J. Membrane Biol. 139, 127–136 (1994)

The Journal of
Membrane
Biology
© Springer-Verlag New York Inc. 1994

Regulation of Gap Junctional Coupling in Isolated Pancreatic Acinar Cell Pairs by Cholecystokinin-Octapeptide, Vasoactive Intestinal Peptide (VIP) and a VIP-Antagonist

A. Ngezahayo[1], **H.-A. Kolb**[2]

[1]University of Konstanz, Faculty of Biology, D-78464 Konstanz, Germany
[2]University of Tübingen, Institut of Physiology I, Gmelinstr. 5, D-72076 Tübingen, Germany

Received: 24 September 1993/Revised: 5 January 1994

Abstract. Cholecystokinin-octapeptide (CCK-OP) induces a time- and dose-dependent decrease of gap junctional conductance in isolated pairs of pancreatic acinar cells. In double whole-cell experiments, the time course could be described by the latency and the half-life time ($t_{1/2}$) of cell-to-cell uncoupling. The latency shows a biphasic dependence on [CCK-OP] with a minimum of about 50 sec at 10^{-9} M CCK-OP. In the presence of vasoactive intestinal peptide (VIP), the biphasic relationship is shifted to lower CCK-OP concentrations. The increase of latency at high concentrations of CCK-OP ($>10^{-9}$ M) was blocked by addition of a VIP-antagonist. $t_{1/2}$ decreases monophasically with increasing [CCK-OP]. Addition of GTPγS to the pipette solution suppresses the [CCK-OP] dependence of the latency and potentiates the uncoupling phase. The kinetic data are discussed in terms of CCK binding to receptors of high and low affinity. Evidence is presented that secretion and cell-to-cell coupling are not related by an all-or-none process, but that for physiological CCK-OP concentrations, gap junctional uncoupling follows secretion.

Key words: Gap junctions — Double whole cell — Cholecystokinin-octapeptide — Vasoactive intestinal peptide (VIP) — VIP antagonist — Pancreatic acinar cells

Introduction

One of the known peripheral physiological functions of the major secretagogue cholecystokinin (CCK) is the stimulation of digestive enzyme release from pancreatic acinar cells (Walsh, 1987). In pancreatic acinar cells from all species examined, CCK has been shown to interact with specific receptors (*cf.* Jensen et al., 1989) stimulating guanine nucleotide-binding protein (G protein)-mediated phospholipase C (PLC) activation (Merritt et al., 1986; Schnefel et al., 1988), resulting in phosphatidylinositol 4,5-bisphosphate breakdown to inositol 1,4,5 triphosphate (IP_3) and 1,2 diacylglycerol (Putney et al., 1983; Berridge, 1987; Berridge & Irvine, 1989; Matozaki & Williams, 1989). The downstream mechanisms affected by this CCK-stimulated cascade are believed to be dependent on a rise in intracellular Ca^{2+} levels (Streb et al., 1984) and the activation of protein kinase C (PKC) (Nishizuka, 1988; Asaoka et al., 1992).

Intercellular communication via the gap junctions of gland cells has been implied in the control of secretion (*cf.* Kolb & Somogyi, 1991). Unspecific closure of gap junctions in pancreatic acinar cells with heptanol has been shown to increase basal release of amylase (Meda et al., 1987). Also, in addition to activating PKC, cholinergic agents like carbachol lead to uncoupling (Iwatsuki & Petersen, 1978). In previous electrophysiological experiments using the double whole-cell patch-clamp technique (Neyton & Trautmann, 1985; *cf.* Kolb, 1992), we have shown that introduction of purified PKC into murine pancreatic acinar cell pairs or exposure to 1-oleoyl-2-acetyl-sn-glycerol causes gap junction channel closure (Somogyi, Batzer & Kolb, 1989). By contrast, 12-*O*-tetradecanoylphorbol-13-acetate (TPA) failed to block the junctional coupling of pancreatic acinar cell pairs, whereas on dispersed acini a TPA-dependent PKC translocation from a cytosolic to a microsomal fraction was observed (Chanson et al.,

Correspondence to: H.-A. Kolb

1989*a*). Furthermore, evidence had been presented that electrical uncoupling of pancreatic acinar cell pairs by the cholinergic agonist carbamylcholine involves activation of PLC via a G protein (Somogyi & Kolb, 1989). More recently, we could demonstrate that CCK-OP in the picomolar range causes Ca^{2+} oscillations and electrical uncoupling (Ngezahayo & Kolb, 1993).

To elucidate the proposed hypothesis of an interaction of secretion and coupling, we analyzed here the kinetics of gap junctional conductance ($g_j(t)$) between isolated pancreatic acinar cells in the double whole-cell configuration as a function of [CCK-OP]. The time course of $g_j(t)$ is described by two phenomenological parameters: the half-life time ($t_{1/2}$) which characterizes the kinetics of the actual uncoupling phase, and the latency which describes the induction phase for a significant decrease of $g_j(t)$. Both parameters were determined as a function of CCK-OP concentration.

To clarify the signal transduction pathways involved in the CCK-OP dependent modulation of $g_j(t)$, the effect of nonhydrolyzable analogues of GTP and GDP such as guanosine 5′-[γ-thio]triphosphate (GTPγS) and guanosine 5′-[β-thio]diphosphate (GDPβS) on CCK-OP-induced electrical uncoupling was investigated, as well as the action of polymyxin B (Wooten & Wrenn, 1984) and staurosporine, which are both known inhibitors of PKC activation. Furthermore, the potentiation of CCK-OP-induced secretion by the vasoactive intestinal peptide (VIP) (Gardner & Jackson, 1977) was investigated on the level of electrical coupling in terms of $t_{1/2}$ and latency.

It is discussed whether CCK-evoked uncoupling and secretion are modulated by common biochemical pathways and are concentration-dependent interacting processes.

Materials and Methods

Cell Preparation

Cells were prepared by collagenase digestion of freshly isolated pancreas of two-to-three-month-old male NMRI mice. The isolation procedure was previously described in detail (Somogyi & Kolb, 1988). The preparation yielded a high ratio of cell pairs to other cells. The cells were stored at 4°C in BME (Basal Medium Eagle-Hanks; Sigma, St. Louis, MO) buffered with 10 mM HEPES at pH 7.4 and could be used up to 36 hr after isolation.

Recording of Junctional Conductance

The cells were placed in a perfusion chamber containing 200 μl of bath solution. After a settlement period of about 10 min, a cell pair was selected and the double whole-cell configuration established (Neyton & Trautmann, 1985). The junctional conductance was determined, as described previously (Somogyi & Kolb, 1988). Rectangular voltage pulses of 100 Hz were applied immediately after sealing to check seal and input resistance. Junctional current records were obtained by applying a rectangular voltage pulse of varying amplitude to one cell while the other was held constant, and reversing the order of the pulsed and resting cell once every minute to exclude one-sided artifacts (*see also* Kolb, 1992).

Data Analysis

Data were recorded and evaluated as described (Somogyi & Kolb, 1988). Whole-cell currents were amplified using two List EPC-7 amplifiers and recorded on a Racal Store 4 DS FM tape recorder at a cut-off frequency of 2.5 kHz.

