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Activation of protein kinase C is not an absolute requirement for amylase release from permeabilized rat pancreatic acini

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The effect of protein kinase C (PKC) on amylase discharge from streptolysin-O-permeabilized rat pancreatic acini was investigated. Addition of phorbol 12-myristate 13-acetate (PMA) to permeabilized cells potentiated Ca^{2+}-stimulated release, but had no effect on discharge at non-stimulatory Ca^{2+} concentrations. PMA markedly shifted the Ca^{2+}-concentration-dependence of amylase discharge to the left, by enhancing the time over which the permeabilized cells release. This effect was inhibited by both staurosporine and PKC-19–31-amide peptide inhibitor, indicating that the effect of PMA was due to its action on PKC. Staurosporine also partially inhibited amylase release at the optimal concentration of Ca^{2+}; this effect was not replicated by the more specific PKC-19–31-amide peptide inhibitor and may be due to an effect on another second-messenger system. PKC appears to be an important modulator of release in pancreatic acini, but its activation is not an absolute requirement for Ca^{2+}-dependent amylase discharge.

INTRODUCTION

The major protein secretagogues for the rat exocrine pancreas are vasoactive intestinal peptide, secretin, cholecystokinin (CCK) and acetylcholine (for review see Hootman & Williams, 1987). Vasoactive intestinal peptide and secretin are believed to mediate exocytosis via cyclic AMP (cAMP), whereas CCK and acetylcholine are believed to elicit secretion by stimulating the hydrolysis of phosphatidylinositol 4,5-bisphosphate to inositol 1,4,5-trisphosphate ($InsP_3$) and diacylglycerol (DAG) (Berridge & Irvine, 1989). $InsP_3$ releases Ca^{2+} from an internal store which is believed to elicit the initial phase of protein discharge, and DAG is believed to prolong secretion subsequently. However, the role of DAG and protein kinase C (PKC) has remained controversial as experiments purportedly implicating its role in exocytosis are dependent on the use of phorbol 12-myristate 13-acetate (PMA) and several inhibitors of questionable specificity. It has been reported that the PKC inhibitor H7 actually potentiates amylase release elicited by CCK and acetylcholine, suggesting that PKC plays an inhibitory role in the control of exocytosis (Pandol & Schoeffield, 1986). Subsequently the more specific inhibitor staurosporine was shown to inhibit secretion partially (Verme *et al.*, 1989; Ederveen *et al.*, 1990), supporting the generally accepted view of PKC as stimulatory in the exocytic pathway. Care should be taken in the evaluation of these data, as staurosporine has been subsequently shown to affect a wide variety of second-messenger systems other than PKC (Rüegg & Burgess, 1989). In particular, staurosporine has been shown to inhibit arachidonic acid release from adrenal chromaffin cells (Morgan & Burgoyne, 1990). Arachidonic acid is released in response to CCK stimulation of pancreatic acini, and it has recently been reported that addition of arachidonic acid potentiates secretion induced by CCK (Pandol *et al.*, 1991).

Several groups have recently independently developed a streptolysin-O (SLO)-permeabilized pancreatic acinar cell system which can be used to alter different components of the intracellular milieu, allowing, for the first time, a dissection of the separate parts of the intracellular mediators that control exocytosis in this cell type (Edwardson *et al.*, 1990; Kitagawa *et al.*, 1990; Padfield *et al.*, 1991). The large pores generated by SLO allow the introduction of a wide variety of peptides and proteins into the cells, including the highly specific peptide inhibitors of protein kinases.

