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Protein kinase C activation inhibits receptor-evoked inositol trisphosphate formation and induction of cytosolic calcium oscillations by decreasing the affinity-state of the cholecystokinin receptor in pancreatic acinar cells

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Abstract — Digital-imaging microscopy of Fura–2-loaded pancreatic acinar cells revealed that the C-terminal octapeptide of cholecystokinin (CCK_8) dose-dependently recruited 94% of freshly isolated acinar cells in terms of receptor-evoked Ca^{2+} mobilization. Maximal and half-maximal cell-recruitment were reached with 0.1 nM and 16.8 pM CCK_8, respectively. The upstroke of the dose-recruitment curve consisted of cells displaying oscillatory changes in free cytosolic Ca^{2+} concentration ($[Ca^{2+}]_i$). After having reached its maximum, the percentage oscillating cells dose-dependently decreased upon further increasing of the CCK_8 concentration. Pretreatment of the acinar cells with 0.1 μM TPA caused a rightward shift of the dose-recruitment curve but did not change the maximal effect of CCK_8 on the recruitment of oscillating cells. Half-maximal recruitment was obtained with 287 pM CCK_8. This observation demonstrates that high levels of protein kinase C activation do not inhibit Ca^{2+} oscillations at a level downstream to receptor activation. Moreover, this observation demonstrates that protein kinase C-mediated inhibition of Ca^{2+} oscillations evoked by submaximai CCK_8 concentrations occurs at the receptor level, converting it from a high-affinity state into a low-affinity state. This conclusion is supported by the observation that TPA completely inhibited the recruitment of acinar cells in response to the high-affinity receptor agonist JMV-180. The inhibitory action of TPA on CCK_8-evoked cell-recruitment was paralleled by an inhibitory effect of the phorbol ester on the CCK_8-evoked peak increase in average inositol trisphosphate concentration in a population of acinar cells. This observation indicates that low concentrations of CCK_8 interact with the high-affinity CCK receptor to increase $[Ca^{2+}]_i$ through the intermediation of inositol trisphosphate.

Abbreviations: CCK_8, C-terminal octapeptide of cholecystokinin; $[Ca^{2+}]_i$, cytosolic free Ca^{2+} concentration; JMV-180, Boc-Tyr(SO^{3-})-Nle-Gly-Trp-Nle-Asp-2-phenylethylester; TPA, 12-O-tetradecanoylphorbol 13-acetate; DAG, 1,2-diacylglycerol; $Ins(1,4,5)P_3$, inositol 1,4,5-trisphosphate.

The C-terminal octapeptide of the pancreatic secretagogue cholecystokinin (CCK_8) interacts with specific receptors located on the basolateral membrane of the acinar cell to stimulate the process of digestive enzyme secretion [1,2]. Initially, triggering of the secretory response is paralleled by a rapid rise in free cytosolic Ca^{2+} concentration ($[Ca^{2+}]_i$), which, in a suspension of acinar cells, is transient by nature [3,4]. We have recently demonstrated that the peak height of this transient is largely proportional to the number of responding cells [5].

Thus far, this Ca^{2+} mobilizing effect of CCK_8 is generally believed to be mediated through the intracellular messenger inositol 1,4,5-trisphosphate [$Ins(1,4,5)P_3$], which is produced upon the receptor-stimulated cleavage of phosphatidylinositol 4,5-bisphosphate [6]. However, recent findings have cast some doubts on the exclusive role of $Ins(1,4,5)P_3$ in this process. First of all, both CCK_8 and its analogue JMV-180 [Boc-Tyr(SO^{3-})-Nle-Gly-Trp-Nle-Asp-2-phenylethylester], which acts as an agonist at the high-affinity CCK receptor [7–10], were demonstrated to increase the average $[Ca^{2+}]_i$ already half-maximal without detectably stimulating the production of $Ins(1,4,5)P_3$ [7,11]. Moreover, $Ins(1,4,5)P_3$ production was only marginally stimulated by JMV-180 concentrations maximally effective in terms of receptor-evoked Ca^{2+} mobilization [7,11]. In contrast, maximally effective CCK_8 concentrations markedly stimulated the production of $Ins(1,4,5)P_3$. It was postulated that only the low-affinity CCK receptor acts through the intermediation of $Ins(1,4,5)P_3$, whereas the high-affinity CCK receptor uses a messenger other than $Ins(1,4,5)P_3$. In part, this idea was supported by the observation that JMV-180, but not the CCK analogue caerulein, stimulated the release of Ca^{2+} from an internal store insensitive to $Ins(1,4,5)P_3$ [12,13]. Also partly in favour of this idea was the observation that the putative phospholipase C inhibitor, U73122, effectively blocked the Ca^{2+} mobilizing effect of CCK_8 without interfering with JMV-180-evoked Ca^{2+} mobilization [14]. Although these findings are compatible with the idea that JMV-180 acts through a messenger other than $Ins(1,4,5)P_3$ they exclude, at the same time, that low concentrations of CCK_8, acting solely through the high-affinity receptor, make use of such an alternative messenger. In contrast to the above findings, some of the latest studies demonstrate that the Ca^{2+} mobilizing effect of JMV-180 can be blocked by intracellular application of the $Ins(1,4,5)P_3$ receptor antagonist heparin, thus providing evidence that $Ins(1,4,5)P_3$ is really involved in the mechanism of action of this CCK_8 analogue [15–17]. This, in turn, suggests that both the high- and the low-affinity CCK receptor are coupled to phospholipase C and that, for hitherto unknown reasons, activation of the high-affinity CCK receptor by JMV-180 only poorly stimulates the production of $Ins(1,4,5)P_3$. In this case, the Ca^{2+} mobilizing effect of JMV-180 can be explained by assuming that local increases in $Ins(1,4,5)P_3$, too small to be detected, are sufficient to trigger the observed expansive rises in $[Ca^{2+}]_i$. Evidence in favour of this idea was recently provided by Muallem and co-workers [17]. Thus far, it is unclear whether activation of the high-affinity receptor by CCK, similarly to JMV-180, only poorly stimulates the formation of $Ins(1,4,5)P_3$.

In a recent study, we have demonstrated that activation of protein kinase C by means of the phorbol ester 12-O-tetradecanoylphorbol 13-acetate (TPA) causes both a rightward shift of the dose-response curve for the stimulatory effect of CCK_8 on the peak increase in average $[Ca^{2+}]_i$ and complete inhibition of JMV-180-evoked Ca^{2+} mobilization [18]. In other words, protein kinase C activation leads to inhibition of Ca^{2+} signaling through the high-affinity receptor, without interfering with Ca^{2+} signaling through the low-affinity receptor. Therefore, TPA can be used to assign the concentrations at which CCK_8 acts exclusively through the high-affinity receptor. In doing so, it can be investigated whether the high-affinity CCK receptor is coupled to phospholipase C to stimulate the production of $Ins(1,4,5)P_3$.

The present study aims to assess the ability of CCK_8 to detectably increase the average $Ins(1,4,5)P_3$ content in a suspension of acinar cells when added at concentrations demonstrated in TPA-treated acinar cells to act solely through the high-affinity receptor. In order to determine which concentrations of CCK are completely blocked by TPA in terms of receptor-evoked Ca^{2+} mobilization, $[Ca^{2+}]_i$ was measured in large numbers of individual acinar cells by means of digital imaging microscopy.