The junctional conductance $g_j(t)$ was calculated by dividing the respective differences of junctional current and junctional potential difference before and after a voltage pulse (Neyton & Trautmann, 1985). The potential difference was corrected by the voltage drop across the series resistances of the pipettes. The junctional current was corrected by the contribution of the current across the nonjunctional membrane (Kolb, 1992).

The following relationship was used to describe the time course of electrical uncoupling: $g_j(t) = g_0 \times \exp(-t/\tau)$, g_o being the extrapolated time-zero parameter of the fit, τ the time constant, and $\tau \times \ln 2$ corresponds to the half-life time, $t_{1/2}$. After agonist perfusion, the time point t after which $g_j(t)$ had decreased by more than 10% of the maximal initial level was considered as an estimate for the latency of agonist action (*compare* Fig. 1*b*).

For the figures, the ratio of the individual $g_j(t)$ values over the maximal value $g_{j\max}$ was presented. The time axis origin was calibrated to the exchange of the bath medium with NaCl-BS-containing agonist. To allow the exchange of the pipette solution with the cytosol, we added the agonist 5–10 min after breaking both patch membranes.

Data are given as mean ± SEM where n denotes the number of different cell pairs.

Electrolyte Solutions

A high KCl buffer containing ATP and db-cAMP of pCa 7 and pH 7.4 was used as the pipette control solution: 145 mM K^+, 10 mM Na^+, 127 mM Cl^-, 0.1 μM $Ca^{2+}{}_{free}$ (3.3 mM $Ca^{2+}{}_{tot}$), 1.0 mM $Mg^{2+}{}_{free}$ (6.0 mM $Mg^{2+}{}_{tot}$), 10 mM glucose, 10 mM HEPES, 5 mM EGTA, 5 mM ATP^{2-}, 0.1 mM db-cAMP. ATP and db-cAMP were purchased from Sigma (St. Louis), HEPES from Serva (Heidelberg). The concentration of $Ca^{2+}{}_{free}$ was determined as given in Somogyi and Kolb (1988). NaCl-BS was used as bath medium (in mM): 145 NaCl, 5 KCl, 1 $CaCl_2$, 1 $MgCl_2$, 10 glucose, 10 HEPES at pH 7.4. The experiments were performed at 20–22°C. Cholecystokinin-octapeptide, vasoactive intestinal peptide and [Ac-Tyr, D-Phe]GRF (1–29) amide, a VIP-antagonist (Waelbroeck et al., 1985), were purchased from Neosystem (Strassbourg) and added to the NaCl-BS bath. Staurosporine (Sigma) and PMB (polymyxin B sulfate, 7700 USP U/mg, Sigma) were dissolved in the pipette control solution.

Results

Gap Junctional Coupling in the Presence of Cholecystokinin-Octapeptide (CCK-OP)

Gap junctional conductance ($g_j(t)$) between isolated acinar cell pairs was monitored in the double whole-cell

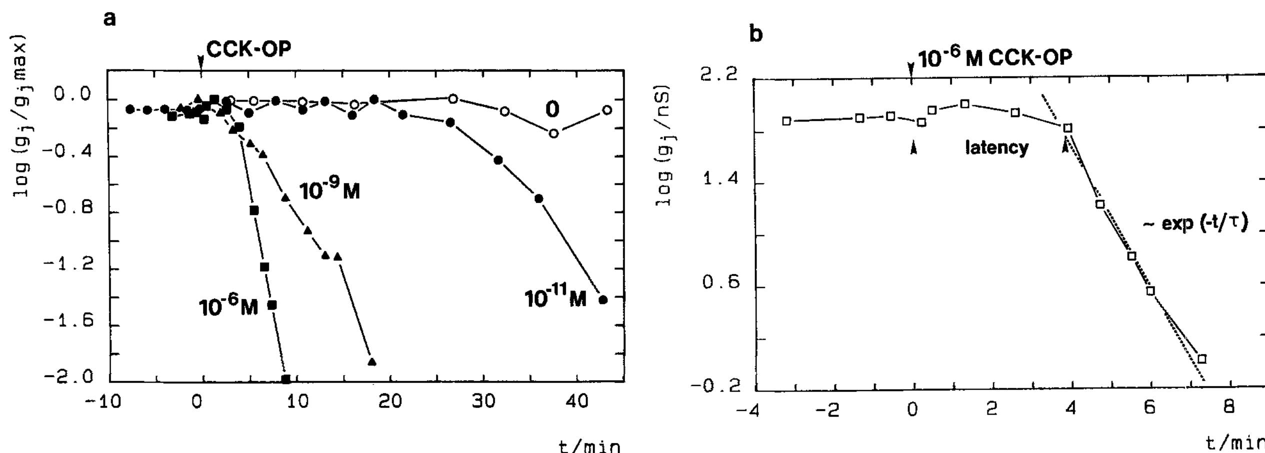

Fig. 1. Effect of cholecystokinin-octapeptide (CCK-OP) on the time course of gap junctional conductance (g_j). (*a*) Influence of increasing CCK-OP concentrations as indicated. For clearer presentation, the ratio of the individual g_j values over the maximal starting value $g_{j_{max}}$ are presented in the different experiments. (*b*) Phenomenological description of CCK-OP-induced electrical uncoupling by a single exponential decrease of time constant τ and a latency after which a significant decrease of g_j starts. For further explanation, *see text.* Pipette control solution was used in the pipette. The origin of the time axes was calibrated to the addition of CCK-OP to the physiological NaCl bath medium.

