quenched after 10 min and the acinar cell cAMP content was expressed as pmol cAMP per mg protein. The values presented are the mean ± SD with the number of experiments given in *parentheses*

Treatment	Acinar cell cAMP content (pmol/mg protein)			
	−IBMX		+ IBMX	
	−Staurosporine	+ Staurosporine	−Staurosporine	Staurosporine
Control	0.9 ± 0.4 (5)	1.2 ± 0.5 (5)	3.0 ± 0.5 (3)	7.9 ± 2.9 (3)
CCK_8	1.8 ± 0.7 (5)	2.0 ± 0.8 (5)	4.2 ± 0.3 (3)	9.8 ± 1.7 (3)
CCK_8 + TPA	0.7 ± 0.3 (5)	1.2 ± 0.6 (5)	3.8 ± 0.8 (3)	11.0 ± 3.1 (3)
Forskolin	12.2 ± 5.4 (5)	27.9 ± 16.0 (5)	89.8 ± 22.8 (3)	159.0 ± 44.9 (3)

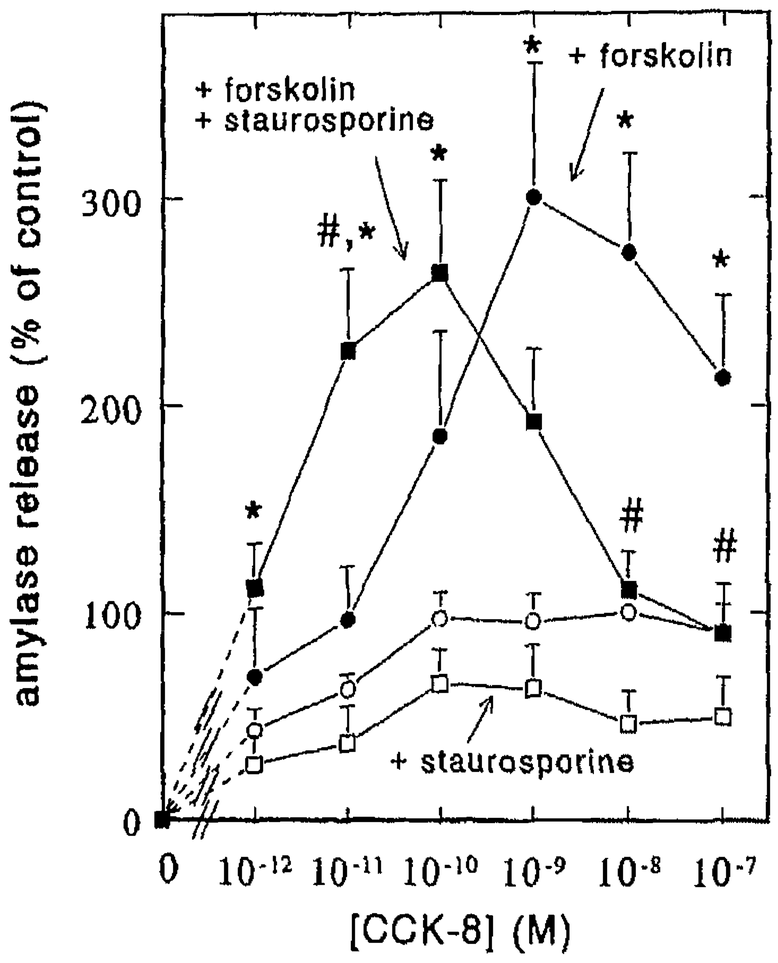


Fig. 8 Effect of forskolin alone and in combination with staurosporine on the dose/response curve for CCK_8-induced amylase secretion from dispersed pancreatic acinar cells. Dispersed rabbit pancreatic acinar cells were resuspended in a HEPES/Tris medium and preincubated in the presence of either dimethylsulphoxide (*circles*) or 1 μM staurosporine (*squares*) for 10 min at 37°C. Aliquots of the suspension were transferred to Eppendorf microtubes containing the indicated concentrations of CCK_8 either alone (*open symbols*) or in combination with 30 μM forskolin (*closed symbols*). Medium amylase activity was determined at 20 min following the onset of stimulation and expressed as a percentage of the initial cellular amylase content. After correction for basal amylase release, the release value obtained with 10 nM CCK_8 alone is set at 100%, to which all other values are related. The data presented are the mean ± SEM of 5 experiments. * Significantly different ($P < 0.05$) from corresponding value for CCK_8-stimulated control cells. # Significantly different ($P < 0.05$) from corresponding value for CCK_8-stimulated forskolin-treated cells

Effect of staurosporine on basal and forskolin- and CCK_8-stimulated cAMP production in dispersed pancreatic acinar cells

The activator of adenylyl cyclase activity, forskolin (30 μM), evoked a substantial (14-fold) increase in acinar cell cAMP content (Table 1). In the presence of the inhibitor of cyclic nucleotide phosphodiesterase activity, IBMX, the effect of forskolin was markedly enhanced (7.4-fold as compared to the effect obtained in the absence of IBMX). IBMX alone induced a 3.5-fold increase of the basal cAMP level. Neither CCK_8 (10 nM) alone nor in combination with TPA (1 μM) evoked a measurable increase in cellular cAMP content even when IBMX was included in the incubation medium. Surprisingly, staurosporine (1 μM) alone caused a 2.5-fold increase in cellular cAMP content provided that IBMX was present. In addition, staurosporine potentiated the stimulatory effect of forskolin both in the absence (2.3-fold) and presence (1.8-fold) of IBMX.