Materials and methods

Materials

Collagenase was purchased from Cooper Biomedical Inc. (Malvern, PA, USA) and Fura–2/AM from Molecular Probes Inc. (Eugene, OR, USA). Hyaluronidase and staurosporine were obtained from Boehringer (Mannheim, Germany) and *t*-butyloxycarbonyl-Tyr(SO^{3-})-Nle-Gly-Tyr-Nle-Asp-2-phenylethyl ester (JMV-180) from Research Plus Inc. (Bayonne, NJ, USA). Poly-L-lysine, bovine serum albumin, soybean trypsin inhibitor, CCK_8, inositol 1,4,5-trisphosphate, trypan blue and TPA were obtained from Sigma Diagnostics (St Louis, MO, USA) and D-*myo*-[^{3}H]-inositol 1,4,5-trisphosphate (51.4 Ci/mmol) from Amersham, UK. All other chemicals were of reagent grade.

Pancreatic acinar cells

Rabbit pancreatic acinar cells were prepared by enzymatic digestion, using collagenase and hyaluronidase, as previously described [5]. At the end of the isolation procedure, the acinar cells were resuspended in a Krebs-Ringer bicarbonate medium (pH 7.4) containing 119 mM NaCl, 3.5 mM KCl, 1.2 mM KH_2PO_4, 25 mM $NaHCO_3$, 1.2 mM $CaCl_2$, 1.2 mM $MgCl_2$, 5.8 mM glucose, 0.2 mg/ml soybean trypsin inhibitor, 0.1% (w/v) bovine serum albumin and an amino acid mixture according to Eagle. For fluorescence measurements, acinar cells were resuspended in a HEPES/Tris 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, an amino acid mixture according to Eagle. 1% (w/v) bovine serum albumin and 10 mM HEPES, adjusted with Tris to pH 7.4 and kept on ice until use.

Fluorescence measurements in individual acinar cells

For fluorescence measurements an aliquot of the chilled suspension was removed, centrifuged and resuspended in 200 µl of the above HEPES/Tris medium containing 1% (w/v) bovine serum albumin. The temperature of the suspension was brought to 37°C and the cells were loaded with Fura–2 in the presence of 5 µM Fura–2/AM for 10 min. After loading, excess Fura–2/AM was removed by washing with 4 ml of the above HEPES/Tris medium containing 0.1% bovine serum albumin (incubation medium). The Fura–2-loaded cells were resuspended in incubation medium and transferred to a thermostated (36°C) incubation chamber. The cells were allowed to attach to a poly-L-lysine-coated glass cover slip, forming the bottom of the chamber, for 1–2 min, after which superfusion was started. Superfusion was at a flow rate of 1 ml/min. The unattached cells were automatically sucked off and the attached cells were allowed to equilibrate for another 10 min before the test substances were added. The chamber was placed on the stage of an inverted microscope and an epifluorescent ×40 magnification oil immersion objective was used to allow simultaneous monitoring of an average of close to 60 individual acinar cells. Dynamic video imaging was carried out as described previously [5].

Ins(1,4,5)P_3 measurements in suspensions of acinar cells

Isolated pancreatic acinar cells resuspended in Krebs-Ringer bicarbonate medium (7.9 mg protein/ml, SEM ± 0.5, n = 31), were incubated in the presence of either dimethylsulphoxide or staurosporine (1 µM) for 5 min at 37°C. At 1 min, either dimethylsulphoxide or TPA (0.1 µM) was added. At 5 min, 100 µl samples were removed and transferred to a micro test tube (Eppendorf) containing the indicated concentrations of CCK_8. The reaction was stopped by the addition of 25 µl 50% trichloroacetic acid at the times indicated. The samples were vigorously mixed and immediately frozen in liquid nitrogen. At the end of the experiment, the samples were thawed and centrifuged for 4 min at 10,000 *g* (Eppendorf minifuge). A 125 µl aliquot of the supernatant was removed and extracted 3 times with 1 ml of water-saturated diethyl ether. A 75 µl aliquot was then removed and 2 µl 50% $KHCO_3$ was added to increase the pH to a value > 7.5. The Ins(1,4,5)P_3 content of the extract was determined by isotope dilution assay as previously described [19]. Protein was determined with a commercial Coomassie Blue kit (Bio-rad, Richmond, CA, USA)

using bovine serum albumin (Hoechst-Behring, Marburg, Germany) as a standard.

Analysis of the data

Half-maximal CCK_8 concentrations for the recruitment of acinar cells in terms of receptor-evoked Ca^{2+} mobilization and maximal recruitment values were calculated by means of the nonlinear regression computer program InPlot (Graphpad Software for Science, San Diego, CA, USA).

Results

Acinar preparation

The acinar preparation used in the present study has been described in detail previously [5]. Briefly, the preparation was demonstrated to consist of single cells (22%) and cells forming part of a doublet (21%), a triplet (34%), or a larger group of (mostly 4–5) cells (22%). Some evidence was found that individual cells responded more readily to stimulation with CCK_8 if forming part of a larger group of cells. On the other hand, it was often observed that individual cells within the same group of cells responded with different sensitivities. Whether the latter situation reflects uncoupling in terms of receptor-mediated Ca^{2+} mobilization needs further investigation. Similarly to the previous study, the data presented in this study include single cells and cells forming part of a doublet, a triplet or a larger group of 4–5 cells.

Effect of TPA pretreatment on the dose-response curve for CCK_8-evoked recruitment of acinar cells in terms of receptor-mediated Ca^{2+} mobilization

Digital-imaging microscopy, allowing simultaneously monitoring of $[Ca^{2+}]_i$ in large numbers of individual acinar cells, revealed that CCK_8 dose-dependently increased the number of responding cells (Fig. 1a). In this particular study, an average of 62 acinar cells (SD = 10, n = 15) was monitored in each individual experiment. Each CCK_8 concentration was tested for at least 300 s. In most of the experiments, however, monitoring time was 600 s. The lowest effective concentration of CCK_8 to induce an increase in $[Ca^{2+}]_i$ was 3 pM. Only 4% of the acinar cells were sensitive to this concentration of the secretagogue. Nonlinear regression analysis revealed that half-maximal recruitment was obtained with 16.8 pM CCK_8. The maximal effect was reached with 100 pM CCK_8. On average, 94% of the acinar cells were recruited upon stimulation with a (supra)maximal concentration of CCK_8. Pretreatment of the acinar cells with a maximal effective concentration of 0.1 μM TPA (*see* [18]) resulted in a rightward shift of the dose-recruitment curve for CCK_8-evoked Ca^{2+} mobilization of approximately one order of a magnitude (Fig. 1a). Nonlinear regression analysis revealed an EC_{50} value of 287 pM CCK_8 and a maximal recruitment level of 89%. The lowest effective hormone concentration for the recruitment of phorbol ester-treated cells was 30 pM. At this concentration, CCK_8 evoked a Ca^{2+} transient in only 2% of the acinar cells. Except for one experiment, in which 25% of the TPA-treated cells responded with a transient increase in $[Ca^{2+}]_i$ to stimulation with 100 pM CCK_8, the number of responding cells remained below 10% upon further increasing the CCK_8 concentration to 175 pM. At 300 pM CCK_8, however, an average of 50% of the cells responded to hormonal activation with a transient rise in $[Ca^{2+}]_i$.