METHODS

Pancreatic acini were prepared by collagenase digestion from starved adolescent Sprague–Dawley male rats weighing 80–100 g, in buffer A [97 mM-NaCl, 5 mM-KCl, 1.2 mM-$MgCl_2$, 2 mM-$CaCl_2$, 20 mM-Hepes, 20 mM-glucose, 0.1 mg of soybean trypsin inhibitor (SBTI)/ml and 0.1% BSA, pH 7.4] as previously described (Bruzzone *et al.*, 1985). The acini were resuspended into buffer B (139 mM-potassium glutamate, 20 mM-Pipes, 0.1 mg of SBTI/ml, pH 6.6) and 50 μl portions of acini were placed into separate tubes. An additional 150 μl of buffer B was added on ice containing EGTA, ATP, Ca^{2+}, Mg^{2+} and SLO (permeabilization buffer) to give final concentrations of 2 mM-EGTA, 2 mM-ATP, 0.2 I.U. of SLO/ml, 2 mM free Mg^{2+} and Ca^{2+} to a value calculated from dissociation constants. Permeabilization was commenced by transferring the tubes to a water bath at 37 °C; amylase release was terminated by transferring the tubes to ice and centrifuging for 2 min at 2000 *g*. A sample of supernatant was removed to measure released amylase activity by a modification of the method of Bernfeld (1955), in which the amylase activity was measured in buffer C (120 mM-NaCl, 0.5 mM-$CaCl_2$ and 20 mM-Mops, pH 6.9). Modification of the assay buffer was necessary, as the EGTA buffers used slightly inhibited amylase activity in phosphate buffer (D. J. Cher, P. J. Padfield, A. J. O'Sullivan and J. D. Jamieson, unpublished work). Total amylase activity was determined by lysis of the acini by sonication at 50 W for 4 s on ice in buffer C containing 0.02% Nonidet P40. Amylase release is expressed as a percentage of total amylase activity.

For experiments involving the PKC peptide inhibitor, acini were permeabilized in the presence of the peptide at the non-stimulatory concentration of 10 nM Ca^{2+} for 10 min before addition of the Ca^{2+}-containing buffer. Staurosporine required preincubation with the acini for 10 min at 37 °C in buffer B before addition of the permeabilization buffer.

Abbreviations used: 4-α-P, 4-α-phorbol; cAMP, cyclic AMP; CCK, cholecystokinin; DAG, diacylglycerol; PKC, protein kinase C; SBTI, soybean trypsin inhibitor; SLO, streptolysin-O; PMA, phorbol 12-myristate 13-acetate.

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Data are expressed as means ± S.E.M. of a typical experiment, carried out on at least four separate portions of cells; all experiments were repeated on at least three different cell preparations.

Collagenase was obtained from Worthington; ATP, EGTA, SBTI and staurosporine were from Boehringer Mannheim. SLO was obtained from Burroughs Wellcome. The PKC-19–31-amide peptide inhibitor was given by Dr. F. S. Gorelick. All other chemicals were obtained from Sigma.

Free Ca^{2+} and Mg^{2+} concentrations were calculated with a computer program kindly supplied by World Precision Instruments, New Haven, CT, U.S.A. The program uses algorithms for calculating free [Ca^{2+}] and [Mg^{2+}] in accordance with Fabiato & Fabiato (1979). EGTA (2 mM) was used in all the experiments, since the results of Ca^{2+}-induced secretion were not significantly different when up to 5 mM-EGTA (at constant [Ca^{2+}] and pH) was in the media (results not shown). Increasing ATP concentrations to 10 mM slightly but non-significantly increased Ca^{2+}-dependent secretion (P. J. Padfield & J. D. Jamieson, unpublished work). In all experiments [ATP] was kept to a more physiological concentration of 2 mM, to avoid the possibility of ATP-induced artifacts.

RESULTS

Fig. 1 shows the effect of various doses of PMA and the inactive phorbol, 4-α-phorbol (4-α-P), on amylase release elicited by the approximately half-maximal dose of 3 μM free Ca^{2+} in SLO-permeabilized cells. PMA significantly potentiates amylase release at concentrations above 10 nM; there was no significant additional release at concentrations greater than 0.1 M. The inactive phorbol 4-α-P causes no significant additional release up to 0.3 μM; thus a dose of 0.1 μM-PMA was used to observe the effects of PKC activation in subsequent experiments. Fig. 2 shows the effect of PMA plus different concentrations of Ca^{2+}. In the presence of 0.1 μM-4-α-P, half-maximal amylase release occurs at 4.8 ± 0.4 μM-Ca^{2+} (mean ± S.E.M.; 5 separate cell preparations). PMA (0.1 μM) shifts half-maximal amylase release to 2.1 ± 0.3 μM-Ca^{2+} (mean ± S.E.M., 5 separate cell preparations) ($P = 0.0005$ by unpaired t test). Fig. 2 also shows that PMA potentiates release induced by the optimal [Ca^{2+}]; significant potentiation at this concentration of Ca^{2+} was observed in 3 out of 5 separate cell preparations.

