Pancreastatin increases free cytosolic Ca^{2+} in rat hepatocytes, involving both pertussis-toxin-sensitive and -insensitive mechanisms

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Freshly isolated rat hepatocytes, loaded with the Ca^{2+} probe Fluo-3, responded to homologous pancreastatin with a sudden increase in free cytosolic Ca^{2+} ($[\text{Ca}^{2+}]_i$) as well as glucose release. Addition of rat pancreastatin ($0.1 \mu\text{M}$) to hepatocytes resulted in an increase in $[\text{Ca}^{2+}]_i$ from 150 nM to 700 nM, which declined back to nearly basal values within 2–3 min. Half-maximal and maximal effects were observed at 0.3 and 100 nM pancreastatin respectively. The increase in $[\text{Ca}^{2+}]_i$ induced by vasopressin and noradrenaline was very similar in extent (from 150 to 800 nM) to that produced by pancreastatin. Neither the α_1 -adrenergic blocker prazosin nor the vasopressin antagonist V_1 modified the increase in $[\text{Ca}^{2+}]_i$ induced by pancreastatin. Pig pancreastatin and its 33–49 C-terminal fragment produced about 65 and 75% of the effect of homologous pancreastatin respectively. Glucose production correlated with changes in $[\text{Ca}^{2+}]_i$ in the same order

of potency: vasopressin > rat pancreastatin > pig 33–49 pancreastatin > pig 1–49 pancreastatin. The effect of pancreastatin on $[\text{Ca}^{2+}]_i$ was decreased by 50% when Ca^{2+} was omitted from the medium, and totally abolished when hepatocytes were depleted of internal Ca^{2+} stores by preincubation without Ca^{2+} and with 2 mM EGTA. When hepatocytes were preincubated for 5 min with PMA, the effects of ATP and noradrenaline were prevented, and those of vasopressin and pancreastatin remained unchanged. The pretreatment of hepatocytes with pertussis toxin diminished the response to pancreastatin and vasopressin. These results suggest that pancreastatin is a new Ca^{2+} -mobilizing glycogenolytic hormone acting through a specific receptor which may involve both pertussis-toxin-sensitive and -insensitive GTP-binding regulatory proteins.

INTRODUCTION

We have previously shown that pancreastatin, a new pancreatic peptide [1], produces hepatic glycogenolysis both *in vivo* and *in vitro* [2,3]. We have also pointed out the Ca^{2+} -dependence of this effect in isolated hepatocytes [3]. The effect of a number of glycogenolytic hormones, such as vasopressin or noradrenaline, results from an increase in cytosolic free Ca^{2+} ($[\text{Ca}^{2+}]_i$) [4,5]. This Ca^{2+} mobilization has been shown to be triggered by $\text{Ins}(1,4,5)\text{P}_3$, which is produced by the hydrolysis of $\text{PtdIns}(4,5)\text{P}_2$ by phospholipase C following hormonal stimulation (for reviews see [6,7]). On the other hand, Ca^{2+} -mobilizing hormones stimulate Ca^{2+} influx across the liver cell plasma membrane [8–10], an effect that is essential for the sustained phase of elevated $[\text{Ca}^{2+}]_i$ [5] and that requires the activation of a G-protein [11], but involves neither protein kinase C [12,13] nor cyclic AMP [14].

The purpose of this study was to determine the effect of pancreastatin on free cytosolic Ca^{2+} . The origin of the Ca^{2+} mobilization involved in the $[\text{Ca}^{2+}]_i$ has been analysed by examining the dependence of the response on external Ca^{2+} . We have also correlated changes in glucose production by hepatocytes and the elevation of $[\text{Ca}^{2+}]_i$. Vasopressin, noradrenaline and ATP were employed as positive controls, since they are well-known Ca^{2+} -mobilizing agents in hepatocytes. The modulation of the Ca^{2+} responses by prazosin, 4 β -phorbol 12 β -myristate 13 α -acetate (PMA) and pretreatment with pertussis toxin was also examined to study further the mechanism of action of pancreastatin.

