Journal of Physiology (1989), **417**, *pp.* 343–359
With 7 text-figures
Printed in Great Britain

ACTIVATION AND DESENSITIZATION MECHANISMS OF MUSCARINIC CURRENT RESPONSE IN SINGLE PANCREATIC ACINAR CELLS OF RATS

BY YOSHIO MARUYAMA

From the Department of Physiology, Jichi Medical School, Minamikawachi-machi, Tochigi-ken, 329-04 Japan

(*Received 27 February 1989*)

SUMMARY

1. In single, enzymatically dissociated, rat pancreatic acinar cells both ACh stimulation and IP_3 (inositol 1,4,5-trisphosphate) injection can evoke Ca^+-dependent transient current responses. However, exogenously applied IP_3 (10 μM) gradually loses its ability to induce the Ca^{2+}-dependent response (an increase in $[Ca^{2+}]_i$) during cell incubation with a saline solution.

2. Administration of IP_4 (inositol 1,3,4,5-tetrakisphosphate, 10 μM) together with the IP_3 (the injection of IP_3–IP_4 mixture) allows partial recovery of the response, but not full replication of the response induced by ACh (0·2 μM). Injection of IP_4 alone never induces the current response.

3. The sensitivity of IP_3 recovers after short-term administration of ACh (0·2 μM), and in turn, the ACh-induced response is augmented by the presence of internal IP_3. These results suggest that a synergism between IP_3 and another ACh-induced substance plays an important role in muscarinic Ca^{2+} signalling.

4. ACh-induced responses are inhibited by pre-incubation (10 min) with an activator of protein kinase C, TPA {12-*O*-tetradecanoylphorbol-13-acetate, 16 nM), or augmented by pre-incubation (10 min) with an inhibitor, H-7 (1-(5-isoquinolinesulphonyl)-2-methylpiperazine, 10 μM), whilst IP_3-induced responses are unaffected by that with both agents. These results indicate that protein kinase C acts negatively on the signalling elements prior to the formation of IP_3.

5. The oscillatory responses, induced by cell dialysis with a nominally Ca^{2+}-free (*ca* 1–10 μM) solution containing GTPγS (100 μM), are unaffected by the pre-treatment with TPA or H-7. In addition, these responses and/or those triggered by short-term stimulation with ACh and internal GTPγS are not influenced by external ACh. On the other hand, the oscillatory responses recorded in acinar cells pre-treated with H-7 are tightly controlled by external ACh.

6. Taken together these results suggest that activation of protein kinase C does not affect the activity of GTP-binding protein, but disconnects the link between the muscarinic ACh receptor and GTP-binding protein, or inhibits ACh binding to the receptor, in rat pancreatic acinar cells.

INTRODUCTION

Activation of muscarinic acetylcholine (ACh) receptors leads to generation of two cellular messengers, inositol 1,4,5-trisphosphate (IP_3) and diacylglycerol (DG). The former releases Ca^{2+} from the intracellular storage site (reviewed by Berridge & Irvine, 1984), while the latter activates protein kinase C (PkC; reviewed by Nishizuka, 1984). Both of these messengers are the products of phosphatydylinositol 4,5-bisphosphate cleavage by phospholipase C (PLC) under the regulation of a type of guanine nucleotide-binding protein (G protein) which couples the receptor to PLC (reviewed by Putney, 1987). There is compelling evidence demonstrating that these signalling elements are involved in ACh receptor stimulation in the acinar cells of exocrine pancreas (Streb, Heslop, Irvine, Schulz & Berridge, 1985; Merritt, Taylor, Rubin & Putney, 1986*a*; Maruyama, 1988). The present paper describes some details of interacting mechanisms amongst the signalling elements in ACh-stimulated rat pancreatic acinar cells. Some of these elements are modified by known activators or inhibitors such as guanosine 5′-[γ-thio]triphosphate (GTPγS, an activator of G protein), 12-*O*-tetradecanoylphorbol-13-acetate (TPA, an activator of PkC; Nishizuka, 1984), 1-(5-isoquinolinesulphonyl)-2-methylpiperazine (H-7, an inhibitor of PkC; Kawamoto & Hidaka, 1984), IP_3 (an inducer of internal Ca^{2+}; Streb, Irvine, Berridge & Schulz, 1984).

Recent evidence suggests that not only IP_3 but also IP_4 is involved in mediating the rise in $[Ca^{2+}]_i$ in a variety of cells (sea urchin eggs: Irvine & Moor, 1986; neuroblastoma cells: Higashida & Brown, 1986; vascular smooth muscle cells: Ohya, Terada, Yamaguchi, Inoue, Okabe, Kitamura, Hirata & Kuriyama, 1988) including exocrine gland acinar cells (lacrimal gland: Morris, Gallacher, Irvine & Petersen, 1987). The question arises whether these inositol phosphates are also effective in pancreatic acinar cells and whether they can replicate ACh-induced increase in $[Ca^{2+}]_i$. This point will be dealt with in the first part of the present paper, in which the patch-clamp whole-cell technique is used to inject inositol phosphates (IP_3 and IP_4) into the cells and monitor Ca^{2+}-dependent membrane currents.

The action of PkC on the muscarinic ACh receptor signalling seems not to be generally present amongst exocrine gland acinar cells. Recently Gray (1988) has shown, using Fura-2 to measure $[Ca^{2+}]_i$ in single parotid acinar cells, that phorbol esters do not affect the response induced by carbachol. On the other hand, in single lacrimal gland acinar cells, Llano & Marty (1987) have shown, using the patch-clamp whole-cell technique, that phorbol esters abolish the response to ACh, and they suggest that PkC phosphorylates the G protein or PLC, disconnecting the signal transduction between them, thus exerting an inhibitory action on the ACh-evoked Ca^{2+} mobilization. In contrast, the evidence described in the present paper indicates that PkC uncouples the ACh receptor–G protein link (presumably phosphorylating the surface receptor) in single pancreatic acinar cells. This point will be described in the second part of the paper.

METHODS

The experimental procedures were similar to those described previously (Maruyama, 1988). Briefly, male Sprague–Dawley rats (150–200 g) were stunned and killed by cervical dislocation.

Single acinar cells were freshly isolated from the pancreas using serial treatment with enzymes, and stored in normal experimental solution at room temperature. Tight-seal whole-cell current recordings were carried out at room temperature (22 °C) on cells immersed in external solution containing (in mM): NaCl, 140; KCl, 4·7; $MgCl_2$, 1·13; $CaCl_2$, 2·5; HEPES–NaOH buffer, 5 (pH 7·2). Unless otherwise indicated, the pipette solution contained (in mM): potassium glutamate, 145; $MgCl_2$, 1·13; EGTA–KOH, 0·07; HEPES–NaOH buffer, 5 (pH 7·2). The EGTA concentration in this solution was chosen to maintain $[Ca^{2+}]_i$ at a low level ($\sim$ 10 nM) in resting cells, while allowing $[Ca^{2+}]_i$ to increase during agonist stimulation (0·2 μM-ACh; IP_3 injection). The cell response to these agonists was abolished by dialysing the cell with pipette solution containing a high dose of EGTA (1 mM). For the cell stimulation, ACh (0·2 μM) dissolved in the external solution was delivered from a nearby pipette (ACh application pipette; positioned within 10 μm of the cell) to the cell using weak continuous positive pressure, or in other experiments (protocols relating to Figs 1 and 2) the cell–pipette assembly was rapidly exposed to the external solution containing ACh (0·2 μM) using a manipulating device (see, Fig. 1*B*; ACh jump).