Fig. 3 shows the effect of PMA on the time course of amylase release in permeabilized cells in response to challenge with 3 μM-Ca^{2+} in the presence of either PMA or 4-α-P. Acini exposed to half-maximal Ca^{2+} plus 4-α-P undergo discharge for up to 2 min; release after 2 min did not occur at a faster rate than in cells challenged with the non-stimulatory concentration of 10 nM-Ca^{2+} plus 4-α-P. In the presence of 0.1 μM-PMA, amylase release is sustained to 6 min; thereafter release occurs at the same rate as in the cells treated with 0.1 μM-4-α-P + 10 nM-Ca^{2+}. Amylase discharge elicited by the optimal dose of 10 μM-Ca^{2+} alone is at approximately the same initial rate as with 3 μM-Ca^{2+}, but continues to stimulate discharge up to 15 min (results not shown).

To determine whether the effects observed were due to the interaction of PMA with PKC, we attempted to inhibit the effects of PMA with a variety of PKC inhibitors. Fig. 4(*a*) shows the effect of various doses of staurosporine on release elicited by half-maximal Ca^{2+} in the presence of PMA. Above 0.1 μM, staurosporine completely abolishes the potentiation observed owing to PMA; the IC_{50} of staurosporine for this inhibition is 50 ± 17 nM (mean ± S.E.M., $n = 3$). *In vitro*, staurosporine inhibits PKC with an IC_{50} of 2.7 nM (Tamaoki *et al.*, 1986). Fig. 4(*b*) shows the lack of effect of staurosporine on release stimulated by 3 μM-Ca^{2+} either alone or in combination with cAMP, whereas the same dose of staurosporine significantly inhibits the PMA potentiation of half-maximal Ca^{2+}-induced amylase discharge. To confirm that staurosporine was blocking PKC, we attempted to confirm the inhibition of the PMA effect with another PKC

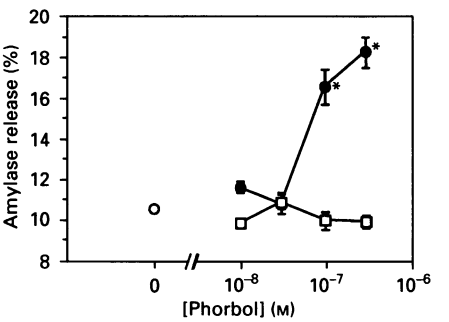

Fig. 1. Effect of PMA on Ca^{2+}-induced amylase release

Permeabilized cells were simultaneously challenged with 3 μM-Ca^{2+} alone (○) or in combination with various concentrations of either PMA (●) or 4-α-P (□). Discharge was terminated after 25 min and amylase release assayed. Data are expressed as means ± S.E.M. ($n = 5$) of a representative experiment: $*P < 0.0001$ by unpaired t test versus 3 μM-Ca^{2+} alone.

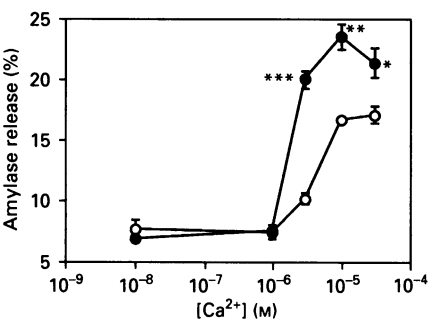

Fig. 2. Effect of PMA on Ca^{2+} dose–response

Permeabilized cells were simultaneously challenged with various concentrations of Ca^{2+} in the presence of either 0.1 μM-PMA (●) or 0.1 μM-4-α-P (○). Release was terminated after 25 min and amylase release assayed. Data are expressed as means ± S.E.M. ($n = 5$) of a representative experiment: $*P = 0.0086$, $**P = 0.0001$, $***P < 0.0001$ by unpaired t test versus Ca^{2+} + 4-α-P.