Effect of TPA pretreatment on the dose-response curve for CCK_8-evoked recruitment of acinar cells displaying cytosolic Ca^{2+} oscillations

Acinar cells display repetitive Ca^{2+} transients when appropriately stimulated with CCK_8. Figure 1b shows the dose-response curve for the recruitment of acinar cells in terms of receptor-mediated induction of oscillatory changes in $[Ca^{2+}]_i$. At the lowest effective concentration of 3 pM, CCK_8 evoked Ca^{2+} oscillations in 4% of the acinar cells. The number of oscillating cells rapidly increased with increasing hormone concentration to reach a maximum of 90% at 100 pM CCK_8. Further increasing of the CCK_8 concentration resulted in a dose-dependent decrease of the number of oscillating cells. At a relatively high CCK_8 concentration of 10 nM, 21% (SEM = 3, n = 4) of the acinar cells displayed sustained oscillatory changes in $[Ca^{2+}]_i$. Pretreatment of the acinar

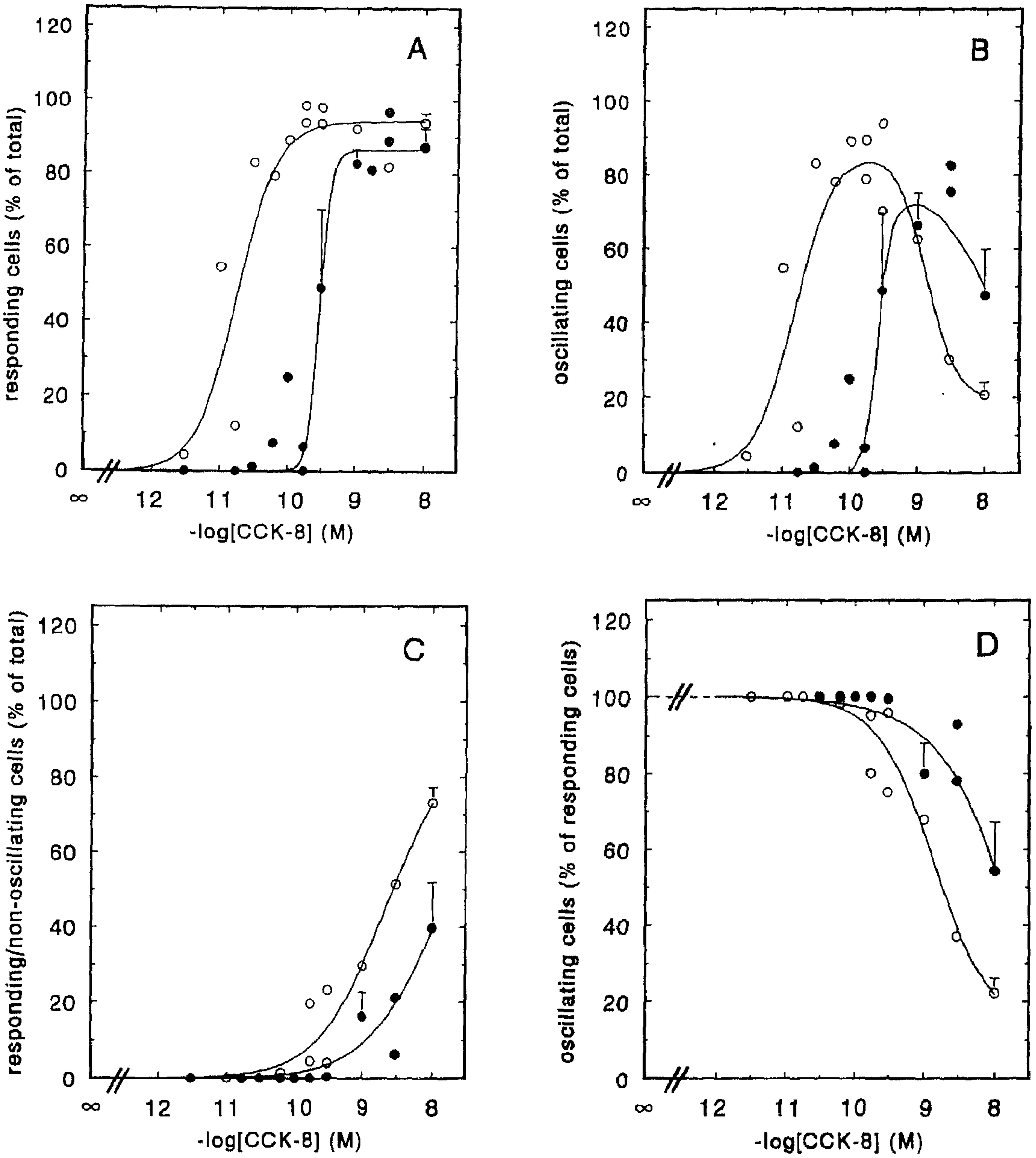

Fig. 1 Effect of TPA pretreatment on the dose-response curve for the effect of CCK_8 on the recruitment of pancreatic acinar cells in terms of receptor-evoked Ca^{2+} mobilization. Isolated pancreatic acinar cells, loaded with Fura–2, were superfused with a medium containing 0 μM (open circles) or 0.1 μM (closed circles) TPA at 37°C. At 5 min, CCK_8 was added at the indicated concentrations to the medium and the cells were superfused for another 5–10 min in the absence or presence of the phorbol ester. The CCK_8-evoked changes in $[Ca^{2+}]_i$ were monitored by digital imaging microscopy. On average, 60 cells were analysed in each individual experiment. In each individual experiment the number of responding (**A**), responding/oscillating (**B**) and responding/non-oscillating (**C**) cells was expressed as a percentage of total. In (**D**), the number of responding/oscillating cells was expressed as a percentage of the number of responding cells.

cells with 0.1 μM TPA resulted in a rightward shift of the dose-response curve for the CCK_8-evoked recruitment of oscillating acinar cells. The lowest effective concentration of CCK_8 to induce Ca^{2+} oscillations in TPA-treated cells was 30 pM. The number of oscillating cells dose-dependently increased with increasing hormone concentration, to reach a maximum of 79% (mean of two independent determinations) at 3 nM CCK_8. Thereafter, the number of oscillating cells dose-dependently decreased. At 10 nM CCK_8, 48% (SEM = 13, n = 5) of the TPA-treated cells displayed oscillatory changes in $[Ca^{2+}]_i$.

Acinar cells could also respond to hormonal stimulation with an immediate but single transient increase in $[Ca^{2+}]_i$ (responding/non-oscillating cells). Figure 1c shows that an average of 12% (mean of 2 independent determinations) of the cells responded with a single Ca^{2+} transient when stimulated with 175 pM CCK_8. The number of responding/ non-oscillating cells dose-dependently increased upon further increasing of the hormone concentration. At 10 nM CCK_8, 73% (SEM = 4, n = 4) of the acinar cells displayed a single Ca^{2+} transient not followed by repetitive increases in $[Ca^{2+}]_i$. In TPA-treated cells, the lowest effective concentration of CCK_8 to evoke a single Ca^{2+} transient was 1 nM. TPA evoked a rightward shift of the dose-recruitment curve for cells displaying a single Ca^{2+} transient in response to CCK_8. At 10 nM CCK_8, 40% (SEM = 12, n = 5) of the acinar cells responded with an immediate but single transient rise in $[Ca^{2+}]_i$.

In order to demonstrate the dose-dependency of the effect of CCK_8 on the recruitment of responding/oscillating and responding/non-oscillating cells more clearly, their numbers were expressed relative to the number of responding cells rather than the total cell number. Figure 1d shows that up to a concentration of 100 pM, CCK_8 recruited solely responding/oscillating cells. Thereafter, the number of responding/oscillating cells rapidly decreased with increasing hormone concentration. The lowest effective CCK_8 concentration to recruit responding/non-oscillating cells was 175 pM. Also in TPA-treated cells. the lower concentrations of CCK_8 recruited solely responding/oscillating cells. However, TPA treatment clearly increased the lowest effective CCK_8 concentration for the recruitment of responding/non-oscillating cells (1 nM in TPA-treated cells versus 175 pM in control cells). At a concentration of 10 nM, CCK_8 evoked Ca^{2+} oscillations in only 22% (SEM = 4, n = 4) of the responding control cells, whereas 54% (SEM = 13, n = 5) of the TPA-treated cells responding to this concentration of CCK_8 displayed oscillatory changes in $[Ca^{2+}]_i$.

