IS PROTEIN CARBOXYMETHYLATION INVOLVED IN STIMULUS–SECRETION COUPLING?

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1. Introduction

Enzyme-catalyzed transfer of methyl-groups from *S*-adenosylmethionine to acidic groups of proteins is well documented [1–4]. From an in vitro demonstration carboxymethylation of proteins in isolated chromaffin granules [5], in vivo studies were designed [6] to observe the effect of stimulation of amylase secretion from parotid glands by isoproterenol: (i) on the activity of *S*-adenosylmethionine–protein carboxylmethyltransferase (PCM); and (ii) on the methyl-group accepting activity of proteins (MAP) in parotid gland tissue. A significant increase was found in both activities with a maximum 30 min after application of isoproterenol [6]. Based on the observations in [5,6], a direct role of protein carboxymethylation in stimulus–secretion coupling has been postulated [7].

This hypothesis requires corroboration as protein carboxymethylation itself has not been studied in an intact secretory system.

We show that stimulation of secretion in the pancreas and parotid glands of the rat in vitro is not associated with changes of protein carboxymethylation nor the activities of PCM or MAP, and that the results in [6], although reproducible, are based on experimental error.

2. Materials and methods

S-Adenosyl-L-[*methyl*-^{14}C]methionine, L-[*methyl*-^{14}C]methionine and [^{3}H]methanol were obtained from Amersham-Buchler (Braunschweig). Ovalbumin, D,L-isoproterenol and carbamylcholine chloride were obtained from Sigma GmbH (Munich), pancreozymin from Kabi Diagnostica, (Studsvik). All other reagents were of analytical grade and obtained from Merck (Darmstadt). Male Wistar rats (140–160 g) came from MUS-Rattus GmbH (Munich).

Protein carboxylmethyltransferase was purified from calf brain as in [8]. The specific activity of our preparation was 10 000 U/mg (1 unit is defined as 1 pmol of protein carboxy[^{3}H]methyl-ester formed/min at 37°C with ovalbumin as substrate).

2.1. *In vivo experiments*

These were done as in [6]. The animals were anesthetized i.p. with 50 mg pentobarbital/kg. They then received i.p. 8 mg D,L-isoproterenol/kg in 1 ml 0.18 M NaCl or the same volume of 0.18 M NaCl. The parotid glands were excised 30 min later and homogenized as in [6]. Aliquots of the homogenates were used for the determination of PCM and MAP activities as described below.

2.2. *In vivo experiments with pancreatic lobules and parotid gland tissue*

Rats were anesthetized i.p. with 50 mg pentobarbital/kg. Pancreatic lobules were prepared as in [9]. Lobules corresponding to ~100 mg wet wt were incubated in 3 ml of KRH-buffer (0.32 M sucrose, 50 mM Tris, 25 mM KCl, 3 mM $MgCl_2$, 2 mM $CaCl_2$, pH 7.4) equilibrated with O_2. Parotid glands were excised and prepared under stereoscopic control. They were cut into small pieces of ~1 mm^3. Portions of ~100 mg wet wt were layered in small nylon baskets and incubated in 1.6 ml O_2-equilibrated Krebs–Ringer–Tris buffer (pH 7.4) containing 5 mM D,L-3-hydroxybutyrate [10].