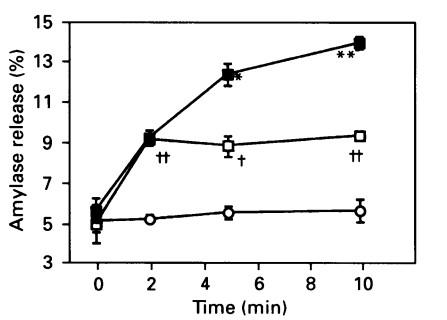

Fig. 3. Effect of PMA on the time course of Ca^{2+}-induced amylase release

SLO-permeabilized acini were challenged with 10 nM-Ca^{2+} + 0.1 μM-4-α-P (○), 3 μM-Ca^{2+} + 0.1 μM-4-α-P (□) or 3 μM-Ca^{2+} + 0.1 μM-PMA (■). The reactions were stopped at the times shown and the supernatants assayed for amylase release. Data are expressed as means ± S.E.M. ($n = 4$) of a representative experiment: $\dagger P = 0.001$, $\dagger\dagger P = 0.0001$, by unpaired t test versus 10 nM-Ca^{2+} + 4-α-P at the same time points; $*P = 0.0042$, $**P = 0.0001$, by unpaired t test versus 3 μM-Ca^{2+} + 4-α-P at the same time point.

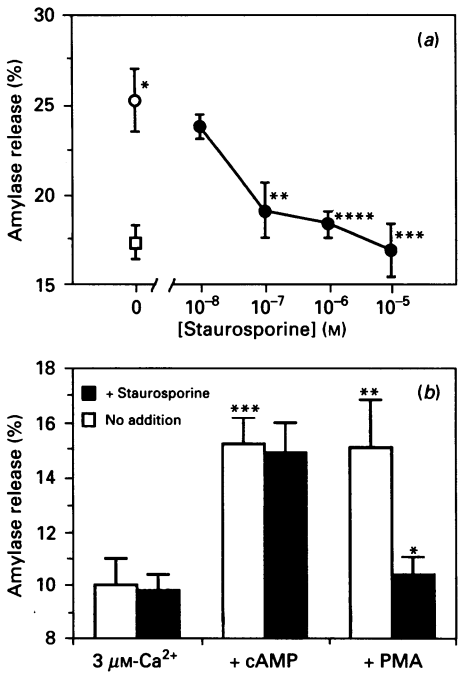

Fig. 4. Effect of staurosporine on Ca^{2+}-, PMA- and cAMP-induced secretion

(*a*) Dose–inhibition curve. Cells were preincubated for 10 min in the absence (○, □) or presence (●) of various concentrations of staurosporine before permeabilization in the presence of 3 μM-Ca^{2+} alone (□), 3 μM-Ca^{2+} + 0.1 μM-PMA (○) or 3 μM-Ca^{2+} + 0.1 μM-PMA + staurosporine (●). Release was terminated after 25 min and the amylase discharge was assayed. Data are expressed as means ± S.E.M. ($n = 5$) of a representative experiment: $*P = 0.0039$ versus Ca^{2+} alone by unpaired t test; $**P = 0.017$, $***P = 0.0038$, $****P = 0.0028$ versus Ca^{2+} + PMA by unpaired t test. (*b*) Effect on PMA- and cAMP-potentiated Ca^{2+}-dependent release. Cells were preincubated for 10 min in the absence or presence of 0.1 μM-staurosporine before permeabilization in the presence of 3 μM-Ca^{2+} alone, 3 μM-Ca^{2+} + 0.1 μM-PMA or 3 μM-Ca^{2+} + 3 μM-cAMP in the absence or presence of 0.1 μM-staurosporine. Discharged amylase was assayed after 25 min release. Data are expressed as means ± S.E.M. ($n = 5$) of a representative experiment: $*P = 0.034$ versus Ca^{2+} + PMA in the absence of staurosporine by unpaired t test; $**P = 0.0175$, $***P = 0.0027$ versus Ca^{2+} alone by unpaired t test.