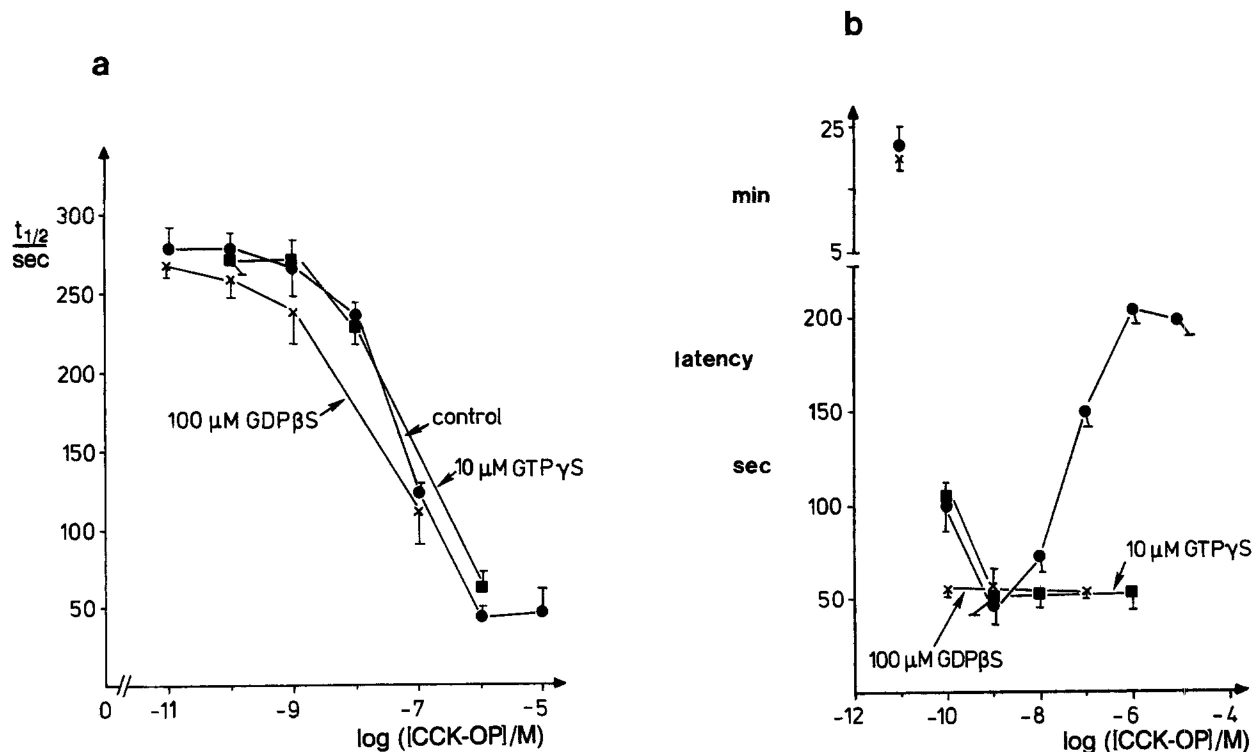

Fig. 2. Phenomenological parameters describing a decrease of gap junctional coupling as a function of [CCK-OP]. As indicated, different additives to the pipette control solution were used: (●) unmodified pipette control; (■) addition of 10 μM GTPγS; (X) addition of 100 μM GDPβS. (*a*) $t_{1/2}$ is the time required for g_j to drop by one-half in the decreasing phase (*see* Fig. 1*b*). (*b*) Latency was determined as the time span after which a 10% decrease (*see* Materials and Methods) of g_j was observed (*see* Fig. 1*b*). Each point represents the mean ± SEM of three or four independently performed experiments.

configuration. A pipette solution containing 0.1 mM cAMP and 5 mM ATP was used for all experiments. As previously shown, these additives are sufficient in suppressing the spontaneously occurring decrease of gap junctional conductance which is caused by cell dialysis with pipette solutions containing pure electrolytes (Somogyi & Kolb, 1988). During the initial phase of recording $g_j(t)$, we obtained under control conditions a value of $g_o = 61 \pm 9$ nS for 155 different cell pairs out of 33 cell preparations. Within a preparation, the corresponding SEM value was smaller by a factor of about five, respectively.

After 5 min of recording $g_j(t)$, the NaCl-BS bath electrolyte was replaced and the cell pair was superfused with the same solution containing CCK-OP. Figure 1*a* shows the corresponding time course of $g_j(t)$ in the absence and after addition of CCK-OP. CCK-OP was varied between 10^{-11} M and 10^{-5} M in different experiments. The figure shows that $g_j(t)$ decreases for [CCK-OP] $\geq 10^{-11}$ M down to the resolution limit of the recording system (2–3 pS, Somogyi & Kolb, 1988). At 10^{-12} M or lower concentrations of CCK-OP, no significant effect on $g_j(t)$ was observed during a typical recording time of 40–50 min. The latter was limited by the stability of the two gigaseals. For clearer presentation, the data derived at 10^{-10} M, 10^{-8} M, 10^{-7} M and 10^{-5} M CCK-OP are not given in Fig. 1*a*. The figure illustrates that the time course of $g_j(t)$ can qualitatively be characterized by two phenomenological parameters, as indicated in Fig. 1*b*. After a certain lag phase, a significant decrease of $g_j(t)$ can be observed. This time span will be denoted as latency. In general, during the phase of electrical uncoupling, $g_j(t)$ follows in a first approximation a single exponential behavior with a time constant, τ. The corresponding half-life time $t_{1/2}$ decreases with increasing CCK-OP concentration (*see* Fig. 2*a*).

In the following, we will compare the two parameters measured as a function of [CCK-OP]. The different preparations had no significant influence on the corresponding SEM values, as it was observed for g_o (*see above*). At [CCK-OP] $\leq 10^{-9}$ M, a $t_{1/2}$ of about 270 sec was found which remains constant within the experimental error and monotonically decreases for increasing [CCK-OP] $> 10^{-9}$ M to about 50 sec (Fig. 2*a*).

The corresponding latency as a function of [CCK-OP] exhibits a biphasic behavior adopting a minimal value of 50 sec at 10^{-9} M (*see* Fig. 2*b*). At lower and higher secretagogue concentration (10^{-12} M $\leq$ [CCK-OP] $\leq 10^{-6}$ M), the latency increases and adopts a plateau value of about 200 sec for [CCK-OP] $\geq 10^{-6}$ M. At [CCK-OP] $\leq 10^{-12}$ M values larger than about 40 min (*see above*) have to be expected. Figure 2*b* shows that a [CCK-OP] of about 10^{-9} M can be considered as a characteristic secretagogue concentration for a fast onset of junctional uncoupling.

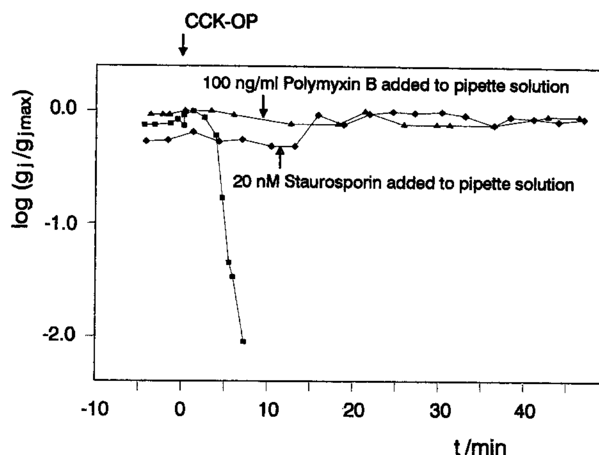

Fig. 3. Suppression of CCK-OP (1 μM) induced gap junctional uncoupling by addition of staurosporine or polymyxin B to the pipette solution. (■) Control curve at 1 μM CCK-OP; (◆) 20 nM staurosporine added to pipette control; (▲) 100 ng/ml polymyxin B added to pipette control.

To elucidate the involved signal-transduction chain yielding the CCK-OP induced electrical uncoupling, we investigated the combined action of CCK-OP and staurosporine or polymyxin B (PMB). The latter polycationic peptide has been determined to be a potent inhibitor of protein kinase C (PKC) activity being more than 100 times more specific for PKC than for the Ca^{2+}/calmodulin system (Wrenn & Wooten, 1984). As an example, Fig. 3 indicates the suppression of CCK-OP-induced electrical uncoupling at 10^{-6} M CCK-OP either by addition of staurosporine ($2 \cdot 10^{-8}$ M) or PMB (100 ng/ml) to the pipette filling solution.

Effect of GTPγS and GDPβS on CCK-OP-induced Electrical Uncoupling

First, we tested whether in the absence of CCK-OP, addition of a weakly hydrolyzable analogue of GTP(GDP), GTPγS(GDPβS), to the pipette filling solution affected $g_j(t)$. Even at a high concentration of 500 μM GTPγS and of 100 μM GDPβS, stable coupling was observed for at least 30 min (*data not shown*).