Dose-response curve for the effect of TPA on JMV-180-evoked recruitment of acinar cells responding in terms of receptor-evoked Ca^{2+} mobilization

We have previously shown that the CCK_8 analogue JMV-180 dose-dependently increases the amplitude of the transient rise in average free cytosolic Ca^{2+} concentration in a suspension of acinar cells [18]. The maximal effect was reached at a JMV-180 concentration of 1 μM. Digital imaging microscopy revealed that JMV-180 at the same concentration of 1 μM mobilized Ca^{2+} in 55% (SEM = 5, n = 5) of the acinar cells (Fig. 2a). A further increase to 71% (mean of 2 independent determinations) was obtained with a 10-fold higher concentration of the analogue. Unexpectedly, 40% (SEM = 10, n = 5) of the acinar cells, responding to 1 μM JMV-180, did not display cytosolic Ca^{2+} oscillations within 10 min following the onset of stimulation with the CCK_8 analogue (Fig. 2b). Preincubation of the acinar cells with 0.1 μM TPA, virtually completely abolished the Ca^{2+} mobilizing effect of 1 μM JMV-180. Only 3.1% of the TPA-treated acinar cells responded to this concentration of JMV-180. Moreover, the response proved to be confined to a single Ca^{2+} transient. The effect of the phorbol ester was dose-dependent (Fig. 2a), in that preincubation with 10 nM TPA reduced the number of responding cells to 36% (SEM = 16, n = 4). At a concentration of 3 nM, TPA did not significantly affect the number of responding cells when compared to the control situation (58%, SEM = 12, n = 3). Interestingly, however, pretreatment with 1 nM TPA further increased the number of responding cells to 79% (SEM = 6, n = 3). The number of responding cells that displayed more than one Ca^{2+} transient (responding/oscillating cells) decreased dose-dependently with increasing TPA concentration (Fig. 2b). In the control experiment 60% (SEM = 10, n = 5) of the responding cells displayed repetitive Ca^{2+} transients, whereas in

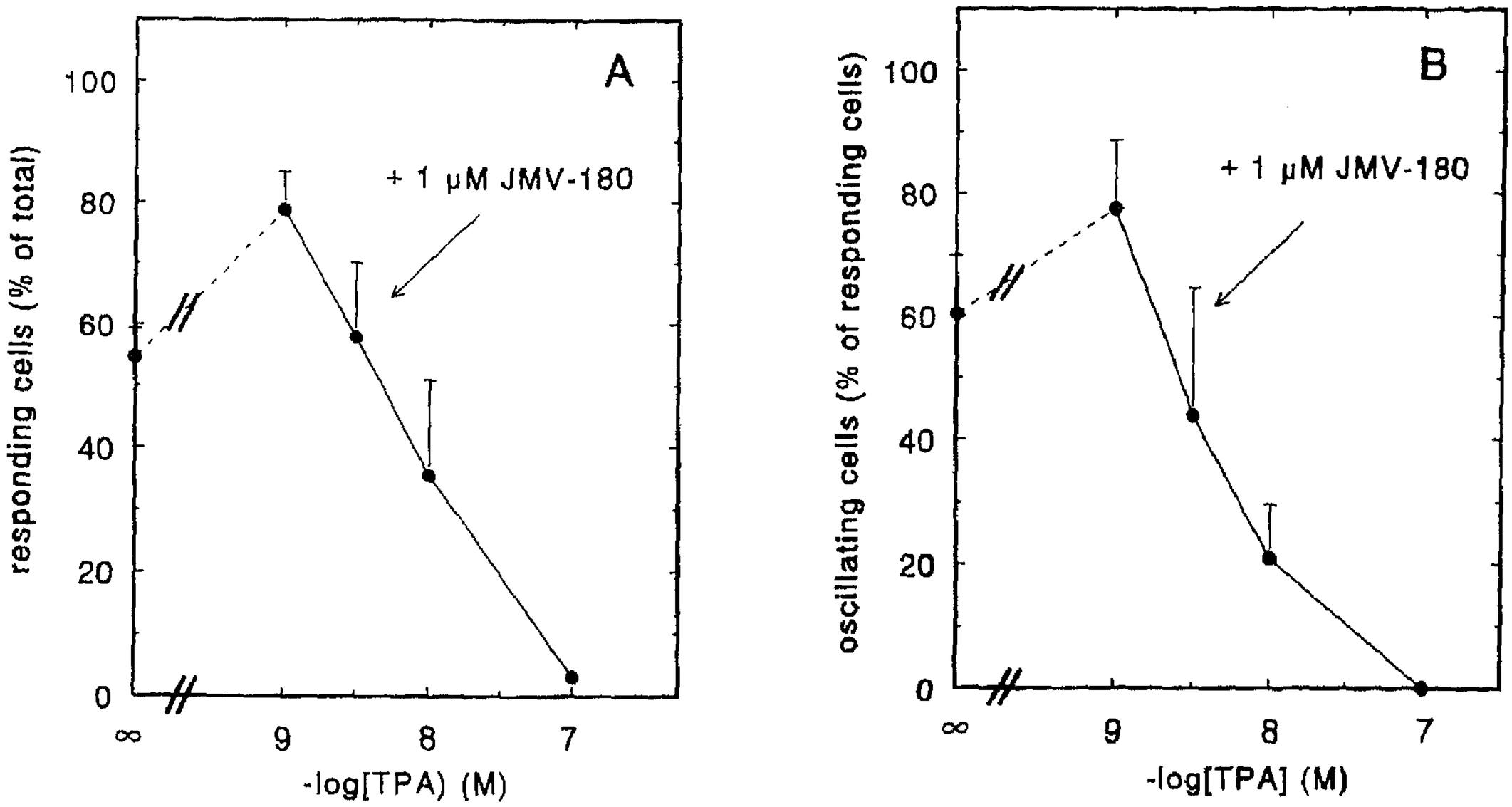

Fig. 2 Dose-response curve for the effect of TPA on the recruitment of pancreatic acinar cells displaying an increase in $[Ca^{2+}]_i$ in response to JMV-180. Isolated pancreatic acinar cells, loaded with Fura–2, were superfused with a medium containing 0 μM (open circles) or 0.1 μM (closed circles) TPA at 37°C. At 5 min, JMV-180 was added at a final concentration of 1 μM and the cells were superfused for another 5–10 min in the absence or presence of the phorbol ester. The JMV-180-evoked changes in $[Ca^{2+}]_i$ were monitored by digital imaging microscopy. In each individual experiment the number of responding (A) cells was expressed as a percentage of total. In (B), the number of responding/oscillating cells was expressed as a percentage of the number of responding cells. With the exception of 0.1 μM JMV-180, which concentration was tested in 1 experiment, the values presented are the mean ± SEM of 3–4 single experiments.

the presence of increasing TPA concentrations this value remained virtually unchanged at first, 77% (SEM = 11, n = 3) at 1 nM TPA, and dose-dependently decreased thereafter, from 44% (SEM = 21, n = 3) at 3 nM TPA, to 21% (SEM = 9, n = 4) at 10 nM TPA, and to 0% (n = 1) at 100 nM TPA.

The above observations demonstrate that TPA pretreatment has two effects, in that it leads to inhibition of JMV-180-evoked Ca^{2+} mobilization and to suppression of JMV-180-evoked oscillatory activity. Thus, pretreatment of acinar cells with a submaximally inhibitory concentration of TPA completely inhibits Ca^{2+} mobilization in one subpopulation of acinar cells (non-responding cells), completely suppresses Ca^{2+} oscillations in a second subpopulation of cells, leaving the initial Ca^{2+} transient unaffected (responding/non-oscillating cells), and finally does not affect JMV-180-evoked Ca^{2+} mobilization in a third subpopulation of cells (responding/oscillating cells).

