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Protein kinase A modulates Ca^{2+}- and protein kinase C-dependent amylase release in permeabilized rat pancreatic acini

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The role of protein kinase A (PKA) in the release of amylase from permeabilized pancreatic acini was investigated. Addition of cyclic AMP (cAMP) to permeabilized acini resulted in a potentiation of Ca^{2+}-dependent amylase release, shifting the Ca^{2+} dose/response curve leftwards. As with protein kinase C (PKC) activation, this is due to an increase in the time of active discharge. The effect of cAMP was shown to be blocked by two inhibitors of PKA, H89 and the PKI-(5–24)-peptide. At low concentration, cAMP synergizes from phorbol 12-myristate 13-acetate (PMA), while at optimal concentrations cAMP and PMA are additive. PKA and PKC appear to work via similar, but not identical mechanisms.

INTRODUCTION

Pancreatic acini are stimulated to exocytose by both phospholipase C- and adenylate cyclase-linked receptors (Hootman & Williams, 1987). Secretin and vasoactive intestinal peptide (VIP) are linked via a G-protein to adenylate cyclase. Occupancy of either the secretin or the VIP receptor leads to activation of the cyclase, generation of cyclic AMP (cAMP) and subsequent activation of protein kinase A (PKA) (Gardner & Jensen, 1986). Both secretin and VIP have been found to elicit a small increase in amylase discharge and to potentiate secretion elicited by the phosphatidylinositol (PI)-linked agonists, cholecystokinin and acetylcholine (Schulz & Stolze, 1980). The site at which the PI- and cAMP-dependent pathways interact has yet to be elucidated. Although it is known that secretin and VIP can potentiate exocytosis stimulated by phorbol 12-myristate 13-acetate (PMA), the interaction of cAMP with the rise in cytosolic free $[Ca^{2+}]$ elicited by PI-mobilizing agonists has yet to be fully investigated. In particular, it has yet to be determined whether cAMP-mediated secretion has an absolute dependence on Ca^{2+}.

The recent development of the permeabilized pancreatic acini allows us for the first time to manipulate independently levels of diacylglycerol, cAMP and $[Ca^{2+}]$ within the cell, as PI turnover does not change on stimulation with Ca^{2+} in the permeabilized acini (Padfield *et al.*, 1991). Permeabilization also allows the introduction of specific peptide inhibitors of protein kinases to determine their role in the secretory pathway of the exocrine pancreas (O'Sullivan & Jamieson, 1992).

METHODS

Pancreatic acini were prepared by collagenase digestion of glands 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-glucose, 20 mM-Hepes, 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 in 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 divided into separate tubes. An additional 150 μl of buffer B was added on ice, containing EGTA, ATP, Ca^{2+}, Mg^{2+} and streptolysin-O (SLO) (permeabilization buffer) to give final concentrations of 2 mM-EGTA, 2 mM-ATP, 0.2 IU 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, and amylase release was terminated by transferring the tubes to ice and centrifugation 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), as previously described (O'Sullivan & Jamieson, 1992). 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 PKI-(5–24)-peptide inhibitor, acini were permeabilized in the presence of the peptide at the non-stimulatory concentration of 10 nM-Ca^{2+} for 7 min before addition of the Ca^{2+}-containing buffer. The action of H89 {*N*-[2-(*p*-bromocinnamoylamino)ethyl]-5-isoquinolinesulphonamide} was found to be affected by the reducing agent found in commercially available SLO; thus SLO was pre-bound to acini at 4 °C for 10 min and washed three times in buffer B before addition of the permeabilization buffer containing H89. Pre-binding of SLO had no effect on amylase release up to the optimal $[Ca^{2+}]$ (results not shown).

Data are expressed as means ± S.E.M. of a typical experiment, carried out on at least four separate samples of cells run in parallel; all experiments were repeated on at least three different acini preparations. Statistical analysis of results was performed by either Student's *t* test or the Mann–Whitney U-test, which was applied to the data in Figs. 2 and 6, which do not have a normal distribution.

Collagenase was obtained from Worthington; ATP, EGTA and SBTI were from Boehringer Mannheim. SLO was obtained from Burroughs Wellcome. The PKI-(5–24)-peptide inhibitor was a gift from Dr. F. S. Gorelick (Yale University Medical School). All other chemicals were obtained from Sigma.