The influence of GTPγS (10^{-5} M) and GDPβS (10^{-4} M) in the cytoplasm on CCK-OP-induced decrease of $g_j(t)$ is given in Fig. 4*a* and *b* at 10^{-9} M and 10^{-6} M CCK-OP, respectively. For clearer presentation, the data obtained at 10^{-10} M and 10^{-11} M CCK-OP are not shown, but the corresponding parameter values are included in Fig. 2. Figure 3*a* shows that in the presence of GTPγS or GDPβS, $g_j(t)$ was left unchanged at the critical CCK-OP concentration of 10^{-9} M, while at high-

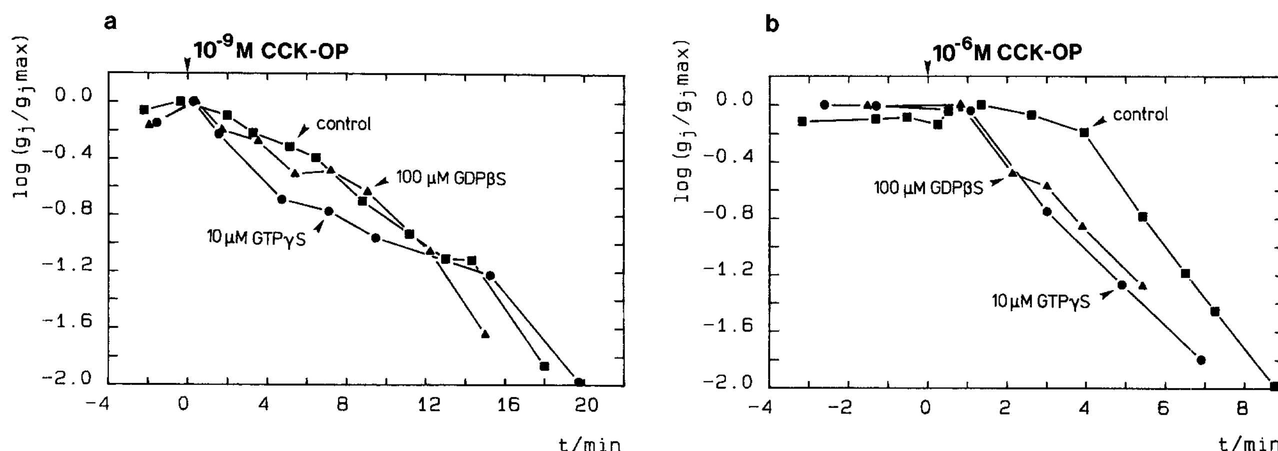

Fig. 4. Effects of intracellular GDPβS (100 µM, filled triangles) and of GTPγS (10 µM, filled circles) on CCK-OP induced change of g_j. For comparison, g_j is presented for unmodified pipette control as well (filled squares). (*a*) Addition of 1 nM or (*b*) 1 µM CCK-OP at t = 0 min to the bath. Individual experiments are presented.

er concentrations an influence is obvious (Fig. 4*b*). Within the statistical error, the presence of GTPγS did not influence $t_{1/2}$ while GDPβS shifted the $t_{1/2}$ *vs*. [CCK-OP] relationship to lower [CCK-OP] by about one order of magnitude. Both nucleotides significantly affected the latency. The corresponding parameter values are summarized in Fig. 2*a* and *b*, respectively. Figure 2*b* shows that, for both nucleotides, the latency becomes at first shorter and then remains constant with increasing [CCK-OP]. At 10^{-10} M CCK-OP, the presence of 10^{-5} M GTPγS and 10^{-4} M GDPβS affected $g_j(t)$ differently. GTPγS had no significant influence whereas GDPβS shortened the latency to the minimal value. The latter was observed in the absence of GTPγS and GDPβS at 10^{-9} M CCK-OP (*compare* Fig. 2*b*).

Common Action of CCK-OP and VIP or a VIP-Antagonist on Electrical Coupling

In the absence of CCK-OP superfusion of a cell pair with 10^{-8} M VIP left $g_j(t)$ unchanged for at least 30 min (Fig. 5). But as the figure shows, simultaneous application of CCK-OP (10^{-9} M) and VIP (10^{-8} M) shifted the time course of $g_j(t)$ obtained in the presence of 10^{-9} M CCK-OP to that observed at 10^{-6} M CCK-OP. $t_{1/2}$ is reduced and the latency prolongated. The corresponding dependence of $t_{1/2}$ on [CCK-OP] becomes about two orders of magnitude more sensitive to CCK-OP (*see* Fig. 6*a*). For the latency, both slopes are similar and thus a shift of the dose-effect curve to lower [CCK-OP] can be assumed (Fig. 6*b*).

To obtain additional information about the step in the proposed signal-transduction chain (*see* Discussion) which might be influenced by VIP, we also used a VIP-antagonist ([Ac-Tyr, D-Phe]GRF (1–29) amide). Figure 7 indicates that at 10^{-7} M this VIP-antagonist had no significant effect on gap junctional coupling, but in the simultaneous presence of CCK-OP it affected differently the two parameters, $t_{1/2}$ and latency. Especially at higher concentrations of CCK-OP, $t_{1/2}$ remained larger by a factor of two, and the latency was independent of [CCK-OP] ≥ 10^{-10} M (*see* Fig. 6*a* and *b*), but was diminished to the minimal value observed in the absence of VIP or VIP-antagonist.

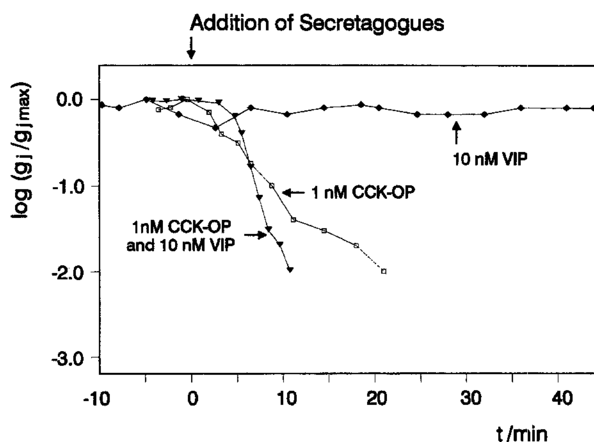

Fig. 5. Simultaneous effect of CCK-OP and of vasoactive intestinal peptide (VIP) on gap junctional conductance. Unmodified pipette control was used in the pipette and 10 nM VIP plus 1 nM CCK-OP (filled triangles) were added to the bath. The data after addition of 1 nM CCK-OP (open squares) or 10 nM VIP (filled diamonds) are given for comparison.