Pancreatic acinar cells from rats possesses two types of Ca^{2+}-dependent ion channels (Maruyama, 1988). One is selective for monovalent cations (Na^+ and K^+; Maruyama & Petersen, 1982) and the other for Cl^- (Randriamampita, Chanson & Trautmann, 1988), and both are poorly dependent on membrane potential between −60 and 60 mV. Increases and decreases in $[Ca^{2+}]_i$ should thus be reflected by the activity of these channels, and in turn, the channel activities (membrane current) can be used as a biological probe for monitoring $[Ca^{2+}]_i$. The holding potential in the whole-cell current measurement was set at 40 mV throughout the present experiments, at which setting the resultant agonist-induced current was outward under the ion gradients present across the membrane. Applying the Ca^{2+} ionophore A23187 (0·1 μM) induced sustained current responses, indicating that ion channels were not inactivated when $[Ca^{2+}]_i$ was maintained at a high level, presumably of the order of 10^{-6} M (Maruyama, 1988).

Cell responses to intracellular injection of IP_3 gradually diminished when cell incubation with the external solution was prolonged (the details will be described in Results). In contrast, the ACh-induced response was intact after up to 5 h incubation, but most of the experiments with ACh stimulation were carried out within 1 h of cell dispersion.

Materials

Acetylcholine-chloride (ACh) was obtained from Sigma (St Louis, MO, USA). GTPγS and A23187 were purchased from Boehringer. H-7 was purchased from Seikagaku Kogyo (Tokyo). D-*myo*-inositol 1,4,5-trisphosphate (IP_3) was purchased from Sigma (prepared by alkaline hydrolysis of bovine brain phosphoinositides, purity approximately 98%). Synthetic D-*myo*-inositol 1,3,4,5-tetrakisphosphate (IP_4) was kindly donated by Dr Ozaki (Ehime University; Ozaki, Kondo, Nakahira, Yamaoka & Watanabe, 1987).

TPA and 4-α-phorbol 12,13-didecanoate were purchased from Sigma. 1-Oleoyl-2-acetyl-glycerol (OAG) was purchased from Nakarai Chemicals (Kyoto). These substances were dissolved in ethanol, and diluted in the experimental solution to a final concentration in which the ethanol was less than 0·01%. Incubation (or pre-treatment) of cells with these agents was performed at 37 °C for 5–10 min for the phorbol esters and H-7, and for 10–40 min for OAG. Unless otherwise stated, the substances were eliminated from the bathing medium by washing the cells several times with the normal external solution before electrical recording.

RESULTS

Responses to the ACh concentration jump

Acetylcholine stimulation induced Ca^{2+}-dependent channel currents in single pancreatic acinar cells, as previously described (Maruyama, 1988). Rapid exposure of the cell to the solution containing ACh (Fig. 1*A*, ACh jump) provides the precise time course of ACh-evoked current. First of all the ACh-induced response was defined in terms of electrical current measurement. Upon stimulation with 0·2 μM-ACh by ACh jump (the first ACh stimulation lasting for 30–40 s), the response abruptly

developed to a peak after a latency of 200–800 ms (470 ± 194 ms, mean ± S.D., $n = 20$; these responses were measured after complete exposure to the ACh-containing external solution labelled 'E'; see trace I_1 in Fig. 1 and legend), with (15/20 experiments) or without a subsequent second peak (5/20 experiments), and decayed to the original level within 30 s (23·4 ± 6·2 s, $n = 20$, from ten animals). The relative size of the first and second peak (if present) varied from cell to cell (mean highest peak amplitude, 461 ± 91 pA; $n = 20$). Prolonged ACh stimulation (120 s) did not induce any subsequent responses or oscillations (10/10 experiments). The initial latency, which was of the same order as reported previously with acinar voltage measurements using glass microelectrodes (Nishiyama & Petersen, 1975), can be interpreted as the time taken for $[Ca^{2+}]_i$ to increase to the effective level. The decay of the current response may be due to recovery of $[Ca^{2+}]_i$ to its original level which should be of the order of 10 nM under present dialysing conditions (70 μM-EGTA and nominally zero Ca^{2+} in the dialysing solution). A second ACh stimulation in the same cell (after the first ACh stimulation and a subsequent incubation with the control solution for 0·5–10 min), induced a smaller response with a longer latency (8·1 ± 2·5 s, $n = 14$) and a decreased rate of rise (Fig. 1, trace I_2), suggesting that a desensitizing mechanism is present in the Ca^{2+}-mobilizing receptor signalling. A third ACh stimulation, similarly repeated, induced no response (8/8 experiments).

Effect of removal of external Ca^{2+} on the response induced by ACh or IP_3

In the next set of experiments, the ACh-jump protocol was carried out in the absence of external Ca^{2+}. Figure 2*A* shows a typical ACh-induced response recorded from a cell that had been exposed to the normal Ca^{2+}-containing solution until the ACh jump was performed with a Ca^{2+}-free (no added Ca^{2+} and 0·2 mM-EGTA) solution. The ACh jump (0·2 μM) induced outward current responses (10/10 experiments, Fig. 2*A*) with a latency of 250–700 ms (420 ± 130 ms, $n = 10$), the value of which was similar to that recorded in the Ca^{2+}-containing solution. In addition, the size of the highest peak did not depend on the presence or absence of external Ca^{2+} (449 ± 59 pA, $n = 10$, in the Ca^{2+}-free condition). It is thus likely that an early part (at least the rising phase) of the response depends mainly on the release of Ca^{2+} from the internal storage pool.

When acinar cells were incubated with the Ca^{2+}-free solution for 20–90 min and the ACh-jump experiment was performed using the same Ca^{2+}-free solution (Fig. 2*B*), it was observed that a smaller response (78 ± 17 pA, $n = 8$) with a longer latency (6·8 ± 2·9 s, $n = 8$) and a decreased rate of rise, resembling that evoked by the repeat ACh stimulation shown by trace I_2 in Fig. 1, occurred (8/8 experiments). The result can be interpreted as the loss of internal Ca^{2+} from the storage site during the cell incubation period in Ca^{2+}-free external medium. When the Ca^{2+}-free incubation period was less than 5 min, the ACh-induced response resembled that shown in Fig. 2*A* (continuous curve).

IP_3 has been reported to release Ca^{2+} from internal storage sites and induce an increase in $[Ca^{2+}]_i$ in acinar cells of rat exocrine pancreas (Streb *et al.* 1984). As a result of this observation, intracellular injection of IP_3 (cell dialysis with internal solution containing IP_3) was tested in terms of the electrical activities of single acinar cells. Experiments were carried out in cells immersed in the Ca^{2+}-free solution (for