Free $[Ca^{2+}]$ and $[Mg^{2+}]$ were calculated by using a computer program supplied by World Precision Instruments, New Haven, CT, USA. This program uses algorithms for determining bivalent-cation concentrations that are based on those published by Fabiato & Fabiato (1979). EGTA (2 mM) was used in all experiments, since the results of Ca^{2+}-induced secretory responses were not significantly different when [EGTA] up to 5 mM (at constant $[Ca^{2+}]$ and pH) was used (results not shown). ATP

Abbreviations used: cAMP, cyclic AMP; PI, phosphatidylinositol; PKA, protein kinase A; PKC, protein kinase C; SBTI, soybean trypsin inhibitor; SLO, streptolysin-O; PMA, phorbol 12-myristate 13-acetate; VIP, vasoactive intestinal peptide.

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concentrations up to 10 mM slightly increased Ca^{2+}-dependent secretion, but this increase was not significant (P. J. Padfield & J. D. Jamieson, unpublished work). In all experiments [ATP] was kept to the more physiological concentration of 2 mM to avoid the possibility of ATP-induced artifacts.

RESULTS

Fig. 1 shows the effect of various concentrations of cAMP on amylase release elicited by a half-maximal concentration of 3 μM-Ca^{2+}. Ca^{2+}-dependent release is maximally potentiated by 3 μM-cAMP, the EC_{50} being 1.7 ± 0.5 μM (mean $\pm$ S.E.M., four separate cell preparations). cAMP, AMP and adenosine had no effect on release up to 10 μM (results not shown). Fig. 2 shows the effect of cAMP on the Ca^{2+} dose/response curve. cAMP shifts the EC_{50} for Ca^{2+} from 4.7 ± 1.1 μM to 3.2 ± 1.0 μM (mean $\pm$ S.E.M., $n = 6$, $P = 0.002$ by paired Student's t test), as has been previously observed with protein kinase C (PKC) activation by PMA under the same conditions (O'Sullivan & Jamieson, 1992). cAMP also induces a small but significant amylase discharge at the non-stimulatory $[Ca^{2+}]$ of 1 μM, unlike PMA, which only potentiates Ca^{2+}-dependent release. cAMP also significantly potentiated release elicited by the optimal dose of $[Ca^{2+}]$. Fig. 3 shows the effect of an optimal dose of cAMP on the time of release elicited by 3 μM-Ca^{2+}. There is no difference in the initial phase of release, but cAMP + Ca^{2+} induce release over a longer period of time than does Ca^{2+} alone.

To determine whether the effect of cAMP on Ca^{2+}-dependent amylase discharge was due to activation of the catalytic subunit of PKA or by some other unknown mechanism, as has been proposed for the effects of cAMP in permeabilized parotid acini (Takuma, 1988), we attempted to inhibit the effects of cAMP with specific inhibitors of PKA. Fig. 4(*a*) shows the effect of increasing concentrations of the PKI-(5–24)-peptide inhibitor on release elicited by 3 μM-cAMP in combination with 3 μM-Ca^{2+}. Half-maximal inhibition of cAMP potentiation is observed at 0.48 ± 0.17 μM (mean $\pm$ S.E.M., four separate acini preparations), approx. 200-fold more than is required *in vitro* (Cheng *et al.*, 1986). Fig. 4(*b*) shows that this effect is specific for PKA, and that the PKI-(5–24) peptide had no effect up to 10 μM on release elicited by either Ca^{2+} alone or in combination with PMA. We attempted to confirm these results with a second inhibitor of PKA, H89, which is competitive with respect to ATP for the kinase. To observe an effect with H89, we decreased the concentration of ATP present in the permeabilization buffer to 100 μM (this significantly decreases Ca^{2+}dependent release, but has no effect on the cAMP potentiation observed). Fig. 5(*a*) shows the effect of increasing doses of H89 on Ca^{2+} + cAMP-stimulated discharge, the IC_{50} for H89 being 5.3 ± 1.3 μM (mean $\pm$ S.E.M., $n = 4$), which is approximately one-tenth of the K_i observed *in vitro* for PKC, myosin light-chain kinase, CAM-kinase II and casein kinases (Chijiwa *et al.*, 1990). Fig. 5(*b*) shows that H89 had no effect on discharge elicited by Ca^{2+} alone or by Ca^{2+} + PMA.