2.3. *Determination of protein carboxymethylation in vitro*

Incubations with [*methyl*-^{14}C]methionine were done as in fig.1,2. They were stopped, after removal of the incubation medium, by immediate addition of 1 ml 10% (w/v) ice-cold trichloroacetic acid, followed by homogenization in a small all-glass Potter homogenizer. The homogenate was spun (10 000 × *g*, 4 min), the supernatant discarded, and the pellet, after 2 additional washes with 10% trichloroacetic acid, suspended in 1 ml 1 M sodium borate (pH 11). [^{3}H]Methanol (0.1 μCi) was added as an internal standard. Then 0.5 ml 50% trichloroacetic acid was added, followed by centrifugation (10 000 × *g*, 10 min). The supernatant was neutralized with 2 N KOH. One ml was transferred into a 2 ml distillation vial, 0.5 ml unlabeled methanol and a drop of octanol (as anti-foaming reagent) were added and the vial connected to a Wheaton micro-distillation apparatus. It was heated at 110°C and the distillate collected in an ice-cooled recipient vial. When the distillation temperature had fallen from 70–55°C, 1 ml methanol was added and the distillation repeated. Aliquots of the combined distillates were counted in a toluene-based scintillator for ^{3}H and ^{14}C radioactivity. The loss of methanol during the whole procedure was calculated from the recovery of [^{3}H]methanol.

2.4. *Determination of MAP activity*

Parotid gland or pancreatic tissue prepared as above was homogenized in 0.3 M sucrose. Aliquots (0.1 ml) of the homogenates were added to 0.2 ml 0.1 M potassium phosphate (pH 6.0) containing 20 μM (final conc.) *S*-adenosyl-[*methyl*-^{14}C]methionine (spec. act. 167 μCi/μmol). The reaction was started by adding 5 units purified protein carboxylmethyltransferase, stopped after 15 min by the addition 1 ml 10% trichloroacetic acid, and the formation of protein carboxymethyl-esters determined as in [11].

2.5. *Determination of PCM activity*

Tissue preparation, incubation and determination of protein carboxymethyl-ester formation were carried out as described for MAP activity, only purified protein carboxylmethyltransferase was omitted and 2 mg ovalbumin added as substrate for the endogenous protein carboxylmethyltransferase.

2.6 *Determination of DNA and α-amylase activity*

DNA was determined as in [12] and α-amylase activity as in [13].