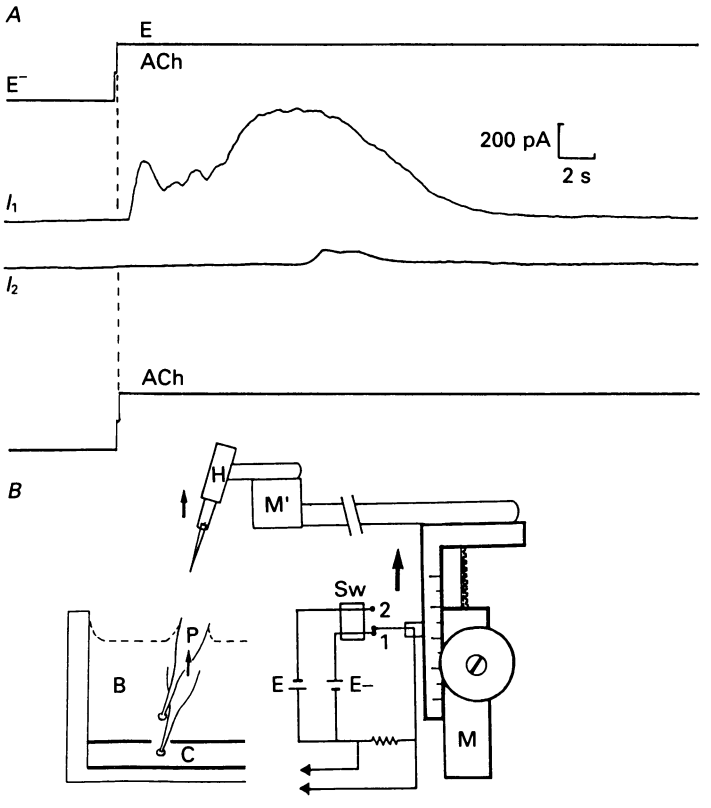

Fig. 1. *A*, time course of ACh-induced outward current response in dialysed single acinar cells of rat exocrine pancreas. The cell was exposed to normal external solution containing 0·2 μM-ACh from that containing no ACh within 100 ms (E− to E, ACh jump). The holding potential was 40 mV, and the ACh-induced outward current was recorded. Traces I_1 and I_2 are the responses evoked by the first and second ACh jumps, respectively. The first and second exposures to ACh-containing solution lasted 34 s with an interruption of 40 s in the control solution. The biological latency of ACh jump was 600 ms in trace I_1, and 11 s in trace I_2. *B* represents the device and procedure used for obtaining ACh jump: M, a manipulator coupled to a switch (Sw); M′, a hydraulic manipulator; H, a head stage of amplifier; P, a patch-pipette; B, an experimental bath (2 ml) having an inlet and outlet for solution exchange; C, a small chamber (70 μl) with a pore of 30 μm in diameter, fabricated from a thin glass pipette, and placed in the bottom of B; Sw, a mechanical switch (having a distance of 1 mm from 'off' to 'on' position), coupled to the up-and-down movement of M (Sw turned on when the tip of the pipette-cell assembly was raised by 1 mm); E and E−, two batteries of opposite polarity. Before the experiment, B and C were filled with the normal external solution. After the establishment of whole-cell recording mode, the tip of a pipette–cell assembly was inserted into C by 200 μm through the pore. The solution in B could be quickly replaced with that containing 0·2 μM-ACh without disturbing the whole-cell mode. After the solution replacement, the tip of the pipette-cell assembly was rapidly withdrawn from C by 1 mm, by hand using M, so that the cell was completely exposed to the bathing solution in B (ACh-containing solution) within 100 ms. At the same instant, the withdrawal turned Sw on, thus giving an indication of exposure (from E− to E). The up-and-down movement of the pipette–cell assembly reduced the access conductance of the whole-cell recording from 66 to 48 nS (66·2 ± 11 to 48·6 ± 6·4 nS, mean ± S.D., $n = 11$). The diffusion of ACh from B into C through the small pore (30 μm) of C, during and 40 s after the solution exchange, was negligible since no current development was observed during the periods when the tip of the assembly was placed in C. Repeating the same procedure, it was possible to rapidly re-immerse the cell several times in the test and/or control solutions placed in B.

less than 8 min) to minimize a possible contribution from external Ca^{2+}. Upon establishment of the whole-cell mode, the cell exhibited a marked current response (8/8 experiments; peak amplitude, 790 ± 140 pA; Fig. 2*C*) after a latency of 0·8–2·8 s ($1{\cdot}5 \pm 0{\cdot}7$ s, $n = 8$) which was presumably the lag due to diffusion of IP_3 into the cell.

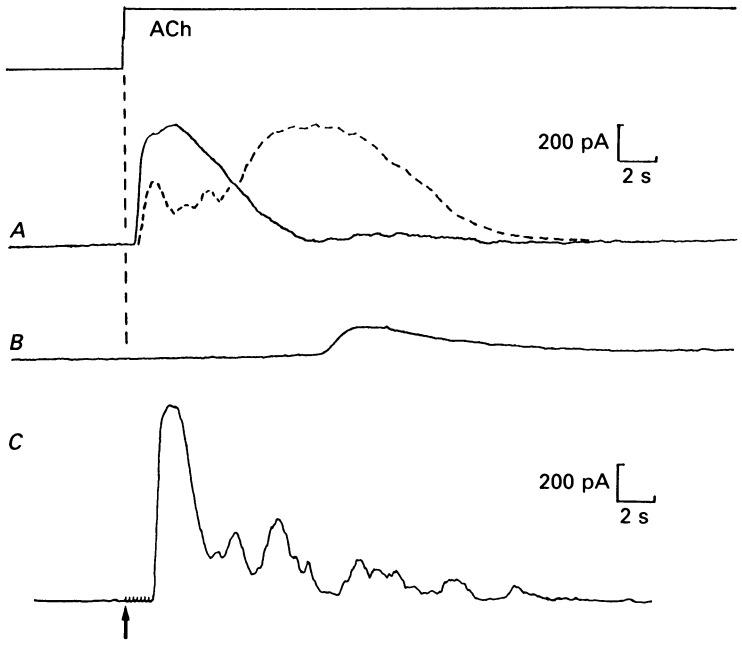

Fig. 2. Current responses induced by ACh jumps and IP_3 injection in Ca^{2+}-free external conditions. Cells were voltage clamped at 40 mV, and the outward current was recorded. *A*, the pipette–cell assembly was rapidly moved from the normal Ca^{2+}-containing solution to Ca^{2+}-free solution (no added Ca^{2+} and 0·2 mM-EGTA) containing 0·2 μM-ACh (continuous trace). The dashed-line trace shows the ACh-induced response recorded in the normal Ca^{2+}-containing solution (shown in Fig. 1, trace I_1), superimposed for comparison. These two traces belong to different cells. *B*, the ACh-induced response recorded in a cell incubated with Ca^{2+}-free solution for 30 min prior to 0·2 μM-ACh jump. *C*, the response induced by cell dialysis with internal solution containing 10 μM-IP_3. Repetitive 10 mV, 100 ms square pulses were applied at intervals of 200 ms. This voltage protocol was interrupted after the initial rapid rising phase was evoked. The whole-cell mode was established at a point indicated by the arrow, which resulted in increases in capacitative surge.

The response reached a peak and then declined to the pre-injection level (transient response) with or without small damped oscillatory waves. These results indicate that both ACh and IP_3 stimulation induce release of Ca^{2+} from internal storage sites. This is consistent with conclusions from a previous study (Streb *et al.* 1984).

Responses induced by internal IP_3 and IP_4 and their comparison with ACh stimulation

Intracellular dialysis with IP_3 (4–10 μM)-containing solution induced a transient current response, which depended on the cell incubation time in external solution prior to the establishment of the whole-cell mode. Figure 3 shows a typical diagram

of the peak amplitude of current responses, induced by injection of IP_3 (10 μM; ● and inset trace *a*), IP_4 (10 μM, △ and inset trace *c*), a mixture of these (10 μM of each, ○ and inset trace *b*), or by ACh stimulation (0·2 μM, + and inset trace *d*), plotted against the incubation time. Each response was recorded from the same batch of cells. In the experiments, single cells immersed in the normal external solution under the inverted microscope were selected randomly and investigated by whole-cell dialysis one after another at intervals of 2–4 min. One set of experiments using a batch of cells usually lasted for 4–5 h, and the bath solution was replaced every 15 min in order to minimize the effect of evaporation. The freshly dispersed cells used in the present experiments showed IP_3-dependent current responses (with a latency of IP_3 injection of $1·6 \pm 0·7$ s, $n = 72$) only within the first 2 h of incubation. The IP_3-induced response was essentially transient and its duration became shorter with longer incubation time.