Given the effects of the PKA inhibitors on Ca^{2+} + PMA-induced release, and the similarity of the effects of PKA and PKC activation on Ca^{2+}-dependent discharge, we investigated the effects of simultaneous PKA and PKC activation on amylase release. Sub-stimulatory doses of 1 μM-cAMP and 30 nM-PMA synergize in the presence of 3 μM-Ca^{2+} to elicit a 75% increase in amylase release above that caused by 3 μM-Ca^{2+}alone ($P < 0.02$ by Mann–Whitney U-test). This effect is entirely dependent on the presence of stimulatory concentrations of Ca^{2+} and is not observed to occur at 10 nM-Ca^{2+} (results not shown). To determine whether PKA and PKC are acting through an identical mechanism to potentiate Ca^{2+}-dependent discharge, we

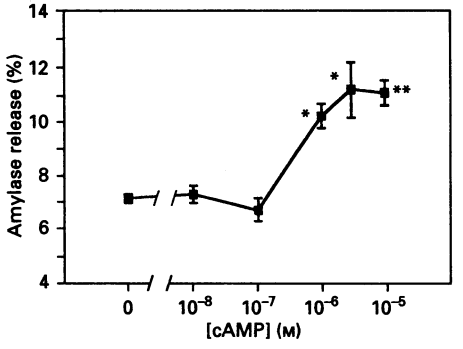

Fig. 1. Effect of cAMP on Ca^{2+}-induced amylase release

Pancreatic acini were permeabilized in the presence of 3 μM-Ca^{2+} and various concentrations of cAMP. Release was terminated after 25 min and amylase discharge was assayed. Data are expressed as means $\pm$ S.E.M. ($n = 5$) of a typical experiment: $*P < 0.005$, $**P < 0.0001$ versus Ca^{2+} alone, by unpaired Student's t test.

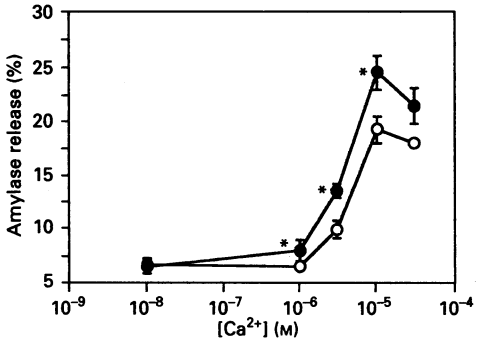

Fig. 2. Effect of cAMP on Ca^{2+} dose/response

Acini were permeabilized in the absence (○) or presence (●) of 3 μM-cAMP and various concentrations of Ca^{2+}. Discharge was terminated after 25 min and amylase release was measured. Data are expressed as means $\pm$ S.E.M. ($n = 4$) of a typical experiment: $*P < 0.05$ versus Ca^{2+} alone, by unpaired Mann–Whitney U-test.

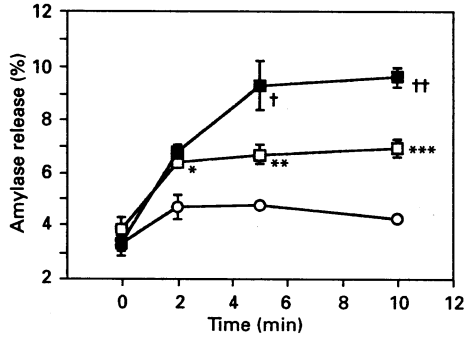

Fig. 3. Time course of amylase discharge in the presence and absence of cAMP

Permeabilized acini were exposed to 10 nM-Ca^{2+} (○), 3 μM-Ca^{2+} (□) or 3 μM-Ca^{2+} + 3 μM-cAMP (■). Release was stopped at various times and amylase activity in the supernatant assayed. Data are expressed as means $\pm$ S.E.M. ($n = 4$) of a typical experiment: $*P < 0.01$, $**P = 0.001$, $***P < 0.0005$ versus 10 nM-Ca^{2+}; $\dagger P < 0.05$, $\ddagger P = 0.001$ versus 3 μM-Ca^{2+} at the same time point, by unpaired Student's t test.

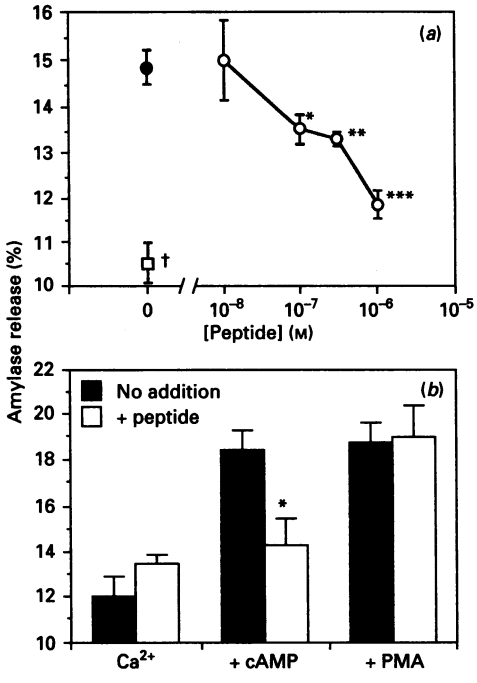

Fig. 4. Effect of PKI-(5–24)-peptide on cAMP- and PMA- potentiated Ca^{2+}-dependent secretion from permeabilized acini