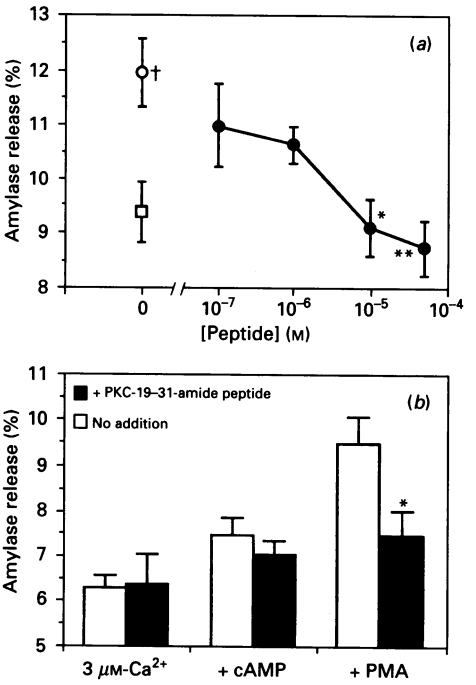

Fig. 5. Effect of PKC peptide inhibitor on Ca^{2+}, PMA- and cAMP-induced secretion

(*a*) Peptide dose–inhibition curve. Cells were permeabilized for 10 min in the absence (○, □) or presence (●) of various doses of PKC-19–31-amide peptide. The cells were subsequently challenged with either 3 μM-Ca^{2+} alone (□) or 3 μM-Ca^{2+} + 0.1 μM-PMA (●, ○). Release was terminated after 25 min and amylase discharge assayed. Data are expressed as means ± S.E.M. ($n = 5$) of a representative experiment: $\dagger P = 0.0072$ versus Ca^{2+} alone; $*P = 0.0042$, $**P = 0.002$ versus Ca^{2+} + PMA by unpaired t test. (*b*) Effect of PKC peptide inhibitor on PMA- and cAMP-potentiated Ca^{2+}-dependent amylase release. Cells were permeabilized for 10 min in the absence or presence of 10 μM-PKC-19–31-amide peptide. The cells were subsequently challenged with 3 μM-Ca^{2+} alone, 3 μM-Ca^{2+} + 3 μM-cAMP or 3 μM-Ca^{2+} + 0.1 μM-PMA in the absence and presence of the peptide. Release was terminated after 25 min and amylase discharge assayed. Data are expressed as means ± S.E.M. ($n = 5$) of a representative experiment: $*P = 0.0194$ versus Ca^{2+} + PMA by unpaired t test.

inhibitor. Fig. 5(*a*) shows the effect of the peptide inhibitor PKC-19–31-amide. Potentiation of half-maximal Ca^{2+}-induced release by TPMA is significantly inhibited at 10 μM peptide; the IC_{50} of the peptide is 2.1 ± 0.4 μM (mean ± S.E.M., $n = 3$). *In vitro* the peptide inhibits PKC with an IC_{50} of 92 nM (House & Kemp, 1987). Concentrations of PKC 19–31 amide required to inhibit PKA and myosin light-chain kinase *in vitro* are at least 10 times higher than the IC_{50} that we observed. Fig. 5(*b*) shows that, as with staurosporine, this effect is specific for PMA and has no effect on discharge caused by half-maximal Ca^{2+} either alone or in combination with cAMP.

In vitro, Ca^{2+} can activate PKC over the range 10 μM in the presence of phospholipid (Nishizuka, 1989). One possible reason for the variable effect of PMA at maximal or supramaximal Ca^{2+} concentrations is that PKC is already activated by Ca^{2+} alone. We investigated this possibility by stimulating permeabilized cells with a range of Ca^{2+} concentrations in the presence and absence of staurosporine. Fig. 6 shows that staurosporine, at a dose which inhibits PMA potentiation of amylase release, significantly inhibits release elicited by the optimal and supra-optimal Ca^{2+} doses. However, the PKC peptide inhibitor had no effect on release induced by any dose of Ca^{2+} (results not shown). One possible reason for the lack of effect of the peptide inhibitor is that the PKC substrate responsible for the staurosporine effect, at optimal Ca^{2+} concentrations, has leaked out of the cell during the preincubation. To rule out this possibility, we preincubated both the staurosporine and PKC-19–31-amide peptide with permeabilized cells. Fig. 7 shows that staurosporine is capable of inhibiting Ca^{2+}-induced release when preincubated with permeabilized cells, whereas the peptide is not. It therefore appears unlikely that the inhibitory effect of staurosporine is entirely due to its effects on PKC. One possibility is that staurosporine may inhibit another second-messenger system involved in the regulation of exocytosis.