MATERIALS AND METHODS

Isolated hepatocytes were prepared from male fed Wistar rats (180–220 g) as described previously [3]. Glucose production was determined by measuring glucose concentration in the

extracellular medium, as described previously [3]. Cells (10 mg dry wt./ml) were loaded with Fluo-3/AM {1-[2-amino-5-(2,7-dichloro-6-hydroxy-3-oxy-9-xanthenyl)-phenoxy]-2-[2-amino-5-methylphenoxy]ethane-*NNN'*-tetra-acetic acid (methyl ester)} [15] as reported elsewhere [16] in modified Krebs–Henseleit buffer (120 mM NaCl, 5.4 mM KCl, 4.2 mM NaHCO_3 , 1.2 mM KH_2PO_4 , 1.3 mM MgSO_4 , 1.3 mM CaCl_2), supplemented with 2.75 mM glucose, 0.1% BSA and 20 mM Hepes, for at least 30 min at room temperature. The cells were washed free of extracellular Fluo-3. A batch of Fluo-3-loaded hepatocytes (equivalent to 1 mg dry wt./ml), suspended in the same buffer, was transferred to a quartz cuvette maintained at 37 °C with constant stirring, and the fluorescence emission was recorded with a spectrofluorimeter (Hitachi F-2000). Excitation and emission fluorescence were at 506 and 526 nm respectively. Cellular autofluorescence accounted for less than 10% of the total signal. The slow leakage of Fluo-3 into the medium was less than 10% over 60 min. The traces shown are representative for at least three different cell preparations. The calibration procedure includes measurement of F_{max} and F_{min} , which were obtained by disruption of the cells with digitonin and subsequent addition of 2 mM EGTA, made up in 0.2 M NaOH, respectively. $[\text{Ca}^{2+}]_i$ was calculated from the equation:

$$[\text{Ca}^{2+}]_i = K_d(F - F_{\text{min}})/(F_{\text{max}} - F)$$

where K_d , the dissociation constant for Ca^{2+} -bound Fluo-3, is 400 nM and F represents the experimental fluorescence.

Pertussis-toxin treatment of hepatocytes was conducted in Krebs–Henseleit buffer containing 20 mM Hepes, 2% BSA and 2 μg of pertussis toxin/ml for 4 h [17] before washing and loading the hepatocytes with Fluo-3. It should be pointed out that, as a measure of the degree of ADP-ribosylation [17], 4 h incubation with pertussis toxin was found to be optimal to block

Abbreviations used: $[\text{Ca}^{2+}]_i$, cytosolic free $[\text{Ca}^{2+}]$; PMA, 4 β -phorbol 12 β -myristate 13 α -acetate.

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completely the inhibitory effect of angiotensin II on cyclic AMP production. Control hepatocytes, incubated under the same conditions but in the absence of the toxin, had the same basal $[Ca^{2+}]_i$ levels as the treated hepatocytes.

Fluo-3/AM was purchased from Molecular Probes (Eugene, OR, U.S.A.). Rat pancreastatin, pig pancreastatin and its 33–49 C-terminal fragment were obtained from Peninsula Laboratories Europe (Merseyside, U.K.), and ATP, noradrenaline, prazosin, $[\beta\text{-mercapto-}\beta\beta'\text{-cyclopentamethylenepropionyl}^1, O\text{-Me-Tyr}^2, \text{Arg}^8]\text{vasopressin}$ (V_1 antagonist) and pertussis toxin from Sigma Chemical Co. (St. Louis, MO, USA). $[\text{Arg}^8]\text{vasopressin}$ was from Boehringer Mannheim (Mannheim, Germany).

RESULTS

Pancreastatin increases $[Ca^{2+}]_i$: effect of extracellular Ca^{2+}

The effects of a maximally effective dose of pancreastatin ($0.1 \mu\text{M}$) on $[Ca^{2+}]_i$, as well as those of other known Ca^{2+} -dependent hormones ($1 \mu\text{M}$ noradrenaline, $0.1 \mu\text{M}$ vasopressin and $10 \mu\text{M}$ ATP) are shown in Figure 1. Pancreastatin increases $[Ca^{2+}]_i$ from basal values (150 nM) to 700 nM within 3 s, with a maximum at 10 s, followed by a slow decrease for at least 2 min, and levels remained above unstimulated values for up to 10 min (results not shown). This pattern was the same as that produced by other Ca^{2+} -dependent hormones, such as vasopressin and noradrenaline. ATP produced a lower increase, which reached a peak at 400 nM and lasted for 1.5–2 min. The effect of extracellular Ca^{2+} on Ca^{2+} transients was studied either by resuspending Fluo-3-loaded hepatocytes in a medium containing no added Ca^{2+} or by preincubation for 15 min in medium lacking Ca^{2+} and containing 2 mM EGTA (Figure 1). In the first case,