(*a*) Dose-dependency for PKI-(5–24)-peptide on cAMP potentiation of Ca^{2+}-induced amylase release. Acini were permeabilized in the presence of various concentrations of the peptide for 7 min before stimulation with 3 μM-Ca^{2+} (□), 3 μM-Ca^{2+} + 3 μM-cAMP (●) or 3 μM-Ca^{2+} + 3 μM-cAMP + peptide (○). Discharge was stopped after 25 min and amylase release measured. Data are expressed as means ± S.E.M. ($n = 5$) of a typical experiment: †$P < 0.0001$ versus 3 μM-Ca^{2+}; *$P < 0.05$, **$P = 0.005$, ***$P < 0.0005$, versus 3 μM-Ca^{2+} + 3 μM-cAMP, by unpaired Student's *t* test. (*b*) Effect of PKI-(5–24)-peptide on cAMP- and PMA-potentiated Ca^{2+}-dependent amylase discharge. Acini were permeabilized in the presence or absence of 1 μM-peptide before stimulation with 3 μM-Ca^{2+}, 3 μM-Ca^{2+} + 3 μM-cAMP or 3 μM-Ca^{2+} + 100 nM-PMA. Release was terminated after 25 min and amylase discharge assayed. Data are expressed as means ± S.E.M. ($n = 5$) of a typical experiment: *$P < 0.005$ versus 3 μM-Ca^{2+} + 3 μM-cAMP by unpaired Student's *t* test.

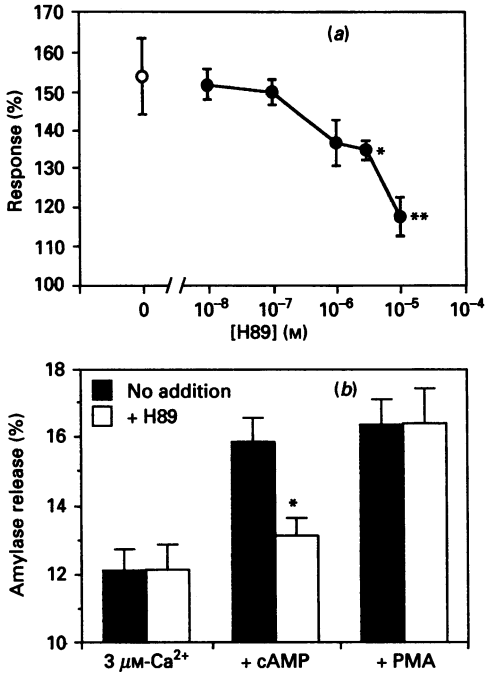

Fig. 5. Effect of H89 on cAMP- and PMA-potentiated Ca^{2+}-dependent secretion from permeabilized acini

(*a*) Dose/inhibition curve of H89. Acini were permeabilized in the presence of 3 μM-Ca^{2+} + 3 μM-cAMP and various doses of H89. Release was stopped after 25 min and amylase discharge measured. Data are expressed as a percentage of the response to 3 μM-Ca^{2+} alone (means ± S.E.M. of four separate cell preparations): *$P = 0.05$, **$P < 0.05$ versus 3 μM-Ca^{2+} + 3 μM-cAMP by unpaired Student's *t* test. (*b*) Effect of H89 on cAMP- and PMA-potentiated Ca^{2+}-dependent amylase discharge. Acini were permeabilized in the absence or presence of 10 μM-H89 with 3 μM-Ca^{2+}, 3 μM-Ca^{2+} + 3 μM-cAMP or 3 μM-Ca^{2+} + 100 nM-PMA. Release was terminated after 25 min and amylase discharge assayed. Data are expressed as means ± S.E.M. ($n = 5$) of a typical experiment: *$P < 0.05$ versus 3 μM-Ca^{2+} + 3 μM-cAMP by unpaired Student's *t* test.

investigated the effect of varying the concentration of PMA in the presence of 3 μM-Ca^{2+} and the optimal concentration of cAMP. Fig. 6 shows that the effect of PMA is additive with that of cAMP, implying that at least part of the effect of the two kinases is due to separate effector mechanisms.