The amplitude of the IP_3-induced response had a clear decreasing trend when plotted against the length of cell pre-incubation although individual responses varied considerably from cell to cell. After 2 h incubation, IP_3 injection finally caused no response (Fig. 3, ●). The amplitude of the IP_3-induced response was 503 pA (503 ± 264 pA, $n = 72$) during the first 2 h incubation period, and it was 12·9 pA ($12·9 \pm 31$ pA, $n = 39$) during the subsequent (second) 2 h incubation period (data were collected from seven different batches of cells). The injection of a mixture of IP_3 and IP_4 (IP_3–IP_4 injection) could induce responses (transient response) after the IP_3 injection became ineffective (i.e. during the second 2 h incubation period; Fig. 3, ○). These responses also gradually declined, and finally disappeared after 4–5 h incubation. The amplitude of the IP_3–IP_4-induced response was 589 pA (589 ± 262 pA, $n = 25$) during the first 2 h incubation, and it was 215 pA (215 ± 175 pA, $n = 16$) during the second 2 h incubation period (data were collected from four different batches of cells). IP_4 injection never caused responses throughout the experiment (Fig. 3, △, $n = 21$). The most striking finding from this experiment was that ACh (0·2 μM) could induce a current response (peak amplitude, 433 ± 63 pA; $n = 14$) at a time when the IP_3 injection had become ineffective (during the second 2 h incubation; Fig. 3, +). ACh could also induce similar sizes of responses after the IP_3–IP_4 injection became ineffective (after 4–5 h incubation, 5/5 experiments).

Thus, the discrepancy between ACh-induced responses and the exogenous phosphoinositide(s)-induced responses became more prominent as the cell incubation time was extended.

Co-operative action of exogenously applied IP_3 and ACh stimulation

In the next series of experiments, possible synergistic effects of ACh and IP_3 were studied in acinar cells which had lost sensitivity to IP_3 injection. The cells were incubated for over 2·5 h, and it was confirmed that exogenously applied IP_3 (10 μM) caused no response in several cells randomly selected under the microscope prior to the experiment. Figure 4*A* shows a typical current trace obtained from such cells. The injection of IP_3 caused no response, but subsequently applied ACh (0·2 μM, from a nearby pipette) induced a marked response composed of several oscillatory waves (19/19 experiments; highest peak amplitude, 1047 ± 180 pA). ACh alone did not induce oscillatory waves but induced transient responses (similar to that shown in

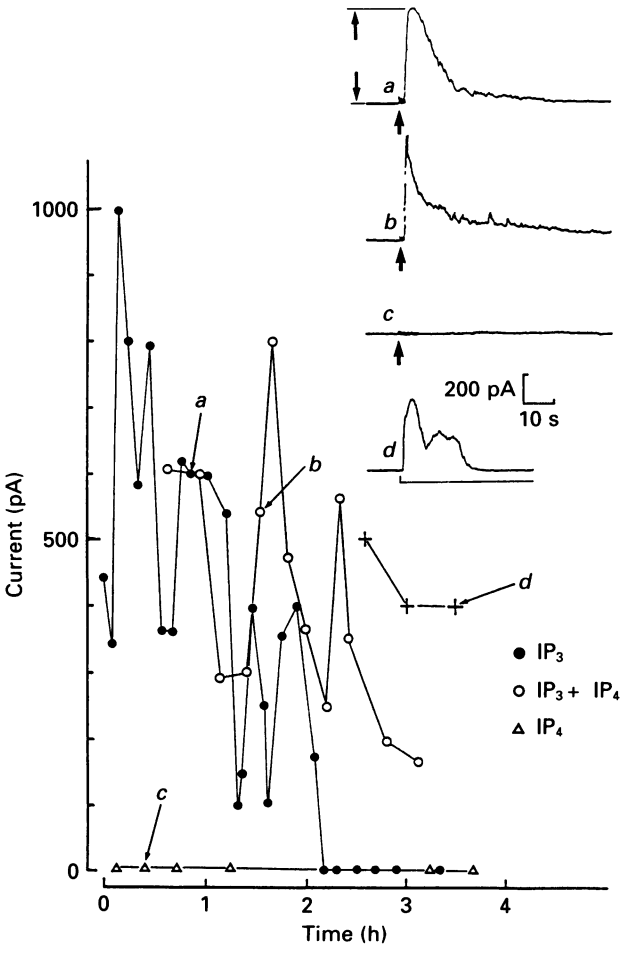

Fig. 3. Polyphosphoinositide-induced current responses in the single dialysed rat pancreatic acinar cells. The peak amplitude of the current responses was plotted against the cell incubation time. The cells were stimulated by either cell dialysis with internal solution containing IP_3 (10 μM, ●, *a*), IP_4 (10 μM, △, *c*), or a mixture of these (10 μM-IP_3 + 10 μM-IP_4, ○, *b*), or by external stimulation with ACh (0·2 μM, +, *d*). Single arrows in inset traces *a*, *b* and *c* stand for the time point when the whole-cell dialysis started, and the bar under trace *d* the period of ACh stimulation. *I* in inset trace *a* shows the peak amplitude of the current response. Data were taken from the same batch of cells.

Fig. 3, inset trace *d*) in these cells (14/14 experiments; peak amplitude, 433 ± 63 pA). In separate experiments on the same batch of cells, an increase in the concentration of IP_3 of up to 30 μM induced no such response (only induced a small transient response, 5/5 experiments), suggesting that the response is not likely to be due to the extra IP_3 which is presumably generated by the ACh stimulation.

The sensitivity to exogenous IP_3 was recovered by a short-term administration of ACh prior to the IP_3 injection. The cell, in the cell-attached condition, was locally perfused with ACh-containing solution using a nearby ACh application pipette, and

then it was subjected to whole-cell dialysis with solution containing 10 μM-IP_3 2–5 s after withdrawal of the ACh application pipette. Figure 4*B* shows a typical response to such IP_3 injection, obtained from the cells perfused for 30–40 s with the solution containing 0·2 μM-ACh. The response was composed of several oscillatory