Discussion

Most studies on the mechanism of action of pancreatic secretagogues have used preparations consisting of either dispersed acinar cells or isolated acini. Dispersed acinar cells are obtained by a procedure employing the enzymatic digestion of pancreatic tissue in combination with mechanical shearing and chelation of divalent cations to break junctional complexes [1]. The acinar cell preparation used in the present study consisted of a mixture of single cells and small cell groups [34]. Acini are prepared in essentially the same way, except that the cation chelating step is omitted; each resulting preparation contains over 50 functionally coupled acinar cells [12, 22]. It is generally recognized that the latter preparation displays a considerably improved secretory response [12]. However, in previous studies using rabbit pancreatic acini, both the efficacy (10% [6] and 26% [32] of total amylase released in 20 min) and the half-maximally effective concentration (75 pM [6] and 1.2 nM [32]) for CCK_8 were found to be highly variable. In contrast, the maximally effective concentration for CCK_8 (10 nM) did not differ between the two studies. The data obtained in the present study

show that in dispersed pancreatic acinar cells the efficacy of CCK_8 was even further reduced to reach a value of 4.1% of total amylase released in 20 min.

The main finding of the present study is that efficacy as well as the half-maximally and maximally effective concentrations for the stimulatory effect of CCK_8 on amylase secretion from dispersed pancreatic acinar cells can be altered by artificially increasing or decreasing the activity of intracellular kinases.

The data presented demonstrate that both forskolin and TPA can markedly (threefold) increase the efficacy of CCK_8 in dispersed rabbit pancreatic acinar cells. In principle, both compounds displayed the same effect on the dose/response curve for CCK_8-stimulated amylase secretion, be it that TPA inhibited the stimulatory effect of the lower concentrations of CCK_8. The fact that the impaired secretory response observed with dispersed acinar cells can be markedly improved by artificial activation of protein kinases suggests that in this particular preparation the intracellular pathway leading from receptor activation to secretion is disturbed at the level of protein kinase activation.

The potentiating effect of both TPA and forskolin on the secretory response to CCK_8 concentrations above 0.1 nM was effectively inhibited by staurosporine, demonstrating the involvement of staurosporine-sensitive protein kinases. In contrast, the potentiating effect of TPA on amylase secretion elicited by CCK_8 concentrations below 0.1 nM was only revealed in the presence of staurosporine. Since staurosporine alone did not affect secretion evoked by these lower concentrations of CCK_8, this observation suggests that TPA acts through a staurosporine-insensitive isotype of PKC. Similarly, staurosporine increased the potentiating effect of forskolin on the secretory response to the lower CCK_8 concentrations, suggesting the involvement of a staurosporine-insensitive isotype of PKA. Assuming that these staurosporine-insensitive protein kinases act in concert with cytosolic free Ca^{2+} to stimulate the secretory process, the potentiating effect of staurosporine might be explained by its previously reported ability to markedly potentiate the Ca^{2+} mobilizing effect of submaximal CCK_8 concentrations [35].

The inhibitory effect of TPA was reversed by staurosporine, indicating the involvement of a staurosporine-sensitive isotype of PKC in the mechanism of action of the phorbol ester.

In order to find an explanation for the pronounced potentiating effect of TPA on the secretory response to the higher CCK_8 concentrations, pancreatic acinar cells were incubated in the combined presence of TPA and thapsigargin, a potent inhibitor of intracellular Ca^{2+}-ATPase activity. Thapsigargin, which evokes a sustained increase in free cytosolic Ca^{2+} concentration [20, 36], only moderately stimulated amylase secretion when added alone (see also [20]). The secretory response to thapsigargin was markedly potentiated by TPA, which by itself caused also a moderate stimulation of the secretory process. However, the maximal effect that could be reached with this combination was 2 times lower than that obtained with the combination of TPA and CCK_8. Differences in the pattern of Ca^{2+} mobilization between CCK_8 and thapsigargin may explain the inability of TPA to potentiate the secretory response to both compounds to the same extent.

Thapsigargin inhibited the secretory response to CCK_8 dose dependently. Inhibitory effects of thapsigargin or the Ca^{2+} ionophore, A23187, on receptor-stimulated amylase secretion have been described for the Ca^{2+}-mobilizing secretagogues [11, 20]. The latter observation might suggest that the inability of TPA to equally potentiate the secretory response to thapsigargin and CCK_8 can be explained by this inhibitory action of thapsigargin. However, no supramaximal inhibition was observed when acinar cells were stimulated with TPA in the presence of increasing concentrations of thapsigargin. Moreover, thapsigargin was not found to inhibit amylase secretion elicited by cAMP-generating secretagogues [20]. These observations argue against an inhibitory action of thapsigargin downstream of the receptor-stimulated production of second messengers.