TPA-induced deceleration and extinction of ongoing JMV-180-evoked cytosolic Ca^{2+} oscillations

Individual acinar cells, displaying ongoing oscillatory changes in free cytosolic Ca^{2+} concentration in the continuous presence of 1 μM JMV-180, in majority responded to the analogue with a broadened and often enlarged Ca^{2+} transient (Fig. 3). Frequently, 1–4 rapidly succeeding Ca^{2+} transients were observed on top of the falling phase of the initial Ca^{2+} transient. In the continuous presence of JMV-180, the frequency of the transients rapidly declined to a remarkably constant value, with $[Ca^{2+}]_i$ returning to prestimulatory levels in between two successive transients. Addition of TPA during the latter,

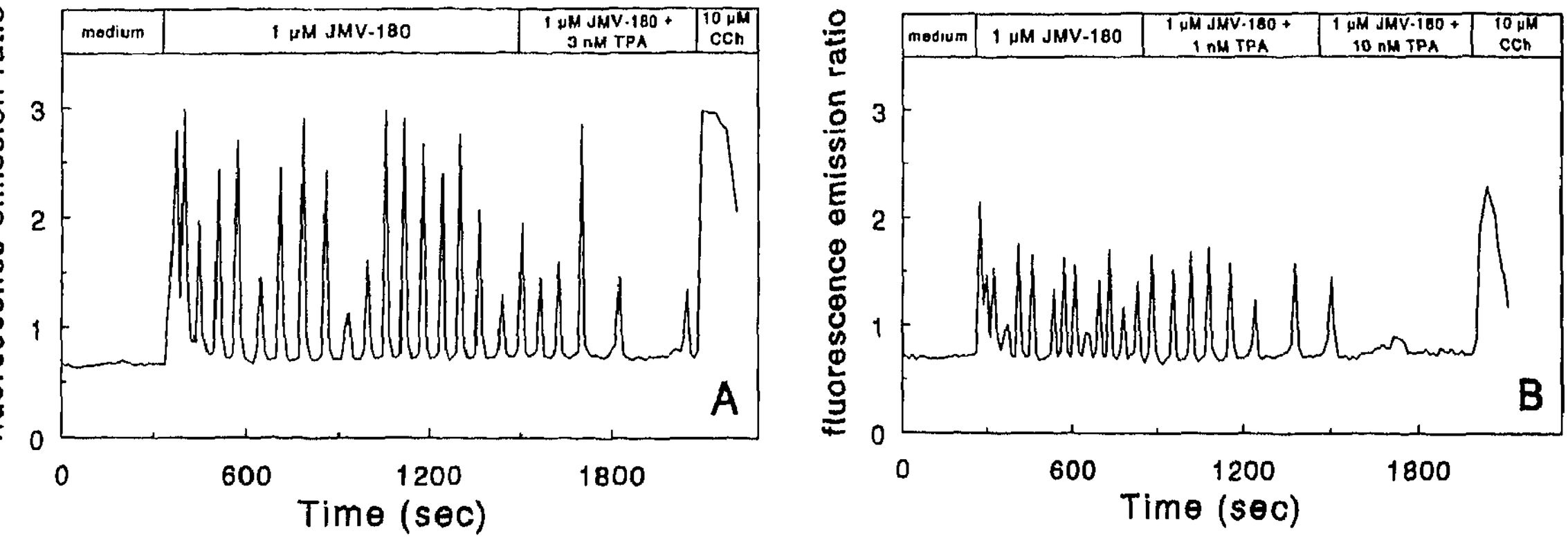

Fig. 3 Inhibitory effect of TPA on ongoing JMV-180-evoked cytosolic Ca^{2+} oscillations in individual pancreatic acinar cells. Isolated acinar cells, loaded with Fura–2, were stimulated with 1 μM JMV-180 for the indicated period of time. Changes in $[Ca^{2+}]_i$ were monitored by digital imaging microscopy of Fura–2 fluorescence from individual acinar cells. TPA was added on top of JMV-180 at the times indicated. At the end of the experiment, the cells were stimulated with a high concentration of carbachol (10 μM).