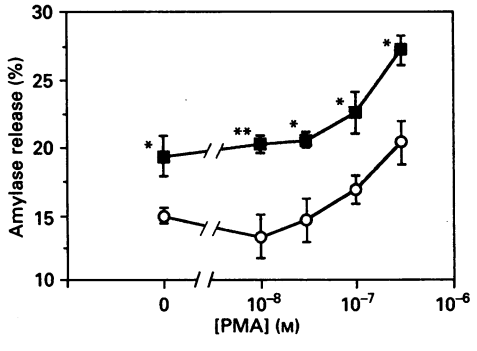

Fig. 6. Additive effect of varying [PMA] on cAMP-stimulated amylase discharge

Pancreatic acini were permeabilized in the presence of 3 μM-Ca^{2+} and various concentrations of PMA in the absence (○) and presence (■) of 3 μM-cAMP. Release was terminated after 25 min and amylase discharge was assayed. Data are expressed as means ± S.E.M. ($n = 4$) of a typical experiment: *$P < 0.05$, **$P < 0.01$, ***$P < 0.005$ versus Ca^{2+} + PMA, by unpaired Student's *t* test.

DISCUSSION

The data in this paper show that cAMP potentiates release of amylase from permeabilized rat pancreatic acini cells, elicited by both Ca^{2+} and PKC. Previous studies in permeabilized acini have reported similar effects, but at much higher concentrations of cAMP (Kitagawa *et al.*, 1991). Those investigators also failed to show whether the effects of cAMP were due to activation of PKA. We have shown that the actions of cAMP on amylase release can be inhibited by two specific inhibitors of the catalytic subunit of PKA. In addition, the KCl-based buffers used in previous studies have raised questions about the possibility of second-messenger-activated Cl^--dependent granule lysis (O'Sullivan & Jamieson, 1992).

It has been proposed that cAMP-mediated exocytosis in the closely related parotid acinar cell is due to the actions of the regulatory rather than the catalytic subunit of PKA (Takuma, 1988). This conclusion was derived from the finding that the PKA inhibitors H8 and PKI-(5–24) blocked cAMP-dependent protein phosphorylation, but had no effect on amylase release. However, the phosphoprotein(s) responsible for regulating

exocytosis remain unknown, and it is possible that, whereas H8 and PKI-(5–24) inhibited most of the phosphorylations, they did not block phosphorylation of the protein or proteins involved in regulating exocytosis. This seems particularly likely in the light of findings implicating a rapid phosphorylation/dephosphorylation event being central to the regulation of secretion in *Paramecium* (Zieseniss & Plattner, 1985), adrenal chromaffin cells (Holz *et al.*, 1989) and pancreatic acini (J. D. Jamieson & P. J. Padfield, unpublished work).

The effects of PKA activation are very similar to those of PKC activation. Both potentiate Ca^{2+}-dependent release, shifting the curve for Ca^{2+}-dependence of discharge leftwards and increasing the time over which the cells release. This similarity of effect is reflected in the fact that cAMP and PMA synergize at sub-optimal doses. One possible cause of this synergism is that the two kinases act on an identical substrate, which subsequently mediates the effect on discharge. This hypothesis is supported by the finding in intact cells that a small number of proteins are phosphorylated in response to both cAMP and PI-mobilizing agonists, and this phosphorylation is synergistic under combined stimulation (Ederveen *et al.*, 1989).

The major difference between PKA and PKC activation is that PKA activation causes amylase release at non-stimulatory [Ca^{2+}], where PKC activation has no effect on amylase release. However, there is an absolute Ca^{2+}-dependence for the effect of PKA activation on amylase release, as cAMP-dependent release is abolished at 10 nM-Ca^{2+}. This appears to reflect a requirement of the exocytotic machinery rather than a requirement for phosphorylation, as PKA is active *in vitro* in the absence of Ca^{2+}.

Both H89 (3 out of 8 acini preparations) and the PKI-(5–24)-peptide inhibitor (1 out of 5 acini preparations) were found to inhibit PMA-potentiated release intermittently. This may be due to residual cAMP in the permeabilized acini, below a concentration to affect amylase release in the presence of Ca^{2+}. The residual cAMP could synergize with PMA, resulting in an apparent inhibition of Ca^{2+}/PKC-mediated release in the presence of either H89 or the PKI-(5–24)-peptide.

Further studies are needed to determine the substrates of PKA and PKC in permeabilized rat pancreatic acini.

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