Figure 1 Measurement of intracellular Ca^{2+} mobilization by using Fluo-3

Isolated rat hepatocytes were loaded with Fluo-3. $[Ca^{2+}]_i$ was calculated from Fluo-3 fluorescence as described in the Materials and methods section. The cells were incubated at 37°C in the stirred cuvette chamber of the fluorimeter for at least 3 min before addition of hormones to give $0.1 \mu\text{M}$ rat pancreastatin (PST; traces in **a**), $10 \mu\text{M}$ ATP (ATP; trace in **b**), $0.1 \mu\text{M}$ vasopressin (VP; traces in **c**), and $1 \mu\text{M}$ noradrenaline (NA; trace in **d**). The incubation medium contained 1.3 mM CaCl_2 . The dependence of pancreastatin and vasopressin on external Ca^{2+} was analysed by measuring Ca^{2+} transients in the presence of 1.3 mM CaCl_2 ($+Ca^{2+}$) or in medium lacking calcium ($-Ca^{2+}$); EGTA refers to hepatocytes preincubated for 15 min in medium lacking Ca^{2+} and containing 2 mM EGTA. The Figures are hand-drawn traces of the actual chart records.

Figure 2 Dose-response of the pancreastatin-induced increase in glucose release and $[Ca^{2+}]_i$

Glucose release was measured as described in the Materials and methods section for 10 min, and $[Ca^{2+}]_i$ changes were measured as described in the legend to Figure 1. The control rate of glucose production was $90 \pm 7 \text{ nmol/10 min per mg of protein}$. The hormones ($\bullet$, vasopressin; $\circ$, rat pancreastatin; $\triangle$, pig 33–49 pancreastatin; $\blacktriangle$, pig 1–49 pancreastatin) were added after 5 min preincubation. In **(a)** the increase in glucose release produced by the indicated concentrations of hormones is shown. In **(b)** the maximal $[Ca^{2+}]_i$ achieved for each dose of hormone is shown. Values are means $\pm$ S.E.M. from four separate experiments.

the amplitude of the response to pancreastatin and the other hormones was decreased to about 50%. Preincubation with 2 mM EGTA and further incubation without external Ca^{2+} completely abolished the response to the hormones. It is assumed that these experimental conditions produce a depletion of the intracellular Ca^{2+} stores.

Dose-response effect on pancreastatin on $[Ca^{2+}]_i$ and glucose production

To study further the changes in $[Ca^{2+}]_i$ caused by pancreastatin and to correlate these changes produced by Ca^{2+} -mobilizing hormones with the glycogenolytic effect, we have determined the dose-response to homologous (rat) pancreastatin and pig pancreastatin (1–49 and 33–49), as well as the dose-response to vasopressin of the maximal $[Ca^{2+}]_i$ values, and the extent of glucose production induced by different concentrations of these hormones. Figure 2(b) shows changes in $[Ca^{2+}]_i$ at 10–15 s in response to increasing concentration of the hormones (from

Table 1 Effect of α_1 -adrenergic antagonist prazosin and V_1 -vasopressin antagonist on hormone-induced changes in $[\text{Ca}^{2+}]_i$

Fluo-3-loaded hepatocytes were preincubated for 1 min in the absence (control) and in the presence of either prazosin ($10 \mu\text{M}$) or V_1 -antagonist ($1 \mu\text{M}$) before addition of $0.1 \mu\text{M}$ rat pancreastatin, $0.1 \mu\text{M}$ vasopressin or $1 \mu\text{M}$ noradrenaline. Results, given in nM Ca^{2+} , are means $\pm$ S.E.M. of at least three hepatocyte preparations.