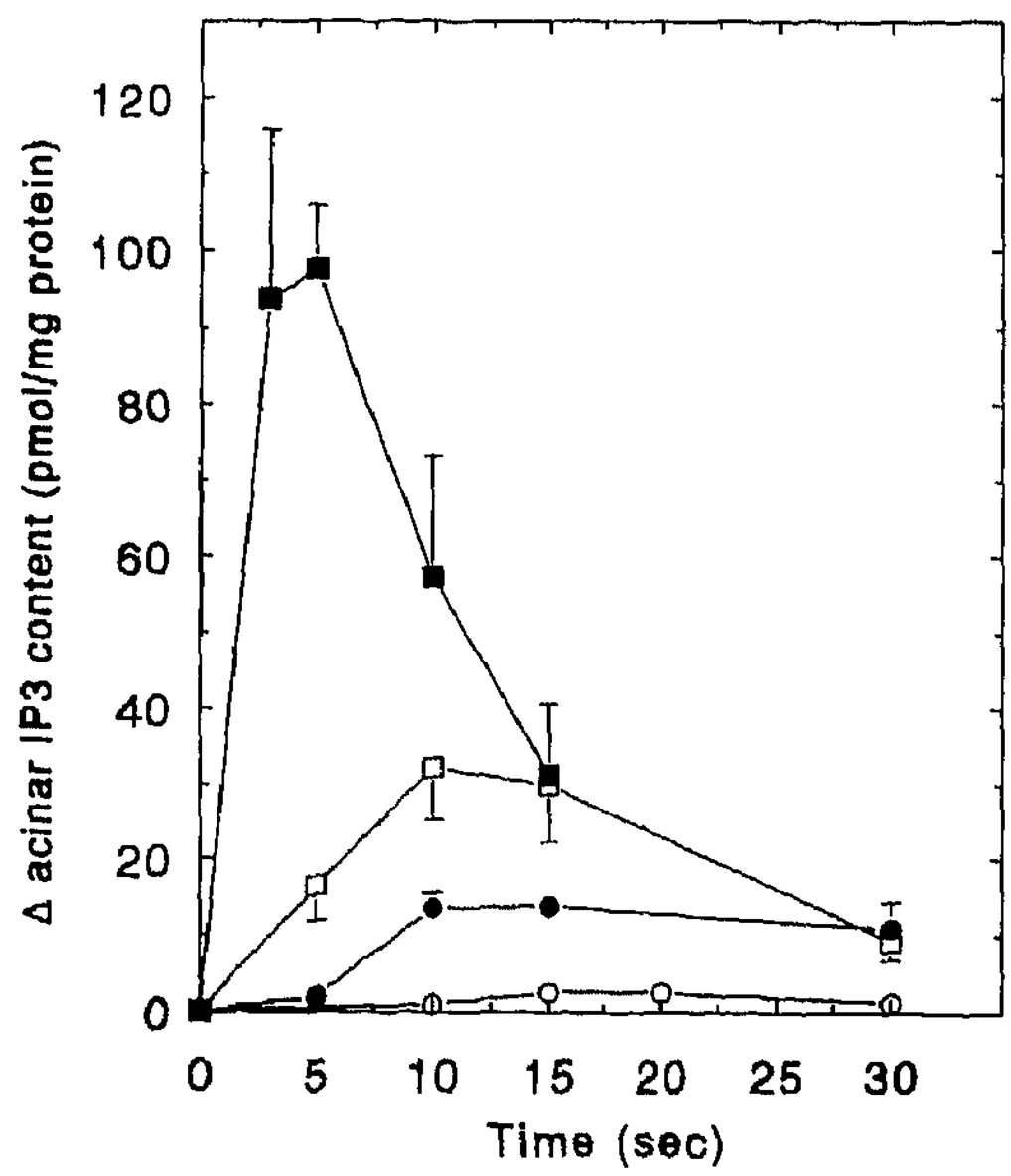

Fig. 4 Time- and dose-dependence of the stimulatory effect of CCK_8 on the average $Ins(1,4,5)P_3$ content in a suspension of pancreatic acinar cells. Aliquots of a suspension of pancreatic acinar cells were transferred to micro test tubes containing either 0 nM (unstimulated control), 10 nM (closed squares), 1 nM (open squares), 0.3 nM (closed circles) or 0.1 nM (open circles) CCK_8. The reaction was stopped by the addition of trichloroacetic acid at a final concentration of 10% (w/v) at the times indicated. $Ins(1,4,5)P_3$ was measured by an isotope dilution assay and was expressed in pmol/mg acinar protein. In each experiment, the values obtained with CCK_8-stimulated acinar cells are corrected for the $Ins(1,4,5)P_3$ content measured in unstimulated control cells. The data presented are the mean ± SEM of 4 experiments.

sustained phase of stimulation resulted in a decrease in frequency of the ongoing Ca^{2+} oscillations (Fig. 3). The effect of TPA was dose-dependent. Thus, the frequency of the oscillations gradually decreased following addition of 1 nM TPA (Fig. 3b), more rapidly decreased following addition of 3 nM TPA (Fig. 3a), and virtually immediately decreased following addition of 10 nM TPA (Fig. 3b).

Effect of TPA pretreatment on the CCK_8-evoked increase of the average inositol 1,4,5-trisphosphate concentration in a suspension of pancreatic acinar cells

The average $Ins(1,4,5)P_3$ content measured in a suspension of unstimulated acinar cells (8.5 mg of protein/ml of suspension, SEM = 0.4, n = 21) amounted to 6.4 pmol/mg of acinar protein (SEM = 0.7, n = 21). Addition of CCK_8 resulted in a rapid and dose-dependent increase in average $Ins(1,4,5)P_3$ content

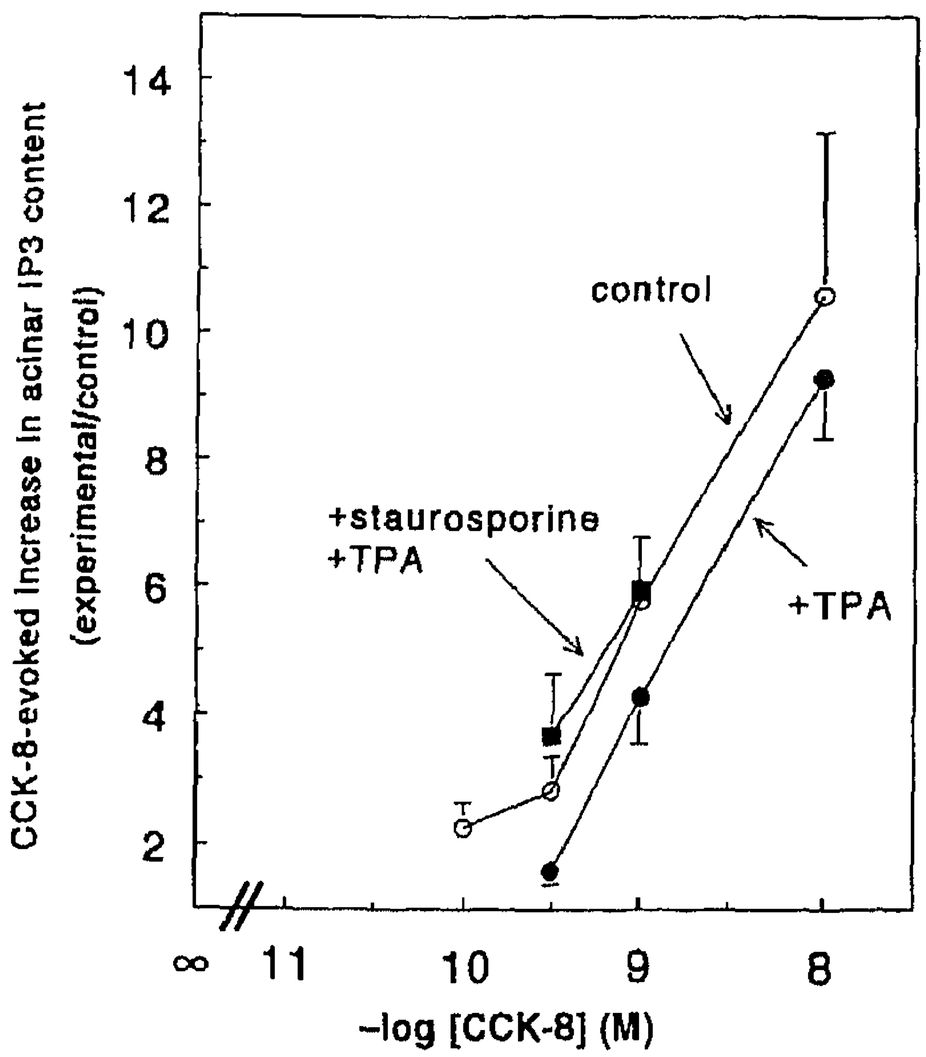

Fig. 5 Effect of TPA pretreatment on the dose-response curve for the stimulatory effect of CCK_8 on the peak increase in average $Ins(1,4,5)P_3$ content in a suspension of pancreatic acinar cells. Pancreatic acinar cells were preincubated in a medium containing either dimethylsulphoxide (open circles), 0.1 μM TPA (closed circles), or 0.1 μM TPA in combination with 1 μM staurosporine (closed squares) for 5 min at 37°C. Aliquots of the suspension were transferred to micro test tubes containing the indicated concentrations of CCK_8. The reaction was stopped by the addition of trichloroacetic acid at a final concentration of 10% (w/v) at 5 s following the addition of 10 nM CCK_8, at 10 s following the addition of 1 nM or 0.3 nM CCK_8 and at 20 s following the addition of 0.1 nM CCK_8. $Ins(1,4,5)P_3$ was measured by an isotope dilution assay and was expressed in pmol/mg acinar protein. The data presented are the mean ± SEM of 3–6 experiments.

(Fig. 4). The time that elapsed for CCK_8 to exert its maximal effect was inversely proportional to the hormone concentration. Thus, 10 nM, 1 nM, 0.3 nM and 0.1 nM CCK_8 evoked a maximal increase at 3–5 s, 10 s, 10–15 s and 15–20 s, respectively. In cells stimulated with 10 nM or 1 nM CCK_8, the average $Ins(1,4,5)P_3$ content rapidly returned to a value slightly higher than the control value, whereas in cells stimulated with 0.3 nM or 0.1 nM CCK_8 the average $Ins(1,4,5)P_3$ content remained maximal for at least 15–20 s. Thus, at 30 s following the onset of stimulation, the average $Ins(1,4,5)P_3$ content in cells stimulated with 1 nM CCK_8 equalled that in cells stimulated with 0.3 nM CCK_8.

In order to investigate the effect of phorbol ester pretreatment on the process of CCK_8-evoked $Ins(1,4,5)P_3$ production, the average $Ins(1,4,5)P_3$ content was determined at 5 s following stimulation with 10 nM CCK_8, at 10 s following stimulation with either 1 nM or 0.3 nM CCK_8, and at 20 s following stimulation with 0.1 nM CCK_8. Figure 5 shows that the CCK_8-evoked increase in average $Ins(1,4,5)P_3$ content was markedly attenuated in cells pretreated with 0.1 μM TPA. The inhibitory effect of the phorbol ester was statistically significant with 0.3 nM and 1 nM CCK_8. In relative terms, the inhibitory effect of TPA decreased with increasing of the CCK_8 concentration. Thus, the response to 0.3 nM CCK_8 was reduced by 70%, that to 1 nM CCK_8 by 30%, and that to 10 nM of the secretagogue by 10%.