The possibility that the potentiating effect of TPA on the secretory response to the higher CCK_8 concentrations was caused indirectly through cAMP-dependent activation of PKA could be ruled out since TPA did not affect the cellular cAMP content in CCK_8-stimulated acinar cells. In contrast, staurosporine caused an additional increase in cAMP content in forskolin-stimulated acinar cells. This potentiating effect of staurosporine was still observed in the presence of IBMX, an inhibitor of cyclic nucleotide phosphodiesterase activity, indicating that the drug exerted its effect at the level of adenylyl-cyclase-catalysed cAMP formation. Effects of staurosporine on basal cAMP levels were only observed in the presence of IBMX.

An alternative explanation for the potentiating effect of TPA might be that higher concentrations of CCK_8 produce a factor other than cAMP, Ca^{2+} or 1,2-diacylglycerol, which becomes only secretory active in the presence of TPA.

Inhibitory effects of TPA on the process of secretagogue-evoked enzyme secretion have been reported before [14, 21, 33]. However, in these studies, using acini rather than acinar cells, TPA was not found to significantly increase the efficacy of the secretagogues [14, 21]. But, whereas the dose/response curves produced in the latter two studies were sharp-pointed, the curve produced in the present study lacked a clear-cut maximum, preceded by a sharp upstroke, occurring over 2 orders of magnitude, and followed by a sharp downstroke. In fact, the dose/response curve for CCK_8 alone, produced in the present study, resembled that obtained with either carbachol or CCK in the presence of staurosporine [21]. In the latter study, the effect of staurosporine was much more pronounced than in the

present study. These observations suggest that, due to an inadequate activation of staurosporine-sensitive protein kinases, CCK_8 concentrations above 0.1 nM were unable to fully develop their secretory capability in the present study.

Staurosporine alone markedly decreased the efficacy of CCK_8. In fact, staurosporine appeared to flatten the dose/response curve by reducing the stimulatory effect of CCK_8 concentrations above 0.1 nM, without affecting the response to concentrations below 0.1 nM. Comparable results have been obtained in rat [21] and guinea-pig acini [29]. In a previous study we have shown that the concentration of 0.1 nM forms the breaking point in the dose/response curve for the effect of CCK_8 on the pattern of Ca^{2+} mobilization in acinar cells. Below this concentration CCK_8 recruits acinar cells displaying oscillatory changes in free cytosolic Ca^{2+} concentration dose dependently. Above this concentration a dose-dependent increase in the size of the initial Ca^{2+} transient is paralleled by a dose dependent decrease in size of the subsequent Ca^{2+} oscillations [34]. Since staurosporine is a potent inhibitor of kinase activity, the present observations suggest that low concentrations of secretagogue stimulate secretion in a kinase-independent manner, most probably by causing periodic local Ca^{2+} rises in the secretory pole [13, 28] sufficiently high to stimulate the process of exocytosis [16, 17].

Binding studies using [^{125}I] Bolton-Hunter-CCK_8 have indicated that pancreatic acinar cells possess CCK-binding sites with either high or low affinity for CCK [10]. Occupancy of the high-affinity state receptor by CCK or its synthetic COOH-terminal heptapeptide analogue, JMV-180 [9], has been associated with the upstroke of the dose/response curve for amylase release, the stimulation of phosphatidylcholine hydrolysis to yield 1,2-diacylglycerol, and the induction of cytosolic Ca^{2+} oscillations with minimal stimulation of phosphatidylinositol 4,5-bisphosphate hydrolysis [18]. In a recent study, pretreatment of rat acini with subnanomolar TPA concentrations, believed to increase the activity of PKC in the physiological range [4], was shown to cause supramaximal inhibition of the secretory response to a phenethyl ester analogue of CCK_8, which by itself did not display supramaximal inhibition [8]. At the same concentrations, the phorbol ester did not affect the secretory response to the lower concentrations of the analogue. Interestingly, the effect of the phorbol ester was found to be mimicked by minimally effective CCK_8 concentrations, acting through high-affinity CCK receptors. These observations suggest that PKC plays a role in supramaximal inhibition. In the present study, pretreatment with staurosporine did not affect CCK_8-evoked supramaximal inhibition, suggesting the involvement of a staurosporine-insensitive isotype of PKC. Moreover, the observation that the CCK dose/response curve was still biphasic in the presence of TPA, shown previously to transform high-affinity CCK receptors to a state of low-affinity [35], demonstrates that supramaximal inhibition occurs also through activation of low-affinity receptors.

In conclusion, the data presented demonstrate that the secretory response of dispersed pancreatic acinar cells to CCK_8 can be considerably improved when simultaneously kinases are artificially activated by the action of either forskolin or TPA. Moreover, they show that in the combined presence of staurosporine and either forskolin or TPA both the maximally and half-maximally effective CCK_8 concentrations tend to shift to lower concentrations of the secretagogue. Detailed information on the secretory activity of dispersed pancreatic acinar cells under the various experimental conditions is of importance with respect to ongoing studies employing the patch-clamp technique to monitor changes in membrane capacitance as a measure of exocytotic secretion.

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