Discussion

Cholecystokinin-octapeptide (CCK-OP) induces a time- and dose-dependent decrease of gap junctional con-

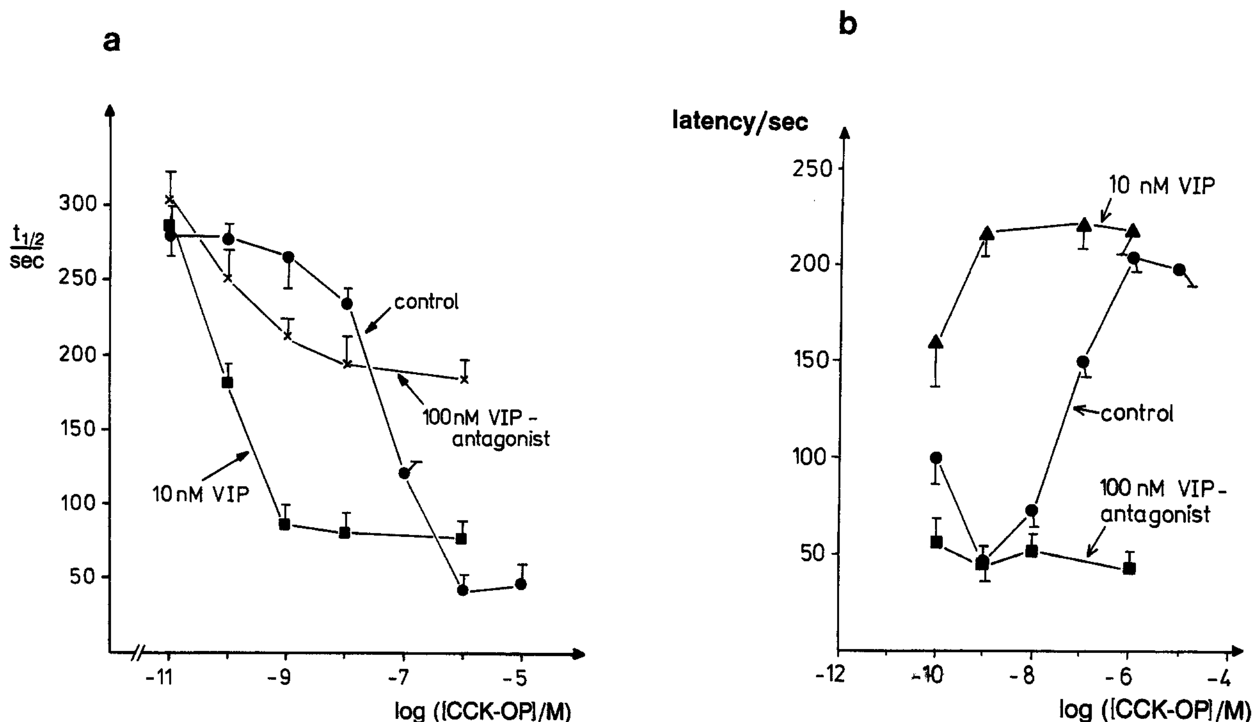

Fig. 6. Phenomenological parameters describing the decrease of gap junctional coupling as a function of [CCK-OP] (filled circles), in the simultaneous presence of 10 nM VIP (filled squares) or of 100 nM VIP-antagonist (X). Unmodified pipette solution was used. (*a*) $t_{1/2}$ and (*b*) latency, determined as described in Fig. 1*b*. Each point represents the mean ± SEM of three or four independently performed experiments.

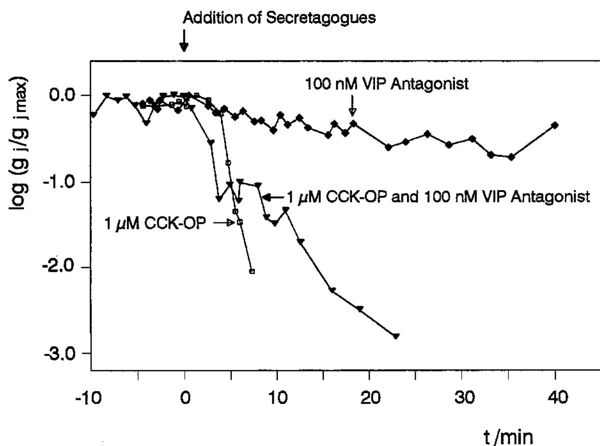

Fig. 7. Effect of a VIP-antagonist (100 nM, [Ac-Tyr, D-Phe] GRF (1–29) amide) on CCK-OP (1 μM) induced decrease of gap junctional coupling (filled triangles). Filled diamonds denote a corresponding control experiment in the absence of CCK-OP. For comparison, the time course of gap junctional conductance after addition of 1 μM CCK-OP (open squares) is shown. Unmodified pipette control was used.

ductance in pairs of pancreatic acinar cells (Fig. 1). The lowest effective concentration of CCK-OP is in the same order of magnitude required for physiological enzyme secretion (*cf.* Jensen et al., 1989). At first we want to discuss the measured [CCK-OP]-dependent parameters of gap junctional uncoupling, the half-life time ($t_{1/2}$) and the latency (for definition, *see* Fig. 1*b*), in terms of the proposed functionally different classes of CCK receptors. Pancreatic acinar cells have been shown to possess three classes of CCK receptors, two with high affinity and one with a low affinity for CCK-OP (Wank et al., 1988; Sato et al., 1989; *cf.* Yu et al., 1990).

Surprisingly, we found on cell pairs that the induction phase or latency follows a biphasic relation as function of [CCK-OP] (Fig. 2*b*). A similar biphasic relationship has been observed for a [CCK-OP]-dependent amount of amylase released from acinar cell suspensions (Gardner & Jensen, 1981; Matozaki et al., 1988, Jensen et al., 1989) and for the closely related [CCK-OP]-induced Cl^- conductance in isolated pancreatic zymogen granules (Piiper et al., 1991*a*). A biphasic dose response of CCK-OP stimulated amylase release could be observed at [CCK-OP] $\geq 10^{-10}$ M down to the shortest incubation time of 5 min (Barlas, Jensen & Gardner, 1982). But the time span during which amylase is actually released has not been determined. On the other hand, the corresponding latencies are shorter than 5 min (Fig. 2*b*). The close parallelism of the biphasic behavior suggests that the time span during which amylase is released and the induction phase of gap junctional uncoupling are related.

The stimulated enzyme secretion has been attrib-

uted to CCK-OP binding to high affinity receptors. The corresponding kinetical parameters of time-dependent gap junctional uncoupling change in parallel. Both the latency and $t_{1/2}$ decrease with increasing [CCK-OP]. Occupation of low affinity receptors at high doses of CCK-OP is believed to inhibit stimulated secretion (Jensen et al., 1989; Yu et al., 1990) and to decrease the Cl^- conductance (Piiper et al., 1991*a*). Opposite changes of latency and $t_{1/2}$ are observed with increasing [CCK-OP] (Fig. 2), the uncoupling is faster, but starts later. For a quantitative kinetical description of CCK-OP-induced uncoupling, the involved molecular mechanism has to be known. It is well established that guanosine-nucleotide binding proteins (G proteins) are involved in the coupling of CCK-OP to phospholipase C (PLC) in pancreatic acinar cells (Merritt et al., 1986; Schnefel et al., 1988). But the dose-dependent biphasic behavior of the latency cannot be explained by a simple chemical reaction scheme which is based on a sequential activation of a G protein and effector protein (PLC) (*compare* Lamb & Pugh, 1992). In addition, inhibitory factors like G_i protein(s) (*see below*) should be taken into account (H.-A. Kolb, *in preparation*); e.g., three inhibitory G_i-like proteins have been identified in plasma membranes from pancreatic acini (Schnefel et al., 1990).