The inhibitory effect of TPA was completely reversed by staurosporine, a general inhibitor of protein kinase activity, added at a concentration of 1 μM 1 min before addition of the phorbol ester (Fig. 5). The average $Ins(1,4,5)P_3$ content measured in acinar cells pretreated with either TPA or staurosporine alone or with the combination of both agents amounted to 6.2 (SEM = 1.0, n = 8), 7.7 (SEM = 1.3, n = 8) and 7.6 (SEM = 1.6, n = 8) pmol/mg of protein, respectively. These values were not significantly different from the control value of 6.8 (SEM = 1.3, n = 8) pmol/mg of protein, indicating that neither treatment affected basal $Ins(1,4,5)P_3$ levels.

Discussion

In this study we investigated the effect of protein kinase C activation on the stimulatory action of CCK_8 on both the formation of $Ins(1,4,5)P_3$ and the recruitment of acinar cells in terms of receptor-evoked Ca^{2+} mobilization in a population of freshly isolated rabbit pancreatic acinar cells. The aim of the study was to establish a possible relationship between these two phenomena.

We have previously demonstrated that CCK_8 dose-dependently recruits freshly isolated pancreatic acinar cells in terms of receptor-evoked Ca^{2+} mobilization [5]. The present study shows that activation

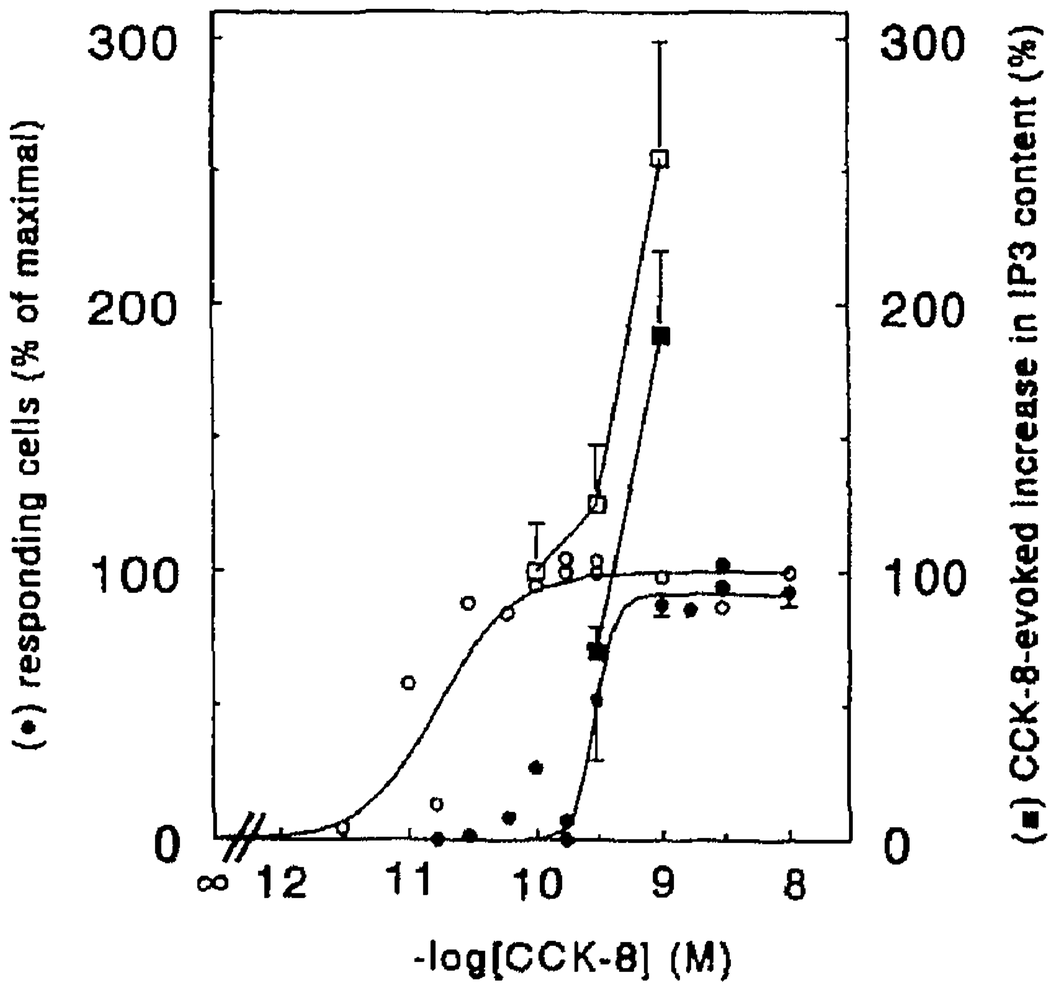

Fig. 6 Comparison of the dose-response curves for the stimulatory effects of CCK_8 on the peak increase in average $Ins(1,4,5)P_3$ content and the recruitment of individual cells in terms of Ca^{2+} mobilization. In this figure, the minimal CCK_8-evoked peak increase in average $Ins(1,4,5)P_3$ content to produce maximal recruitment of untreated cells is set at 100%, to which all other values are related. Circles represent the effect of CCK_8 on the recruitment of control (open circles) and TPA-treated (closed circles) cells. Squares represent the effect of CCK_8 on the peak increase in average $Ins(1,4,5)P_3$ content in control (open squares) and TPA-treated (closed squares) cells.

of protein kinase C by means of a phorbol ester results in a rightward shift of the dose-recruitment curve. The hormone was 17-fold less potent in recruiting TPA-treated cells (EC_{50} = 287 pM) than untreated control cells (EC_{50} = 16.8 pM). By contrast, the efficacy of the hormone remained largely unaltered by TPA pretreatment. Thus, CCK_8 maximally recruited 94% of untreated control cells and 89% of TPA-treated cells. In addition, the present study shows that pretreatment of the acinar cells with TPA at a concentration of 0.1 μM can completely inhibit the recruitment of acinar cells by the CCK_8 analogue JMV-180, which acts as an agonist at the high-affinity CCK receptor [7–10]. The inhibitory effect of TPA was not reversed by further increasing of the JMV-180 concentration. Thus, at a concentration of 10 μM, JMV-180 recruited 5% (SEM = 2, n = 3) of the TPA-treated cells.

The latter observation suggests that in the rabbit, in contrast to the mouse [8], JMV-180 does not act as an agonist at the low-affinity CCK receptor since otherwise an increase in the number of responding cells, similarly to that observed with higher CCK_8 concentrations, would have been expected. Moreover, when a suspension of TPA-treated rabbit pancreatic acinar cells was stimulated with a supramaximal concentration of CCK_8 subsequent to the addition of JMV-180, which by itself did not increase the average $[Ca^{2+}]_i$, a marked reduction of the CCK_8-evoked increase in average $[Ca^{2+}]_i$ was observed, indicating that in this species JMV-180 acts as an antagonist at the low-affinity CCK receptor. From these observations it can be concluded that in the rabbit, similarly to the rat [9], JMV-180 acts as an agonist at the high-affinity CCK receptor and as an antagonist at the low-affinity CCK receptor.