	$[\text{Ca}^{2+}]_i$ (nM)		
	Pancreastatin	Vasopressin	Noradrenaline
Control	730 ± 40	500 ± 30	690 ± 36
Prazosin	725 ± 30	740 ± 32	158 ± 15
V_1 -antagonist	727 ± 21	150 ± 12	698 ± 23

Figure 3 Effect of PMA on the hormone-induced increase in hepatocyte $[\text{Ca}^{2+}]_i$

Fluo-3-loaded hepatocytes were preincubated with $1 \mu\text{M}$ PMA for 5 min before addition of $0.1 \mu\text{M}$ rat pancreastatin (PST), $0.1 \mu\text{M}$ vasopressin (VP), $1 \mu\text{M}$ noradrenaline (NA), or $10 \mu\text{M}$ ATP (ATP). Further experimental details are described in the legend to Figure 1.

Figure 4 Effect of pretreatment of hepatocytes with pertussis toxin on pancreastatin-induced increase in hepatocyte $[\text{Ca}^{2+}]_i$

For experimental details of pretreatment with pertussis toxin see the Materials and methods section. Traces show the changes in $[\text{Ca}^{2+}]_i$ after addition of $0.1 \mu\text{M}$ rat pancreastatin (PST) or $0.1 \mu\text{M}$ vasopressin (VP) to Fluo-3-loaded hepatocytes incubated in the presence (Ptx) or in the absence (Control) of pertussis toxin.

glucose production with similar half-maximal effective concentrations: 0.2 nM vasopressin, 0.3 nM rat pancreastatin, 0.8 nM 33–49 pancreastatin and 1 nM 1–49 pancreastatin.

Effects of the vasopressin antagonist V_1 , the α_1 -adrenergic blocker prazosin and PMA on pancreastatin-stimulated increases in $[\text{Ca}^{2+}]_i$

The effects of V_1 -antagonist ($10 \mu\text{M}$) and prazosin ($10 \mu\text{M}$) on the pancreastatin ($0.1 \mu\text{M}$)-induced increase in $[\text{Ca}^{2+}]_i$ were examined in Fluo-3-loaded hepatocytes, and compared with the effects on the noradrenaline ($10 \mu\text{M}$)- and vasopressin ($0.1 \mu\text{M}$)-induced increase in $[\text{Ca}^{2+}]_i$. When $1 \mu\text{M}$ V_1 -antagonist is present 1 min before addition of vasopressin, the increase in Ca^{2+} is completely abolished, whereas the vasopressin antagonist failed to modify the effect of pancreastatin. When $10 \mu\text{M}$ prazosin is present 1 min before addition of noradrenaline, the increase in Ca^{2+} is completely abolished, whereas the increases in Ca^{2+} caused by pancreastatin and vasopressin were not affected by prazosin (see Table 1).

The addition of the activator of protein kinase C [18] PMA to Fluo-3-loaded hepatocytes produces an inhibition of the ATP- as well as the noradrenaline-induced increase in $[\text{Ca}^{2+}]_i$. Figure 3 shows that treatment with $1 \mu\text{M}$ PMA for 5 min completely abolished the ability of ATP and noradrenaline to increase $[\text{Ca}^{2+}]_i$. However, the effect of pancreastatin, like that of vasopressin, was only slightly affected by treatment with PMA.

Effects of pertussis-toxin pretreatment on pancreastatin-stimulated increase in $[\text{Ca}^{2+}]_i$

It has been reported that vasopressin-stimulated Ca^{2+} entry involves a pertussis-toxin-sensitive GTP-binding regulatory protein [11]. Incubation of hepatocytes for 4 h with pertussis toxin ($2 \mu\text{g}/\text{ml}$) had no effect on basal $[\text{Ca}^{2+}]_i$ but inhibited in part the response of hepatocytes to pancreastatin and vasopressin. Figure 4 shows the time course of $[\text{Ca}^{2+}]_i$ after addition of pancreastatin or vasopressin, and the effect of pertussis-toxin pretreatment, which inhibited the response to pancreastatin and vasopressin to almost 50 %, without modifying basal levels of $[\text{Ca}^{2+}]_i$. When hepatocytes were resuspended in medium lacking Ca^{2+} , the Ca^{2+} transients stimulated by pancreastatin and vasopressin were not affected by pretreatment with the toxin (results not shown).