We found similar effects with GDPβS and GTPγS on the [CCK-OP] response of both $t_{1/2}$ and latency (Figs. 2 and 4). In other cell systems, it has been shown that GDPβS counteracts the stimulatory effect of GTPγS by inhibition of G proteins (Barrowman et al., 1986; Schwiebert et al., 1990). One explanation might be that GDPβS is phosphorylated to GTPγS by an endogenous GDP kinase (Ohatsuki et al., 1986; Seifert et al., 1988). A further explanation is based on the stimulation of inhibitory G proteins for PLC. Such an explanation was given for the GDPβS-dependent thrombin-receptor coupled activation of PLC in human platelets (Oberdisse & Lapetina, 1987). Similar effects with GDPβS and GTPγS on both enzyme secretion from permeabilized acini and Cl^- conductance in pancreatic zymogen granules have been reported (Piiper et al., 1991*a*). The authors concluded that if inhibitory G proteins couple to both high and low affinity CCK receptors, the stimulatory effect of CCK-OP at low concentrations could be increased by GDPβS. This would be in line with our findings (*see* Fig. 2*b*), if we consider a minimal latency at submaximal CCK-OP concentration as an indicator of stimulated enzyme secretion. However, the role of PLC and/or unknown metabolites in high-dose inhibition of CCK-OP-stimulated enzyme secretion is not understood. Therefore, the stimulatory influence of GDPβS on the latency at high [CCK] will not be considered any further.

Under the assumption that the binding of CCK-OP to different receptors (binding sites) is reflected in the kinetics of gap junctional uncoupling, we discuss the effect of the simultaneous presence of CCK-OP and VIP as well as of the VIP-antagonist on $t_{1/2}$ and the latency (Fig. 6). It is well known that VIP alone has no stimulating effect on amylase release but potentiates the secretory response to Ca^{2+}-mediated agents (Gardner & Jensen, 1981; Hootman, Brown & Williams, 1985). In accordance with this finding, VIP alone had no effect on gap junctional coupling (Fig. 5) and the simultaneous presence of VIP and CCK-OP shifted the latency and $t_{1/2}$ to lower [CCK-OP]. It appears that VIP interacts with the inhibitory pathway stimulated by high concentrations of CCK-OP. This hypothesis is supported by the finding that the simultaneous presence of the VIP-antagonist hinders the CCK-OP effect attributed to the occupancy of low affinity receptors.

Piiper et al. (1991*b*) provided evidence that the interaction of the two agonists CCK-OP and epidermal growth factor in stimulating the Cl^- conductance occurs on the G protein level. Since in our experiments the known VIP-dependent stimulation of cAMP-dependent protein kinase A was most probably masked by the clamped cAMP concentration (0.1 mM in the pipette solution), it is likely that the observed interaction of VIP and CCK-OP on gap junctional coupling occurs at this molecular level, e.g., by VIP-induced inhibition of inhibitory G proteins involved in the coupling of CCK-OP receptors to PLC.

Previous work has indicated that CCK-OP receptor-mediated stimulation of PLC leads to breakdown of PtdIns(4,5)P_2 to IP_3 and diacylglycerol. IP_3-induced intracellular Ca^{2+} mobilization and activation of Ca^{2+}-activated phospholipid-dependent PKC by diacylglycerol act synergistically to cause digestive enzyme secretion (Pandol et al., 1985). PLC activity as well as the concentrations of both second messengers, IP_3 and diacylglycerol, show a monophasic dependence on [CCK-OP] (*cf.* Jensen et al., 1989) and no inhibition at high [CCK-OP] (Nakanishi et al., 1988) which was also found for the decrease of gap junctional conductance as expressed in terms of $t_{1/2}$. Previously, we have shown that addition of purified PKC to the pipette solution uncouples pancreatic acinar cell pairs (Somogyi et al., 1989). In addition, we could show that inhibitors of PKC like polymyxin B (Wrenn & Wooten, 1988) block carbachol (Somogyi & Kolb, 1989) as well as CCK-OP-evoked uncoupling (Fig. 3). These findings are in accord with studies on permeabilized pancreatic acinar cells and isolated plasma membranes, which demonstrate that acetylcholine and CCK receptors functionally couple via different G proteins to PLC in pancreatic acinar cells (Merritt et al., 1986; Matozaki et al., 1988, Schnefel et al., 1988, 1990; *see also* Ashkenazi et al., 1989) which finally results in activation of PKC.

Despite the presence of putative phosphorylation sites on the subunits (connexins) of the cell-to-cell

channels forming the gap junctions (Loewenstein, 1985; Saez et al., 1986; *cf.* Kolb & Somogyi, 1991), it cannot be excluded that besides PKC-dependent phosphorylation phosphatases and further unknown metabolites are involved also in gap junctional uncoupling; e.g., it has been demonstrated that dephosphorylation of various proteins correlates best with stimulated amylase secretion (Burnham et al., 1988).

The proposed interaction of cell-to-cell coupling and secretion (Meda et al., 1987; Chanson et al., 1989*b*) raises numerous questions for the investigated CCK-OP-mediated uncoupling: Does this interaction depend on CCK-OP concentration, and which degree of uncoupling is necessary and sufficient to affect secretion? Which process becomes stimulated at first? Which type of biochemical pathway is affected by change of coupling and modifies secretion?

To discuss the first two questions, we want to consider the known biochemical pathway of CCK-OP-evoked secretion, including the concentration dependence, and compare it with the derived concentration-dependent parameters of gap junctional uncoupling.

It seems to be obvious that the onset of both processes differs. At physiological [CCK-OP], a significant change of the steps involved in secretory pathways, like the increase of cytoplasmic-free calcium, can be observed within less than a minute (Pandol et al., 1985; Toescu et al., 1992; Yule & Williams, 1992; Ngezahayo & Kolb, 1993) down to 15 sec (Hootman et al., 1985). Furthermore, the stimulated amylase secretion occurs in less than 5 min (Peikin et al., 1978; Barlas et al., 1982), whereas the cells are still well coupled (*see* Figs. 1*a* and 2*b*).

Despite the different size of cell aggregates used for measurement of amylase release and for double whole-cell experiments, it is striking that maximal amylase release (Jensen et al., 1989) and minimal latency of uncoupling seem to occur at similar [CCK-OP]. This close parallelism suggests that amylase release and uncoupling are causally related, but not by an all-or-none relationship. Therefore it is tempting to suggest that at submaximal concentrations of [CCK-OP]—occupancy of the high affinity receptors—gap junctional uncoupling potentiates secretion. This would be in line with the observation that heptanol-induced uncoupling increases basal amylase release (Meda et al., 1987; Chanson et al., 1989*b*). However, at supramaximal [CCK-OP], where uncoupling has a longer latency but faster rate (Fig. 2), amylase release is inhibited (Jensen et al., 1989). At this latency range, then, uncoupling correlates with inhibition of secretion. It seems likely that at this unphysiological range of [CCK-OP]—with binding to the low affinity receptor(s)—the corresponding biochemical pathways (which are not yet known in detail) become overstimulated (Matozaki et al., 1990) and inhibit both secretion (Pandol & Schoeffield, 1986) and gap junctional coupling, as well as their interaction. Although the presented observations provide support for the hypothesis that uncoupling occurs after the onset of secretion and terminates the secretory process, the available evidence is still circumstantial. Simultaneous measurements of time-resolved secretion (Chow et al., 1992), electrical coupling and cytoplasmic Ca^{2+} (Ngezahayo & Kolb, 1993) will help to clarify an inhibitory and/or stimulatory role of coupling in the stimulus-secretion relationship as well as shed light on the unknown molecular pathway linking the stimulus-secretion and stimulus-gap junctional uncoupling in pancreatic acinar cells.