The inhibitory effects of TPA described here are consistent with those originally reported by Honda et al. [20] and support the idea that protein kinase C, directly or indirectly, converts the CCK receptor from a high-affinity state into a low-affinity state [18]. Moreover, they demonstrate that the TPA-evoked reduction in peak increase of the average $[Ca^{2+}]_i$ observed with acinar cells in suspension [18] reflects a decrease in number of responding cells rather than a generalized decrease in $[Ca^{2+}]_i$.

CCK_8 both time- and dose-dependently increased the average $Ins(1,4,5)P_3$ content in a suspension of acinar cells. But, whereas cell-recruitment was completed with 0.1 nM CCK_8, the peak increase in average $Ins(1,4,5)P_3$ content further increased with increasing hormone concentration. Pretreatment of the acinar cells with TPA significantly attenuated the stimulatory effect of CCK_8 on the peak increase in average $Ins(1,4,5)P_3$ content. Again, the peak increase in average $Ins(1,4,5)P_3$ content continued to increase with further increasing of the hormone concentration after cell-recruitment was completed. Comparison of the dose-response curves for the stimulatory effects of CCK_8 on the peak increase in average $Ins(1,4,5)P_3$ content and the recruitment of individual cells in terms of receptor-evoked Ca^{2+} mobilization (Fig. 6), reveals that the relative effect of 0.3 nM CCK_8 on the peak increase in average $Ins(1,4,5)P_3$ content in TPA-

treated cells coincides with the percentage of phorbol ester-treated cells recruited in terms of receptor-evoked Ca^{2+} mobilization. This observation supports a correlation between the CCK_8-evoked peak increase in average $Ins(1,4,5)P_3$ content and the number of cells responding with an increase in free cytosolic Ca^{2+} concentration. Thus, from the data presented in this study, it can be concluded that CCK_8, at concentrations acting through the high-affinity receptor, increases the cellular $Ins(1,4,5)P_3$ content to mobilize Ca^{2+} from intracellular stores. Therefore, the present findings are in support of the idea that $Ins(1,4,5)P_3$ is really involved in JMV-180-evoked Ca^{2+} signaling [15–17].

As previously reported, the upstroke of the dose-recruitment curve consists of cells displaying repetitive increases in $[Ca^{2+}]_i$. At 0.1 nM CCK_8, virtually all cells responded and they did so by displaying oscillatory changes in $[Ca^{2+}]_i$. However, with increasing CCK_8 concentration, the number of oscillating cells rapidly decreased whereas the number of responding cells remained constant. Similarly, in TPA-treated cells, the upstroke of the dose-recruitment curve consisted of oscillating cells. The maximum was reached with 3 nM CCK_8, at which concentration virtually all of the responding cells displayed oscillatory changes in $[Ca^{2+}]_i$. Beyond this concentration, the number of oscillating cells rapidly declined, leaving the number of responding cells unaltered. This observation demonstrates that TPA causes a rightward shift of the biphasic dose-response curve for the recruitment of oscillating cells in its entirety. Moreover, this observation demonstrates that addition of TPA to acinar cells displaying ongoing Ca^{2+} oscillations in response to a submaximal CCK_8 concentration causes their complete cessation (*see* also [21,22]) by desensitization of the receptor and not by inhibition of the 'oscillator' itself.

However, it should be noted that this conclusion is not supported by the observation that phorbol esters can effectively block ongoing Ca^{2+} oscillations evoked by the G-protein activating agent mastoparan [23]. Furthermore, the observation that Ca^{2+} oscillations do occur in TPA-treated cells demonstrates that protein kinase C is not directly involved in the mechanism by which higher CCK_8 concentrations prevent the occurrence of oscillatory changes in $[Ca^{2+}]_i$. Finally, the observation that CCK_8 can induce Ca^{2+} oscillations in TPA-treated cells, in which signaling is believed to occur through the low-affinity CCK_A receptor, demonstrates that their induction is not mediated solely by the high-affinity CCK_A receptor as has been suggested in the literature [7].

On average, 55% of the acinar cells (57% of the CCK_8-recruitable acinar cells) responded with an increase in $[Ca^{2+}]_i$ upon stimulation with 1 μM JMV-180. In approximately 60% of the cells, this initial increase in $[Ca^{2+}]_i$ was followed by periodic rises in $[Ca^{2+}]_i$. Interestingly, pretreatment of the acinar cells with a relatively low concentration of 1 nM TPA increased (1.4-fold) the number of responding cells. Moreover, it increased (1.3-fold) the number of responding cells displaying oscillatory changes in $[Ca^{2+}]_i$. But, whereas low-level protein kinase C activation appeared to increase the number of (oscillating) cells recruitable by 1 μM JMV-180, it gradually decreased the frequency of ongoing Ca^{2+} oscillations. Thus, on the one hand low-level protein kinase C activation sensitizes acinar cells with respect to receptor-evoked Ca^{2+} mobilization, whereas, on the other hand, it slows down the periodicity of subsequent oscillatory changes in $[Ca^{2+}]_i$. A similar observation has recently been reported by Gaisano et al. [24]. At concentrations beyond 1 nM, the phorbol ester dose-dependently decreased the percentage of responding cells. On top of that, TPA dose-dependently decreased the percentage of oscillating cells. Thus, at 10 nM TPA only 20% of the responding cells displayed repetitive changes in $[Ca^{2+}]_i$, whereas at 1 nM TPA this value amounted to 80%. This observation demonstrates that TPA inhibits the initial Ca^{2+} transient less effectively than the subsequent Ca^{2+} oscillations. In view of the above conclusion that TPA does not inhibit the 'oscillator' itself, the latter finding can be explained by assuming that TPA may desensitize the high-affinity receptor to such an extent that the initial JMV-180-evoked rise in $[Ca^{2+}]_i$ remains largely unimpaired but that the subsequent induction of $[Ca^{2+}]_i$ oscillations is inhibited by protein kinase C acting in conjunction with a receptor kinase activated independently of protein kinase C. Evidence in support of the existence of such a protein kinase C-independent receptor kinase has been provided by Klueppelberg et al.

[25]. On the other hand, the above observation might also point to the existence of a subpopulation of cells, about 25% of the cell population, displaying a single Ca^{2+} transient upon stimulation with 1 μM JMV-180.

We have previously proposed that TPA inhibits CCK_8-evoked Ca^{2+} mobilization by lowering the affinity state of the CCK receptor [18]. Evidence was provided by the observation that the putative high affinity receptor agonist JMV-180 was unable to increase the average $[Ca^{2+}]_i$ in a suspension of TPA-treated acinar cells. The present study confirms this observation in that it shows that TPA can completely prevent the recruitment of acinar cells in terms of JMV-180-evoked Ca^{2+} mobilization. Many studies have questioned a role of $Ins(1,4,5)P_3$ in the mechanism of action of JMV-180 and thus whether the high affinity CCK receptor is coupled to phospholipase C [7,11–13]. The present study demonstrates that CCK_8 can increase both $[Ca^{2+}]_i$ and the cytoplasmic $Ins(1,4,5)P_3$ level at concentrations not effective in phorbol ester-treated cells and therefore thought to interact solely with high affinity CCK receptors. Moreover, the present study shows that the relatively small increase in $Ins(1,4,5)P_3$ evoked by CCK_8, acting through the high affinity receptor, is comparable to that reported in the literature for JMV-180 concentrations maximally effective in increasing the average $[Ca^{2+}]_i$ [7,11].

In conclusion, the data presented are in agreement with a model recently put forward by Muallem and co-workers [17] according to which activation of the high-affinity CCK receptor by either JMV-180 or CCK_8 results in local increases in $Ins(1,4,5)P_3$, too small to be detected but large enough to trigger an expansive rise in $[Ca^{2+}]_i$, whereas activation of the low-affinity CCK receptor leads to a marked increase in $Ins(1,4,5)P_3$ production.

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