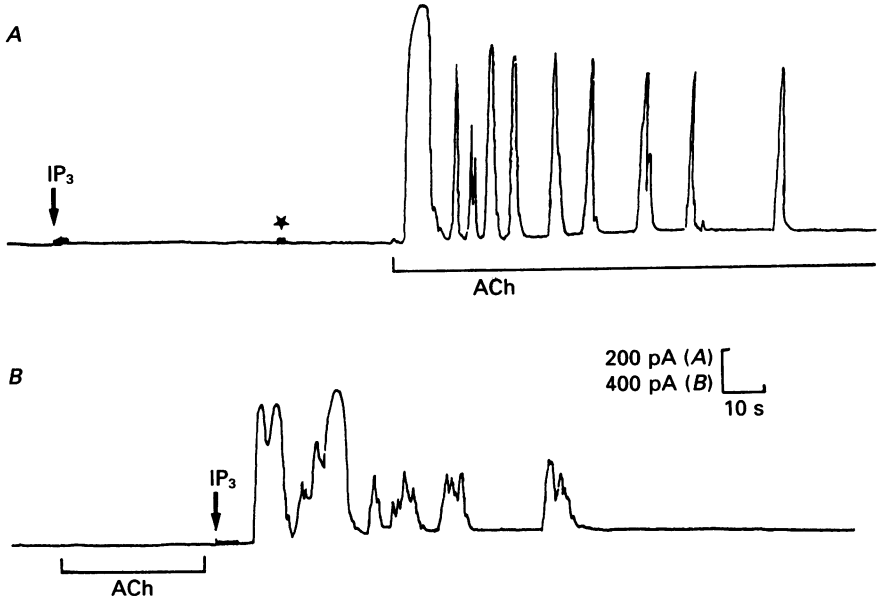

Fig. 4. Synergistic effects of ACh and IP_3. The pancreatic acinar cells were incubated with external solution for 2·5 h in order to eliminate the response induced by IP_3 injection (10 μM-IP_3). *A*, ACh (0·2 μM)-induced response in the cell injected with IP_3 (10 μM). *B*, IP_3 10 μM)-induced response in a cell perfused with solution containing ACh (0·2 μM) from a nearby ACh application pipette for 33 s in the cell-attached mode. The interval between the withdrawal of the ACh application pipette and the start of whole-cell dialysis was 2·5 s. Arrows indicate the point when the whole-cell recording mode was established. Small notches in the trace, indicated by arrows and a star, were the leakage current and capacitative surge of whole-cell recording evoked by 10 mV, 100 ms square voltage pulses (200 ms interval). Such voltage pulses were constantly applied until the establishment of whole-cell mode was confirmed.

waves (highest peak amplitude was 1110 ± 156 pA, $n = 10$) and resembled that induced by sequential stimulation with IP_3 and ACh (Fig. 4*A*). The latency of the response induced by such IP_3 injection became longer ($7{\cdot}8 \pm 1{\cdot}9$ s, $n = 10$) than that induced by IP_3 injection alone in cells sensitive to exogenous IP_3 (see Fig. 3 and relevant text; $1{\cdot}6 \pm 0{\cdot}7$ s, $n = 72$). The access conductance (52–75 nS) and the cell size (the input capacitance, 7·4–9·3 pF) of the whole-cell dialysis in this particular type of experiment were in the usual range (54 ± 9 nS, $n = 52$ and $7{\cdot}3 \pm 1{\cdot}5$ pF, $n = 85$), suggesting that the prolonged latency is not due to artifacts. Whole-cell dialysis performed under the same protocol without IP_3 induced no response, indicating that ACh administration as well as injection of IP_3 are needed to evoke the response. This type of IP_3-induced response depended on the length of ACh administration (in cell-attached mode). As the perfusion time with the ACh-containing solution was

increased, IP_3-induced responses became smaller (roughly half-size after 17 min perfusion), and were finally abolished after a 30 min perfusion (3/3 experiments).

Thus, a type of synergism, presently unknown, between exogenous IP_3 and ACh exists, and it can be postulated that ACh stimulation generates a short-lived (of the order of 10 min) substance(s) which acts synergistically with IP_3.

Effects of protein kinase C modifiers on the response induced by IP_3 and ACh

Llano & Marty (1987) have demonstrated that protein kinase C activators (TPA or OAG) inhibit ACh-induced current responses in rat lacrimal acinar cells. Their experimental protocol was repeated in the pancreatic acinar cells, and similar results were obtained. In brief, pre-treatment with TPA (16 nM for 5–10 min) made ACh (0·2 μM) completely ineffective (10/10 experiments), while the same pre-treatment with 4-α-phorbol 12,13-didecanoate (an inactive phorbol ester) did not affect the ACh-induced response (peak amplitude 411 ± 145 pA, $n = 5$). Reducing TPA concentration to 4 nM gave strongly reduced ACh-induced responses (peak amplitude, $76{\cdot}7 \pm 19{\cdot}0$ pA, $n = 6$), and 0·8 nM-TPA caused responses not markedly different (peak amplitude 432 ± 125 pA, $n = 6$) from those observed in the control condition. OAG also caused inhibitory effects on ACh-induced responses. Pre-treatment with OAG (25 μg/ml) for 30–40 min failed to produce any effect on ACh-induced responses when OAG was washed from the bath prior to the whole-cell recording (peak amplitude, 445 ± 165 pA; $n = 5$). However, the presence of OAG (25 μg/ml) before (pre-treatment for 10 min) and during the recording period, reduced the ACh-induced response or made ACh ineffective (peak amplitude, 15 ± 42 pA; $n = 10$). Incubation with OAG (25 μg/ml) for more than 60 min usually made cells fragile, and rupture of the patch membrane (whole-cell procedure) induced a type of transient current development (5/5 experiments). This effect was not observed in acinar cells treated with TPA. Therefore, TPA was mainly used as a protein kinase C (PkC) activator in the experiments described in later sections.

A known PkC inhibitor, H-7, in pancreatic acinar cells (Pandol & Schoeffield, 1986) caused opposite effects to those of TPA on the ACh-induced response: that is, pre-treatment of cells with H-7 (10 μM for 5–10 min) eliminated the spontaneous decay of the ACh-induced response. Details of this will be described in a later section relating to Fig. 6.

A known Ca^{2+} ionophore, A23187 (0·1 μM), induced sustained current responses in acinar cells pre-treated with TPA or H-7 (current amplitude, 555 ± 89 pA; $n = 4$), indicating that these agents did not affect Ca^{2+}-evoked ion channel opening.

Based on the observations of PkC modification described in the previous two paragraphs, the effects of IP_3 injection (10 μM) and subsequent application of ACh were studied in batches of cells pre-treated with TPA or H-7 (pre-treatment with 16 nM-TPA for 10 min, Fig. 5*B*; with 10 μM-H-7 for 10 min, Fig. 5*C*). In these experiments, the cells were used for whole-cell recording within 60 min of cell dispersion, during which time the IP_3 injection was expected to be effective. The IP_3 injection induced current responses equally in cells pre-treated with or without PkC modifiers (Fig. 5*A*, *B* and *C*). The amplitude of 10 μM-IP_3-induced responses was 497 pA (497 ± 97 pA, $n = 12$) in cells without PkC modifiers, 501 pA (501 ± 105 pA, $n = 12$) in those treated with TPA, and 554 pA (554 ± 137 pA, $n = 12$) in those

treated with H-7 (data were collected from four batches of cells). However, ACh subsequently applied (0·2 μM) evoked different responses. It evoked no response in the cells pre-treated with TPA (12/12 experiments), and with H-7 it induced a large damped oscillation, the shape of which was clearly different from the oscillation with-