DISCUSSION

Our results show that PMA potentiates Ca^{2+} induced release from permeabilized pancreatic acini through its effects on PKC. It has recently been suggested that the effects of PMA on pancreatic acini are not due to its effects on PKC, but rather through an effect on some other unknown mechanism (Pandol & Schoeffield, 1986). The PKC inhibitor H7 was found to potentiate, rather than inhibit, secretion elicited by either CCK or

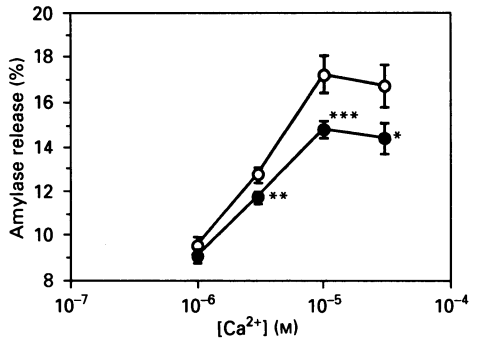

Fig. 6. Effect of staurosporine on Ca^{2+} dose–response curve

Cells were preincubated for 10 min without (○) or with (●) 0.1 μM-staurosporine before permeabilization in the presence of various concentrations of Ca^{2+} in the absence or presence of staurosporine. Discharged amylase was assayed after 25 min release. Data are expressed as means ± S.E.M. ($n = 5$) of a representative experiment: $*P = 0.0402$, $**P = 0.0286$, $***P = 0.0111$ versus release elicited by an identical Ca^{2+} dose in the absence of staurosporine, by unpaired t test.

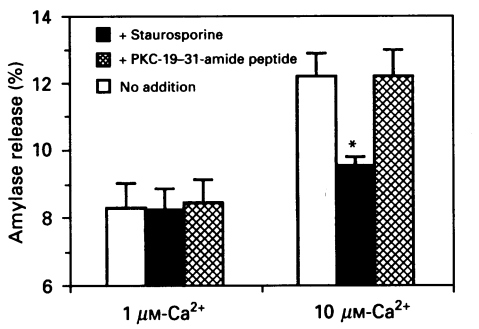

Fig. 7. Effect of PKC inhibitors on amylase release induced by optimal and sub-optimal Ca^{2+} doses

Cells were permeabilized for 10 min in the presence of 0.1 μM-staurosporine, 10 μM-PKC-19–31-amide peptide or with no addition. The cells were subsequently challenged with either 1 μM- or 10 μM-Ca^{2+}. Release was terminated after 25 min and amylase discharge assayed. Data are expressed as means ± S.E.M. ($n = 4$) of a representative experiment: $*P = 0.007$ versus 10 μM-Ca^{2+} with no addition, by unpaired t test.

PMA in intact acini, and it was concluded that the results rule out any significant role for PKC in the control of exocytosis from the pancreas. Subsequently the effects of H7 were found not to be in accord with the effects of the PKC inhibitor staurosporine, which inhibited both PMA- and CCK-induced release of amylase (Verme *et al.*, 1989; Ederveen *et al.*, 1990). Recent evidence has suggested that staurosporine may not be as specific an inhibitor as was first thought. Staurosporine has been shown to inhibit tyrosine kinase (Fallon, 1990), cAMP-dependent protein kinase (Tamaoki *et al.*, 1986) and arachidonic acid release (Morgan & Burgoyne, 1990) at concentrations previously thought to be specific for PKC. Staurosporine has also been shown to have complex effects on protein phosphorylation in platelets, both stimulating and inhibiting several different protein kinases (Kocher & Clemetson, 1991). As both serine/threonine and tyrosine kinases have been implicated in the control of exocytosis from pancreatic acinar cells (Jena *et al.*, 1991), the question of whether and how PKC controls exocytosis in the exocrine pancreas remains in doubt.