The authors would like to thank Dipl. Biol. F. Mendez for his support in software development for analysis of gap junctional conductance. The work was supported by the Graduiertenkolleg Biochemische Pharmakologie, the Herrmann und Lilly Schilling Stiftung and the Sonderforschungsbereich 156 of the Deutsche Forschungsgemeinschaft.

References

Asaoka, Y., Yoshida, K., Oka, M., Shinomura, T., Koide, H., Ogita, K., Kikkawa, U., Nishizuka, Y. 1992. The family of protein kinase C in transmembrane signaling for cellular regulation. *J. Nutr. Sci. Vitaminol.* **Spec No.:** 7–12

Ashkenazi, A., Peralta, E.G., Winslow, J.W., Ramachandran, J., Capon, D.J. 1989. Functionally distinct G proteins selectively couple different receptors to PI hydrolysis in the same cell. *Cell* **56:**487–493

Barlas, N., Jensen, R.T., Gardner, J.D. 1982. Cholecystokinin-induced restricted stimulation of pancreatic enzyme secretion. *Am. J. Physiol.* **242:**G464–G469

Barrowman, M.M., Cockroft, S., Gomperts, B.D. 1986. Two roles for guanine nucleotides in the stimulus-secretion sequence of neutrophils. *Nature* **319:**504–507

Berridge, M.J. 1987. Inositol triphosphate and diacylglycerol: two interacting second messengers. *Annu. Rev. Biochem.* **56:**159–193

Berridge, M.J., Irvine, R.F. 1989. Inositol phosphates and cell signaling. *Nature* **341:**197–205

Burnham, D.B., Sung, C.K., Munowitz, P., Williams, J.A. 1988. Regulation of protein phosphorylation in pancreatic acini by cyclic AMP-mediated secretagogues: interaction with carbamylcholine. *Biochim. Biophys. Acta* **969:**33–39

Chanson, M., Bruzzone, R., Bosco, D., Meda, P. 1989*b*. Effects of n-alcohols on junctional coupling and amylase secretion of pancreatic acinar cells. *J. Cell. Physiol.* **139:**147–156

Chanson, M., Bruzzone, R., Spray, D.C., Regazzi, R., Meda, P. 1988. Cell uncoupling and protein kinase C: correlation in a cell line but not in a differentiated tissue. *Am. J. Physiol.* **255:**C699–C704

Chanson, M., Meda, P., Bruzzone, R. 1989*a*. Increase in pancreatic secretion during uncoupling:evidence for a protein kinase C-independent effect. *Exp. Cell Res.* **182:**349–357

Chow, R. H., von Rüden, L., Neher, E. 1992. Delay in vesicle fusion revealed by electrochemical monitoring of single secretory events in adrenal chromaffin cell. *Nature* **356:**60–63

Gardner, J.D., Jackson, M.J. 1977. Regulation of amylase release from dispersed pancreatic acinar cells. *J. Physiol.* **270:**439–454

Gardner, J.D., Jensen, R.T. 1981. Regulation of pancreatic exocrine

secretion in vitro: the action of secretagogues. *Philos. Trans. R. Soc. Lond.* **296:**17–26

Hootman, S.R., Ochs, D.L., Williams, J.A. 1985. Intracellular mediators of Na^{+}-K^{+} pump activity in guinea pig pancreatic acinar cells. *Am. J. Physiol.* **249:**G470–G478

Iwatsuki, N., Petersen, O.H. 1978. Pancreatic acinar cells: acetylcholine-evoked electrical uncoupling and its ion dependency. *J. Physiol.* **274:**81–96

Jensen, R.T., Wank, S.A., Rowley, W.H., Sato, S., Gardner, J.D. 1989. Interaction of CCK with pancreatic acinar cells. *Trends Pharmacol. Sci.* **10:**418–423

Kolb, H.-A. 1992. Double whole cell patch clamp technique. *In:* Practical Electrophysiological Methods: A Guide for In Vitro Studies in Vertebrate Neurobiology. H. Kettenmann, R. Grantyn, Editors. pp. 289–298. John Wiley & Sons, New York

Kolb, H. A., Somogyi, R. 1991. Biochemical and biophysical analysis of cell-to-cell channels and regulation of gap junctional permeability. *Rev. Physiol. Biochem. Pharmacol.* **118:**2–47

Lamb, T.D., Pugh, E.H. 1992. G-protein: gain and kinetics. *Trends in Neurosci.* **15:**291–298

Loewenstein, W.R. 1985. Regulation of cell-to-cell communication by phosphorylation. *Biochem. Soc. Symp. Lond.* **50:**43–58

Matozaki, T., Göke, B., Tsunoda, Y., Rodriguez, M., Martinez, J., Williams, J.A. 1990. Two functionally distinct cholecystokinin receptors show different modes of action on Ca^{2+}-mobilization and phospholipid hydrolysis in isolated rat pancreatic acini. *J. Biol. Chem.* **265:**6247–6254

Matozaki, T., Sakamoto, C., Nagao, M., Nishizaki, H., Baba, S. 1988. G protein in stimulation of Pl hydrolysis by CCK in isolated rat pancreatic acinar cells. *Am. J. Physiol.* **255:**E652–E659

Matozaki, T., Williams, J.A. 1989. Multiple sources of 1,2-diacylglycerol in isolated rat pancreatic acini stimulated by cholecystokinin. *J. Biol. Chem.* **264:**14729–14734

Meda, P., Bruzzone, R., Chanson, M., Bosco, D., Orci, L. 1987. Gap junctional coupling modulates secretion of exocrine pancreas. *Proc. Natl. Acad. Sci. USA* **84:**4901–4904

Merritt, J.E., Taylor, C.W., Rubin, R.P., Putney, J.W., Jr. 1986. Evidence suggesting that a novel guanine nucleotide regulatory protein couples receptors to phospholipase C in exocrine pancreas. *Biochem. J.* **236:**337–343

Nakanishi, H., Ohyanagi, H., Takeyama, Y., Onoyama, H., Saitoh, Y., Kikuchi, A., Takai, Y. 1988. Mode of inhibitory action of cholecystokinin in amylase release from isolated rat pancreatic acini—inhibition of secretory process post to protein kinase C-calcium ion systems. *Biochem. Biophys. Res. Commun.* **154:**1314–1322

Neyton, J., Trautmann, A. 1985. Single-channel currents of intracellular junction. *Nature* **317:**331–335