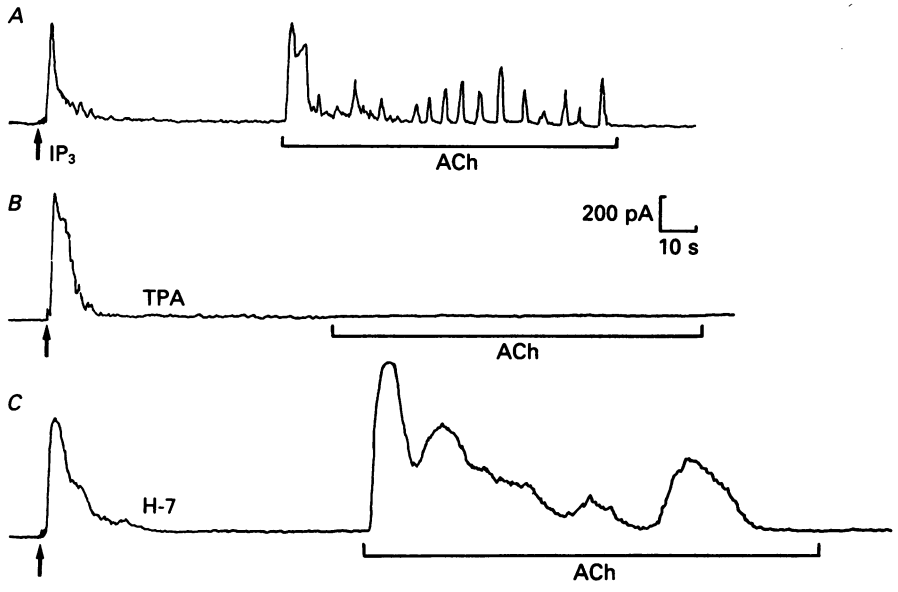

Fig. 5. Effects of protein kinase C modifiers (TPA and H-7) on the response induced by IP_3 and/or ACh. Experiments were carried out within 60 min after cell dispersion (i.e. within 60 min incubation with the external solution). IP_3 (10 μM) was injected into the cells (same batch of cells) pre-treated with TPA or H-7 (*A*, control; *B*, 16 nM-TPA for 10 min, *C*; 10 μM-H-7 for 10 min). ACh (0·2 μM) was subsequently applied after the IP_3-induced response had declined to the resting level. Arrows show the point when the whole-cell dialysis was established. Bars under traces show periods when 0·2 μM-ACh was present.

out H-7 (12/12 experiments; compare the ACh-induced current trace in Fig. 5*C* to that in *A*). The peak amplitude of the response induced by such ACh stimulation, on top of the IP_3 injection, in cells treated with H-7 was 1045 pA (1045 $\pm$ 200 pA, $n = 12$), which was similar to that obtained from the cells without H-7 (982 $\pm$ 175 pA, $n = 12$). The result indicates that TPA and H-7 do not influence Ca^{2+} release from IP_3-sensitive storage sites, but interfere with the signalling pathway prior to the IP_3 generation.

The ACh-dependent oscillatory response in acinar cells treated with H-7

When acinar cells were treated with H-7, ACh induced a characteristic current response which completely depended on the presence of ACh (12/12 experiments). Figure 6 shows typical current traces recorded consecutively from a H-7-treated cell (cell incubation with 10 μM-H-7-containing solution for 5–10 min prior to the experiment). Upon stimulation with ACh (0·2 μM), an oscillatory response was seen (highest peak amplitude, 474 $\pm$ 125 pA; $n = 12$). The response persisted as long as ACh was present. Discontinuation of ACh-stimulation immediately abolished the

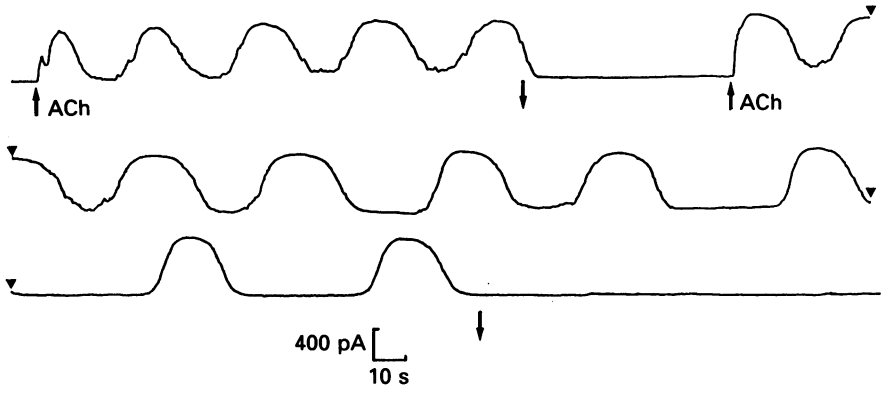

Fig. 6. Effects of H-7 on the response induced by ACh. The cell was pre-treated with 10 μM-H-7 for 10 min prior to the experiments. ACh (0·2 μM) was continuously applied through a nearby application pipette. Upward arrows show the start of ACh stimulation, and downward arrows the end of stimulation. ▼ show continuity of the record.

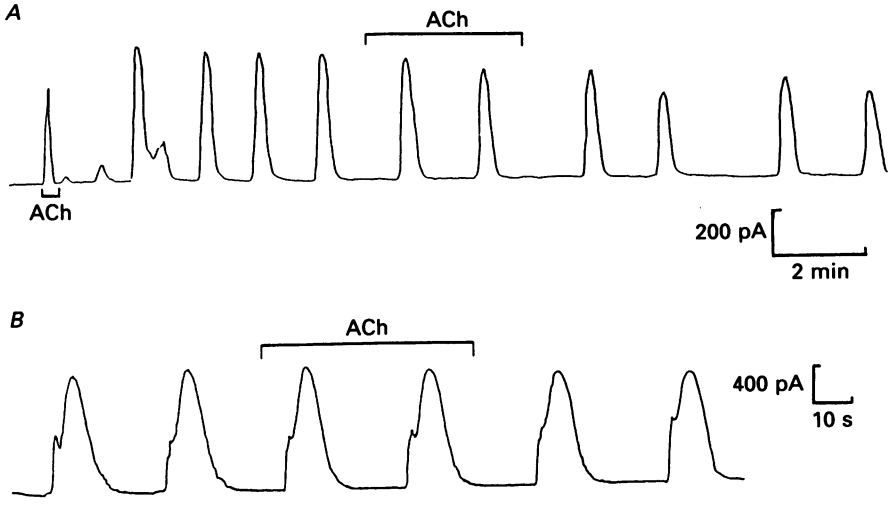

Fig. 7. GTPγS-induced responses and the escape from the influence of ACh stimulation. The pancreatic acinar cells were dialysed with internal solution containing GTPγS (100 μM). Internal Ca^{2+} was loosely buffered with 70 μM-EGTA (*A*), or loosely adjusted to micromolar levels with no added EGTA and Ca^{2+} (*B*). ACh (0·2 μM) was continuously applied through a nearby pipette during the period indicated by bars above the traces.

response. Repeated ACh stimulation after a rest period again induced a response although the intervals between the waves became longer.

Escape from the influence of external ACh in oscillatory response induced by internal GTPγS

An oscillatory response, resembling that observed in H-7-treated cells, could be triggered by ACh stimulation lasting for 20–30 s (which was enough time to induce

an initial cycle of oscillations) in acinar cells dialysed with a solution containing 100–200 μM-GTPγS for 4–8 min. This oscillatory response, once triggered, was uninfluenced by external ACh (8/8 experiments; Fig. 7*A*). Repeated ACh stimulations caused no change in frequency and size of the oscillatory waves.

The oscillatory waves could also be induced, without ACh, by cell dialysis for several minutes with a nominally Ca^{2+}-free (*ca* 1–10 μM) solution containing GTPγS, as previously described (Maruyama, 1988). Once the oscillations began, externally applied ACh was without effect in all the cells tested (8/8 experiments; for example, see Fig. 7*B*). This type of oscillatory response could equally be observed in acinar cells pre-treated with TPA (16 nM for 10 min; 12/12 experiments) or H-7 (10 μM for 5 min; 10/10 experiments). The highest current amplitude and the frequency of the response obtained during the first 2 min of oscillations from the control cells (without PkC modifiers) were 602 pA (602 ± 301 pA, $n = 8$) and 44·3 s ($44{\cdot}3 \pm 13{\cdot}7$ s, $n = 8$), respectively (each sample of the frequency was the mean of intervals obtained from several oscillatory waves in one acinar cell). In the TPA-treated cells, the corresponding values were 572 pA (572 ± 239 pA, $n = 12$) and 42·2 s ($42{\cdot}2 \pm 14{\cdot}0$ s, $n = 12$). In the H-7-treated cells, they were 776 pA (776 ± 285 pA, $n = 10$) and 46·4 s ($46{\cdot}4 \pm 15{\cdot}0$ s, $n = 10$). Thus, it is reasonable to conclude that PkC modifiers do not affect the GTPγS-induced current oscillations.