The use of SLO-permeabilized cells allows the introduction of specific peptide inhibitors of PKC to determine the role of PKC in Ca^{2+}-stimulated exocytosis. We have demonstrated that activation of PKC by PMA potentiates amylase release by increasing the sensitivity of the system to Ca^{2+}, as is observed in a wide variety of other exocytotic systems (Knight & Scrutton, 1986). In pancreatic acini, increasing doses of Ca^{2+} and potentiation of Ca^{2+}-dependent release by PMA appear to have little effect on the initial rate of release, but increase the length of time over which cells undergo discharge. This is in agreement with the widely held view that DAG is responsible for the second sustained phase of exocytosis in intact cells stimulated with CCK (Hootman & Williams, 1987). The data presented are broadly in agreement with the findings by Kitagawa *et al.* (1991), who demonstrated that PMA at 1 μM enhances the time over which permeabilized cells release amylase, but has no effect on the initial rate of discharge. Thus PMA appears to elicit a second phase of amylase release after the initial Ca^{2+}-dependent phase. The initial rate of discharge is similar for both 3 μM-Ca^{2+} and 10 μM-Ca^{2+} in the presence and absence of PMA. It is possible that the activation of PKC causes the retention of a cytosolic protein, required for exocytosis, within the permeabilized cell. A possible candidate is annexin II (calpactin I), which has been shown to be necessary for exocytosis in the permeabilized bovine adrenal chromaffin cell (Ali *et al.*, 1989). Pretreatment of chromaffin cells with PMA leads to an increase in membrane bound annexin II, retarding its loss on permeabilization with digitonin (Sarafian *et al.*, 1991). A similar mechanism may exist in pancreatic acinar cells. However, given the potentiating effect of PMA on ionophore-induced secretion in intact acini, it seems unlikely that this fully accounts for the effect of PMA in this system.

PKC-19–31-amide peptide was found to have no effect on amylase discharge elicited by Ca^{2+} alone when used under conditions which blocked PMA-induced potentiation of Ca^{2+}-dependent release. However, the PKC inhibitor staurosporine partially inhibited amylase release elicited by the optimal Ca^{2+} concentration of 10 μM-Ca^{2+} (compare Figs. 5*b* and 6). Ca^{2+}-dependent release appears to consist of two components, staurosporine-sensitive and -insensitive. Given the known specificity problems of staurosporine, and the lack of effect of the peptide inhibitor, it is probable that the effects seen on Ca^{2+}-dependent release are due to some other second-messenger system.

The major problem with the permeabilized cell system is the very high levels of Ca^{2+} required to elicit protein discharge. We observe maximal release at 10 μM-Ca^{2+}, as has been widely observed in a variety of other exocytotic systems (Knight & Scrutton, 1986), which is at least 10-fold higher than is observed by fluorimetric measurements with fura-2 or quin-2. Williams and co-workers have reported that SLO-permeabilized mouse pancreatic acini undergo maximal release at 1 μM-Ca^{2+} (Kitagawa *et al.*, 1990). This may be due to either species differences or their use of a NaCl-based buffer rather than a potassium glutamate-based buffers (Churcher & Gomperts, 1990). In addition, zymogen granules are known to lyse in both NaCl and KCl buffers cells, compared with the more widely used potassium glutamate-based buffers (Churcher & Gomperts, 1990). In addition, zymogen granules are known to lyse in both NaCl and KCl buffers (Burwen & Rothman, 1972). The effects of cAMP and PMA to increase amylase release would be consistent with Cl^--mediated granule lysis, as the Cl^--permeability of the granules is known to be enhanced by both protein kinase A and PKC activity (Fuller *et al.*, 1989). The use of pH 7 buffer is also of concern, as the zymogen granules are known to become increasingly fragile at higher pH (Hokin, 1955; Holtzer & Van Lancker, 1963).

The reason for the very high levels of Ca^{2+} required to elicit release from permeabilized cells remains unclear. It is possible that cytosolic components of the exocytotic machinery leak out of the cells on permeabilization. However, reconstitution of

exocytosis in permeabilized cells with cytosol does not shift the Ca^{2+}-dependency of release (Wu & Wagner, 1991). An alternative possibility is that the measurements of $[Ca^{2+}]$ in stimulated cells are incorrect. Recent experiments have revealed that the rise in cytosolic free $[Ca^{2+}]$ elicited by phosphoinositol-mobilizing agonists is initially restricted to the apical pole, near the exocytotic site (Kasai & Augustine, 1990). This raises the possibility that rises in cytosolic free $[Ca^{2+}]$ at the site of exocytosis are actually much greater than those previously observed in spatially averaged measurements.

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