DISCUSSION

Glycogen metabolism is regulated by hormonal signals, which are mediated by two distinct mechanisms involving either cyclic AMP or Ca^{2+} (see [6] for review). We have recently described the glycogenolytic effect of pancreastatin, which is independent of the production of cyclic AMP and dependent on Ca^{2+} [3]. This study describes for the first time the mechanisms involved in the glycogenolytic effect of the peptide hormone pancreastatin on isolated rat hepatocytes. Indeed, we have monitored the changes in $[\text{Ca}^{2+}]_i$ under conditions which stress the glycogenolytic effect of this hormone in rat hepatocytes, providing experimental basis for the above statement.

The experimental evidence presented here strongly suggests that pancreastatin mediates an increase in the permeability of the plasma membrane of hepatocytes to extracellular Ca^{2+} , as well as a release of Ca^{2+} from internal stores. The increase in $[\text{Ca}^{2+}]_i$ following pancreastatin stimulation of isolated hepatocytes is very rapid and transient, and returned slowly to near-basal levels. The rapid first component of the response to Ca^{2+} -mobilizing hormones has been described as being caused by both

10 pM to $10 \mu\text{M}$), and Figure 2(a) shows changes in glucose production by the increasing concentration of the same hormones. Dose-response changes in $[\text{Ca}^{2+}]_i$ paralleled those of

the Ca^{2+} mobilization from internal stores, an action that is known to be linked to $\text{Ins}(1,4,5)\text{P}_3$ and by the entry of extracellular Ca^{2+} [7,8], whereas the slow second component is maintained by the stimulation of Ca^{2+} influx across the plasma membrane [19] and a Ca^{2+} -efflux component [20].

The fact that Ca^{2+} transients and the glycogenolytic response of isolated hepatocytes to pancreastatin, illustrated in the present work, are abolished after depletion of intracellular Ca^{2+} suggests that pancreastatin behaves as a Ca^{2+} -dependent glycogenolytic hormone. Results in Table 1 show that the effect of pancreastatin could be mediated by interaction with specific receptors, since the increase in $[\text{Ca}^{2+}]_i$ induced by pancreastatin was not inhibited by either the vasopressin antagonist V_1 or by the α -adrenergic antagonist prazosin, whereas they inhibited the response to vasopressin and noradrenaline, as expected [21]. On the other hand, the effects of pancreastatin on $[\text{Ca}^{2+}]_i$ and glucose release are comparable with those caused by vasopressin. The effect of treatment with PMA further supports this similarity, since PMA inhibited the response to ATP and noradrenaline, which is in accordance with previous studies [22–24], whereas a minor effect was observed in the response to vasopressin, as previously described [23,25] and to pancreastatin (see Figure 3).

The results obtained with pertussis toxin are quite different, depending on whether Ca^{2+} was present in or absent from the medium bathing the hepatocytes. In the presence of external Ca^{2+} pertussis toxin partially inhibited Ca^{2+} transients induced by both pancreastatin and vasopressin, indicating that a GTP-binding regulatory protein could be involved. The effect of pertussis toxin on vasopressin-stimulated Ca^{2+} transients is in agreement with previous work [11,26] and indicates that vasopressin stimulates receptor-activated Ca^{2+} inflow involving a G-protein, suggesting a similar mechanism for pancreastatin action. By contrast, in the absence of external Ca^{2+} , the response to both vasopressin and pancreastatin was not affected by the pertussis toxin, so suggesting a pertussis-toxin-insensitive mechanism. Pancreastatin stimulated PtdInsP_2 -phospholipase C (V. Sánchez-Margalet and R. Goberna, unpublished work), and the possibility remains to be studied that pertussis-toxin-insensitive and sensitive mechanisms can be ascribed to InsP_3 -induced Ca^{2+} release and InsP_3 - or InsP_4 -induced Ca^{2+} entry [27].

On the basis of the relative potency of rat pancreastatin and its related peptides in producing an increase in $[\text{Ca}^{2+}]_i$ and glycogenolysis in isolated rat hepatocytes (rat pancreastatin > pig 33–49 pancreastatin > pig 1–49 PST), it could be pointed out that pancreastatin may interact with specific receptors on the plasma membrane of hepatocytes. There is evidence to support the fact that pancreastatin stimulation of isolated hepatocytes results in mobilization of Ca^{2+} , involving both pertussis-toxin-

sensitive and -insensitive mechanisms. Further investigations are now in progress to characterize the pancreastatin receptor in rat liver membranes.

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