DISCUSSION

The present study has given new insight into the mechanisms of activation and desensitization of muscarinic ACh receptor signalling in pancreatic acinar cells. The possible interactions of signalling elements have been examined with their modifiers by monitoring Ca^{2+}-dependent current.

Inositol polyphosphates and the increase in $[Ca^{2+}]_i$

There is no doubt that IP_3 is one of the cellular messengers leading to Ca^{2+} release from intracellular storage sites in rat pancreatic acinar cells following muscarinic receptor activation (Streb *et al.* 1984; Putney, 1987). In the present study, IP_3 injection induced a Ca^{2+}-dependent membrane current response in the absence of external Ca^{2+}, and seemed to replicate the effect of ACh in acinar cells incubated with saline solution for a short time. However, the major discrepancy between the effect of IP_3 and ACh, is that ACh stimulation was still effective after the cells lost their sensitivity to IP_3 (and also to IP_3–IP_4) during prolonged cell pre-incubation. The sensitivity to IP_3, however, could be restored by a short-term administration of ACh. One interpretation of these results is that ACh stimulation generates a short-lived substance(s), which is somehow wasted away during cell incubation, synergistically acting with IP_3 to increase $[Ca^{2+}]_i$.

IP_3 injection never induced a sustained current response although it was expected to be continuously introduced from the pipette into rat pancreatic acinar cells (present results). In rat lacrimal acinar cells, however, it has been reported that the IP_3-induced response does not decay over a time course of several tens of seconds (Evans & Marty, 1986), and the decay is only observed in Ca^{2+}-free external solution (Llano, Marty & Tanguy, 1987). Although this discrepancy between rat pancreatic

and lacrimal acinar cells is not clear at present, it is suggested, at least in pancreatic acinar cells, that a type of desensitizing mechanism is present in the action of IP_3 on the Ca^{2+} stores. On the other hand, IP_4 on its own never induced Ca^{2+}-dependent current responses in the pancreatic acinar cells. This is consistent with reports observed in mouse lacrimal acinar cells where, however, a marked synergism of IP_3 and IP_4 in activation of Ca^{2+}-dependent K^+ channels was demonstrated (Morris *et al.* 1987). In rat pancreatic acinar cells, IP_4 seemed to potentiate or temporarily restore the effect of IP_3 in acinar cells which had lost their sensitivity to IP_3. It may be that IP_4 somehow reduces the threshold of the IP_3 receptor site, and potentiates the effect of IP_3 in this particular type of cell.

Desensitization induced by PkC and its possible phosphorylation site

The ACh-induced response recorded with whole-cell dialysis was essentially transient, which is consistent with previous observations (Maruyama, 1988), and a second application of ACh induced a reduced response with a longer latency (Fig. 1). The results can be interpreted as a desensitization of muscarinic ACh stimulation, and two explanations, at least, can be proposed. One is that desensitization is due to the reduced sensitivity of the IP_3 receptor site for IP_3, discussed in the previous paragraph. The other is the negative regulatory action of PkC on the signalling elements prior to the formation of IP_3. This possibility is supported by the evidence obtained from the present study: (1) the PkC activators, TPA or OAG, abolished or reduced the ACh-induced response (Fig. 5), consistent with a previous report on muscarinic receptor activation of lacrimal acinar cells (Llano & Marty, 1987); (2) the inhibitor H-7 augmented the ACh-induced response and made repeated applications effective (Fig. 6); (3) neither of these PkC modifiers influenced the IP_3-induced response (Fig. 5).

Pandol & Schoeffield (1986) have reported that H-7 augmented amylase release induced by CCK or ACh receptor stimulation, indicating that PkC action does not have a stimulatory role in pancreatic stimulus–secretion coupling but an inhibitory one. Their findings are consistent with the present results of electrical measurements, and can be explained by the PkC-induced desensitization of Ca^{2+}-mobilizing receptor signalling. On the other hand, Merritt, Taylar, Rubin & Putney (1986*b*) have reported that carbachol-induced formation of $[^3H]IP_3$ is not affected by a phorbol diester, 4-β-phorbol 12,13-dibutyrate (PDBu, 10 μM), in rat pancreatic acinar cells. Their result is inconsistent with the present result if IP_3 and DG are formed from PIP_2 (phosphatidylinositol 4,5-bisphosphate) at the same time and this signalling pathway is the only one induced by ACh receptor stimulation. This seemingly discrepant observation remains unexplained.

The site of PkC phosphorylation may be neither the G protein, presumably coupling the surface receptor to PLC, nor the PLC, because TPA (or H-7) treatment had no influence on the response induced by internal GTPγS (internal solution nominally free of Ca^{2+}). Thus, the site of PkC action is likely to be the surface ACh receptor. The increase in $[Ca^{2+}]_i$ and DG formation, both activating PkC, may uncouple the receptor from the G protein or inhibit binding of ACh to the receptor. The latter interpretation is supported by the finding that a heterologous desensitization of amylase secretion was caused by carbachol as well as chol-

ecystokinin (CCK) (Abdelemoumene & Gardner, 1980), and TPA or carbachol inhibited binding of CCK to its receptor in pancreatic acinar cells (Honda, Adachi, Noguchi, Sato, Onishi, Aoki & Torizuka, 1987).

The GTPγS-induced response was independent of external ACh application (Fig. 7). This can be explained by the formation of sufficient DG, during the GTPγS-induced oscillations, to activate PkC. In contrast, the response observed in the cells treated with H-7 was completely under the control of external ACh (Fig. 6). This is interpreted as H-7 inhibiting the activity of PkC and thereby preventing the PkC-dependent phosphorylation of the surface receptor.

The oscillatory response and the role of PkC

A variety of cells show oscillatory $[Ca^{2+}]_i$ changes, e.g. mouse oocytes (Cuthbertson & Cobbold, 1985), L cells (Ueda, Oiki & Okada, 1986), rat hepatocytes (Woods, Cuthbertson & Cobbold, 1987), endothelial cells (Jacob, Merritt, Hallam & Rink, 1988), acinar cells of lacrimal gland, salivary gland and pancreatic gland (Evans & Marty, 1986; Gray, 1988; Maruyama, 1988). In the pancreatic acinar cells from the present study, elimination of PkC activity, by treatment with H-7, caused the ACh-induced response to become oscillatory (Fig. 6). This result suggests that one physiological role of PkC is to prevent oscillations by forming a negative feedback loop between DG formation and the surface ACh receptor. Activation (or inhibition) of PkC by treatment with TPA (or H-7) did not influence the GTPγS-induced oscillation (in nominally zero Ca^{2+}, Fig. 7*B*). This result suggests that PkC is not involved in the mechanism of the GTPγS-induced oscillation, and that the oscillatory response is due to the activity of feedback loops formed in the signalling pathway involving G protein, PLC, phosphoinositide sensitive Ca^{2+} storage-sites, and cytosolic Ca^{2+} eliminators.

Phorbol esters enhance amylase release in a Ca^{2+}-dependent manner in the acinar cells of exocrine pancreas (Knight & Koh, 1984; Kimura, Imamura, Eckhardt & Schulz, 1986). Phorbol esters may therefore have a dual effect on stimulus–secretion coupling in pancreatic acinar cells, one of which is desensitization (negative feedback) of receptor signalling, and the other enhancement of exocytosis acting rather directly on the exocytotic machinery (also see Nishizuka, 1988).