Ngezahayo, A., Kolb, H.-A. 1993. Gap junctional conductance tunes phase difference of cholecystokinin evoked calcium oscillations in pairs of pancreatic acinar cells. *Pfluegers Arch.* **422:** 413–415

Nishizuka, Y. 1988. The molecular heterogeneity of protein kinase C and its implications for cellular regulation. *Nature* **334:**661–665

Oberdisse, E., Lapetina, E.G. 1987. GDPβS enhances the activation of phospholipase C caused by thrombin in human platelets: evidence for involvement of an inhibitory GTP-binding protein. *Biochem. Biophys. Res. Commun.* **144:**1188–1196

Ohatsuki, K., Ikeuchi, T., Yokoyama, M. 1986. Characterization of nucleoside-diphosphate kinase-associated guanine nucleotide-binding proteins from HeLa S3 cells. *Biochim. Biophys. Acta* **882:**322–330

Pandol, S.J., Schoeffield, M.S. 1986. 1,2 diacylglycerol, protein kinase C, and pancreatic enzyme secretion. *J. Biol. Chem.* **261:** 4438–4444

Pandol, S.J., Thomas, M.W., Schoeffield, M.D., Sacks, G., Shmeul, M. 1985. Role of free calcium in secretagogue-stimulated amylase release from dispersed acini from guinea pig pancreas. *J. Biol. Chem.* **260:**10081–1086

Peikin, S.R., Rottman, A.J., Batzri, S., Gardner, J.D. 1978. Kinetics of amylase release by dispersed acini prepared from guinea pig pancreas. *Am. J. Physiol.* **235:**E743–E749

Piiper, A., Plusczk, T., Echhardt, L., Schulz, I. 1991*a*. Effects of cholecystokinin, cholecystokinin JMV-180 and GTP analogs on enzyme secretion from permeabilized acini and chloride conductance in isolated zymogen granules of rat pancreas. *Eur. J. Biochem.* **197:**391–398

Piiper, A., Pröfrock, A., Schulz, I. 1991*b*. Effects of epidermal growth factor and calcium omission on cholecystokinin-stimulated Cl^- conductance in rat pancreatic zymogen granules. *Biochem. Biophys. Res. Commun.* **181:**827–832

Putney, J.W., Burgess, G.M., Halenda, S.P., McKinney, J.S., Rubin, R.P. 1983. Effects of secretagogues on [^{32}P]phosphatidylinositol 4,5-bisphosphate metabolism in the exocrine pancreas. *Biochem. J.* **212:**483–488

Saez, J.C., Spray, D.C., Nairn, A.C., Hertzberg, E., Greengard, P., Bennett, M.V.L. 1986. cAMP increases junctional conductance and stimulates phosphorylation of the 27-kD principal gap junction polypeptide. *Proc. Natl. Acad. Sci. USA* **83:**2473–2477

Sato, S., Stark, H.A., Martinez, J., Beaven, M.A., Jensen, R.T., Gardner, J.D. 1989. Receptor occupation, calcium mobilization. and amylase release in pancreatic acini: effect of CCK-JMV-180. *Am. J. Physiol.* **257:**G202–G209

Schnefel, S., Banfic, H., Eckhardt, L., Schultz, G., Schulz, I. 1988. Acetylcholine and cholecystokinin receptors functionally couple by different G-proteins to phospholipase C pancreatic acinar cells. *FEBS Lett.* **230:**125–130

Schnefel, S., Pröfrock, A., Hinsch, K.D., Schulz, I. 1990. Cholecystokinin activates G_i1-, G_i2-, G_i3- and several G_s-proteins in rat pancreatic acinar cells. *Biochem. J.* **269:**483–488

Schwiebert, E.M., Light, D.R., Fejes-Toth, G., Naray-Fejes-Toth, A., Stanton, B.A. 1990. A GTP-binding protein activates chloride channels in a renal epithelium. *J. Biol. Chem.* **265:**7725–7728

Seifert, R., Rosenthal, W., Schultz, G., Wieland, T., Gierschick, P., Jakobs, K.H. 1988. The role of nucleoside-diphosphate kinase reactions in G protein activation of NADPH oxidase by guanine and adenine nucleotides. *Eur. J. Biochem.* **175:**51–55

Somogyi, R., Batzer, A., Kolb, H.-A. 1989. Inhibition of electrical coupling in pairs of murine pancreatic acinar cells by OAG and isolated protein kinase. C. *J. Membrane Biol.* **108:**273–282

Somogyi, R., Kolb, H.-A. 1988. Cell-to-cell channel conductance during loss of gap junctional conductance in pairs of pancreatic acinar cells and chinese hamster ovary cells. *Pfluegers Arch.* **412:**54–65

Somogyi, R., Kolb, H.-A. 1989. A G-protein mediates secretagogue-induced gap junctional channel closure in pancreatic acinar cells. *FEBS Lett.* **258:**216–218

Streb, H., Bayerdörffer, E., Haase, W., Irvine, R.F., Schulz, I. 1984. Effect of inositol,4,5-triphosphate on isolated subcellular fractions of rat pancreas. *J. Membrane Biol.* **81:**241–253

Toescu, E.C., Lawrie, A.M., Petersen, O.H., Gallacher, D.V. 1992. Spatial and temporal distribution of agonist-evoked cytoplasmic Ca^{2+} signals in exocrine cells analysed by digital image microscopy. *EMBO J.* **11:**1623–1629

Waelbroeck, M., Robberecht, P., Coy, D.H., Camus, J.C., De Neef, P., Christophe, J. 1985. Interaction of growth hormone-releasing factor (GRF) and 14 GRF analogs with vasoactive intestinal peptide (VIP) receptors of rat pancreas. Discovery of(N-AC-Tyrl, D-Phe2)-GRF(1–29)-NH2 as a VIP antagonist. *Endocrinology* **116:**2643–2649

Walsh, J.W. 1987. *In:* Physiology of the Gastrointestinal tract (L.R. Johnson, editor. Vol. 1, pp. 195–206. Raven, New York

Wank, S.A., Jensen, R.T., Gardner, J.D. 1988. Kinetics of binding of cholecystokinin to pancreatic acini. *Am. J. Physiol.* **255:**G106–G112

Wooten, M.W., Wrenn, R.W. 1988. Linoleic acid is a potent activator of protein kinase C type III-α isoform in pancreatic acinar cells; its role in amylase secretion. *Biochem. Biophys. Res. Commun.* **153:**67–73

Wrenn, R.W., Wooten, M.W. 1984. Dual calcium-dependent protein phosphorylation system in pancreas and their differential regulation by polymyxin B1. *Life Sci.* **35:**267–276

Yu, D.H., Huang, S.C., Wank, S.A., Mantey, S., Gardner, J.D., Jensen, R.T. 1990. Pancreatic receptors for cholecystokinin: evidence for three receptor classes. *Am. J. Physiol.* **258:**G80–G85

Yule, D.I., Williams, J.A. 1992. U73122 inhibits Ca^{2+} oscillations in response to cholecystokinin and carbachol but not to JMV-180 in rat pancreatic acinar cells. *J. Biol. Chem.* **267:**13830–13835