The author would like to thank Professor O. H. Petersen (Liverpool) for critical reading of the manuscript. The present study was supported by a Grant-in-aid for Scientific Research on Priority Areas from the Ministry of Education, Science and Culture of Japan.

REFERENCES

ABDELEMOUMENE, S. & GARDNER, J. D. (1980). Cholecystokinin-induced desensitization of enzyme secretion in dispersed acini from guinea pig pancreas. *American Journal of Physiology* **239**, G272–279.

BERRIDGE, M. J. & IRVINE, R. F. (1984). Inositol trisphosphate, a novel second messenger in cellular signal transduction. *Nature* **312**, 315–321.

CUTHBERTSON, K. S. R. & COBBOLD, P. H. (1985). Phorbol ester and sperm activate mouse oocytes by inducing sustained oscillations in cell Ca^{2+}. *Nature* **316**, 541–542.

EVANS, M. G. & MARTY, A. (1986). Potentiation of muscarinic and α-adrenergic responses by an

analogue of guanosine 5'-triphosphate. *Proceedings of the National Academy of Sciences of the USA* **83**, 4099–4103.

GRAY, P. T. (1988). Oscillations of free cytosolic calcium evoked by cholinergic and catecholaminergic agonists in rat parotid acinar cells. *Journal of Physiology* **406**, 35–53.

HIGASHIDA, H. & BROWN, D. A. (1986). Membrane current responses to intracellular injection of inositol 1,3,4,5-tetrakisphosphate and inositol 1,3,4-trisphosphate in Ng108-15 hybrid cells. *FEBS Letters* **208**, 283–286.

HONDA, T., ADACHI, H., NOGUCHI, M., SATO, S., ONISHI, S., AOKI, E. & TORIZUKA, K. (1987). Carbachol regulates cholecystokinin receptor on pancreatic acinar cells. *American Journal of Physiology* **252**, G77–83.

IRVINE, R. F. & MOOR, R. M. (1986). Micro-injection of inositol 1,3,4,5-tetrakisphosphate activates sea urchin eggs by a mechanism dependent on external Ca^{2+}. *Biochemical Journal* **240**, 917–920.

JACOB, R., MERRITT, J. E., HALLAM, T. J. & RINK, T. (1988). Repetitive spikes in cytoplasmic calcium evoked by histamine in human endothelial cells. *Nature* **335**, 40–45.

KAWAMOTO, S. & HIDAKA, H. (1984). 1-(5-Isoquinolinesulfonyl)-2-methylpiperazine (H-7) is a selective inhibitor of protein kinase C in rabbit platelets. *Biochemical and Biophysical Research Communications* **125**, 258–264.

KIMURA, T., IMAMURA, K., ECKHARDT, L. & SCHULZ, I. (1986). Ca^{2+}-phorbol ester-, and cAMP-stimulated enzyme secretion from permeabilized rat pancreatic acini. *American Journal of Physiology* **259**, G698–708.

KNIGHT, D. E. & KOH, E. (1984). Ca^{2+} and cyclic nucleotide dependence of amylase release from isolated pancreatic acinar cells rendered permeable by intense electric fields. *Cell Calcium* **5**, 401–418.

LLANO, I. & MARTY, A. (1987). Protein kinase C activators inhibit the inositol trisphosphate-mediated muscarinic current responses in rat lacrimal cells. *Journal of Physiology* **394**, 239–248.

LLANO, I., MARTY, A. & TANGUY, J. (1987). Dependence of intracellular effects of GTPγS and inositol trisphosphate on cell membrane potential and on external Ca ions. *Pflügers Archiv* **409**, 499–506.

MARUYAMA, Y. (1988). Agonist-induced changes in cell membrane capacitance and conductance in dialysed pancreatic acinar cells of rats. *Journal of Physiology* **406**, 299–313.

MARUYAMA, Y. & PETERSEN, O. H. (1982). Cholecystokinin activation of single-channel currents is mediated by internal second messenger in pancreatic acinar cells. *Nature* **300**, 61–63.

MERRITT, J. E., TAYLOR, C. W., RUBIN, R. P. & PUTNEY, J. W. JR (1986*a*). Evidence suggesting a novel guanine nucleotide regulatory protein couples receptor to phospholipase C in exocrine pancreas. *Biochemical Journal* **236**, 337–343.

MERRITT, J. E., TAYLOR, C. W., RUBIN, R. P. & PUTNEY, J. W. JR (1986*b*). Isomers of inositol trisphosphate in exocrine pancreas. *Biochemical Journal* **238**, 825–829.

MORRIS, A. P., GALLACHER, D. V., IRVINE, R. F. & PETERSEN, O. H. (1987). Synergism of inositol trisphosphate and tetrakisphosphate in activating Ca^{2+}-dependent K^+ channels. *Nature* **330**, 653–655.

NISHIYAMA, A. & PETERSEN, O. H. (1975). Pancreatic acinar cells: ionic dependence of acetylcholine-induced membrane potential and resistance change. *Journal of Physiology* **244**, 431–465.

NISHIZUKA, Y. (1984). The role of protein kinase C in cell surface signal transduction and tumour promotion. *Nature* **308**, 693–698.

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

OHYA, Y., TERADA, K., YAMAGUCHI, K., INOUE, R., OKABE, K., KITAMURA, K., HIRATA, M. & KURIYAMA, H. (1988). Effects of inositol phosphates on the membrane activity of smooth muscle cells of the rabbit portal vein. *Pflügers Archiv* **412**, 382–389.

OZAKI, S., KONDO, Y., NAKAHIRA, H., YAMAOKA, S. & WATANABE, Y. (1987). Synthesis of D-myo-inositol 1,3,4,5-tetrakisphosphate. *Tetrahedron Letters* **28**, 4691–4694.

PANDOL, S. J. & SCHOEFFIELD, M. S. (1986). 1,2-Diacylglycerol, protein kinase C, and pancreatic enzyme secretion. *Journal of Biological Chemistry* **261**, 4438–4444.

PUTNEY, J. W. JR (1987). Formation and actions of calcium-mobilizing messenger, inositol 1,4,5-trisphosphate. *American Journal of Physiology* **252**, G149–157.

RANDRIAMAMPITA, C., CHANSON, M. & TRAUTMANN, A. (1988). Calcium and secretagogue-induced conductances in rat exocrine pancreas. *Pflügers Archiv* **411**, 53–57.

STREB, H., HESLOP, J. P., IRVINE, R. F., SCHULZ, I. & BERRIDGE, M. J. (1985). Relationship between secretagogue-induced Ca^{2+} release and inositol polyphosphate production in permeabilized pancreatic acinar cells. *Journal of Biological Chemistry* **260**, 7307–7315.

STREB, H., IRVINE, R. F., BERRIDGE, M. J. & SCHULZ, I. (1984). Release of Ca^{2+} from a nonmitochondrial intracellular store in pancreatic acinar cells by inositol-1,4,5-trisphosphate. *Nature* **306**, 67–69.

UEDA, S., OIKI, S. & OKADA, Y. (1986). Oscillations of cytoplasmic Ca^{2+} and K^+ in fused L cells. *Journal of Membrane Biology* **91**, 56–72.

WOODS, N. M., CUTHBERTSON, K. S. R. & COBBOLD, P. H. (1987). Repetitive transient rises in cytoplasmic free calcium in hormone-stimulated hepatocytes. *Nature* **319**, 600–602.