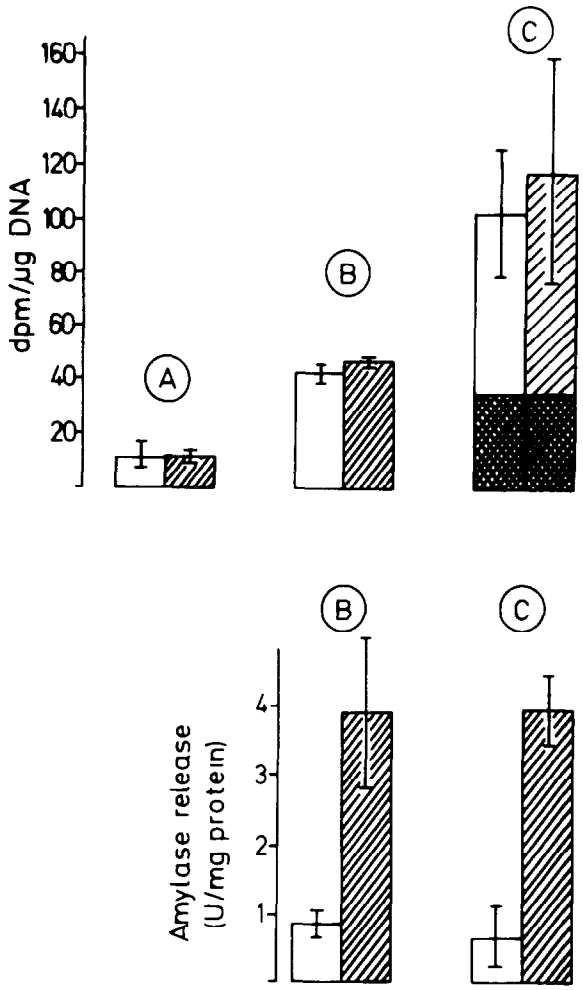

Fig.1. Formation of protein carboxy[^{14}C]methyl-esters from L-[*methyl*-^{14}C]methionine and release of α-amylase by isolated pancreatic lobules from overnight fasted rats. In groups (A) and (B), 5 μCi L-[*methyl*-^{14}C]methionine were added to the medium at zero-time. The final concentration of L-methionine was 0.1 mM, the specific radioactivity 16.7 μCi/μmol. When a stimulus (10^{-6} M pancreozymin) was added (hatched columns) it was added immediately after the addition of the radioactive methionine. The degree of protein carboxy[^{14}C]-methyl-ester formation was measured 5 min (A) or 30 min (B) after addition of the radioactive methionine. The cumulative release of α-amylase after 30 min is likewise shown. In group (C), the pancreatic lobules were first preincubated for 20 min with the same amount of L-[*methyl*-^{14}C]methionine as in (A) and (B). Thereafter the experiment was continued for another 20 min in the presence (hatched column) or absence (open column) of 10^{-6} M pancreozymin. The formation of protein carboxy[^{14}C]methyl-esters during the preincubation is indicated by the black columns. The cumulative release of α-amylase during the 20 min period is likewise shown (C). The data represent mean values ± SD; n = 3 for each time point and each condition.

3. Results and discussion

Figures 1 and 2 indicate that in vitro stimulation of amylase secretion from rat parotid glands or exocrine pancreas was not accompanied by a significant increase in protein carboxymethylation, irrespective of whether an early time point (5 min) or a later time point (20–30 min) after addition of the stimulus was selected. In vitro stimulation by carbamylcholine did not significantly affect the activities of PCM or MAP in pancreatic lobules, although amylase secretion was significantly enhanced (fig.3).

The in vivo experiments seem to confirm [5] in that they show a significant increase of PCM and MAP activities of the parotid glands following isoproterenol treatment (fig.4). However, this is so only if referring

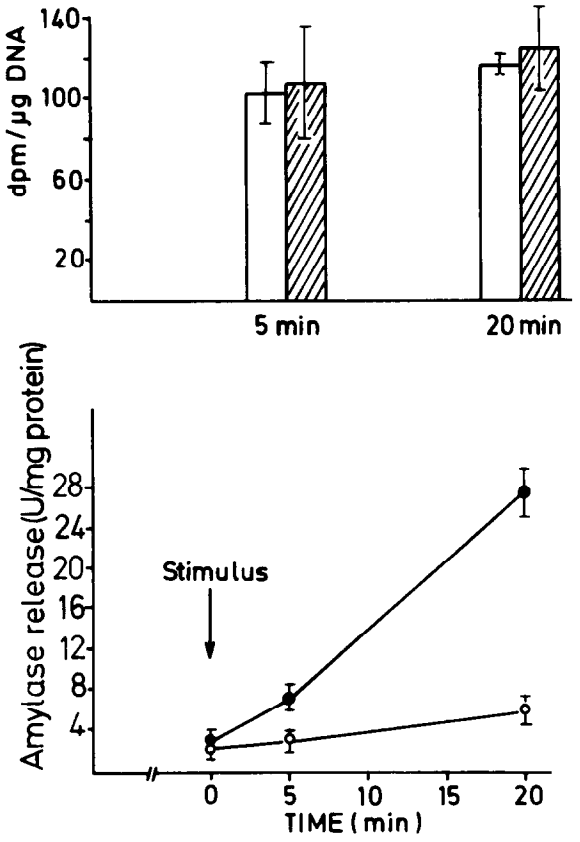

Fig.2. Effect of isoproterenol on protein carboxymethylation in parotid gland slices. Parotid gland slices were preincubated at 37°C for 30 min in the presence of 0.06 mM L-[*methyl*-^{14}C]methionine (spec. act. 53 µCi/µmol). Thereafter, isoproterenol (2×10^{-5} M final conc.) was added, and secretion of α-amylase and protein carboxymethylation determined 5 and 20 min later using method (B): open columns and closed circles, stimulated; open columns and open circles, controls; data represent mean values ± SD; $n = 3$ for each condition.

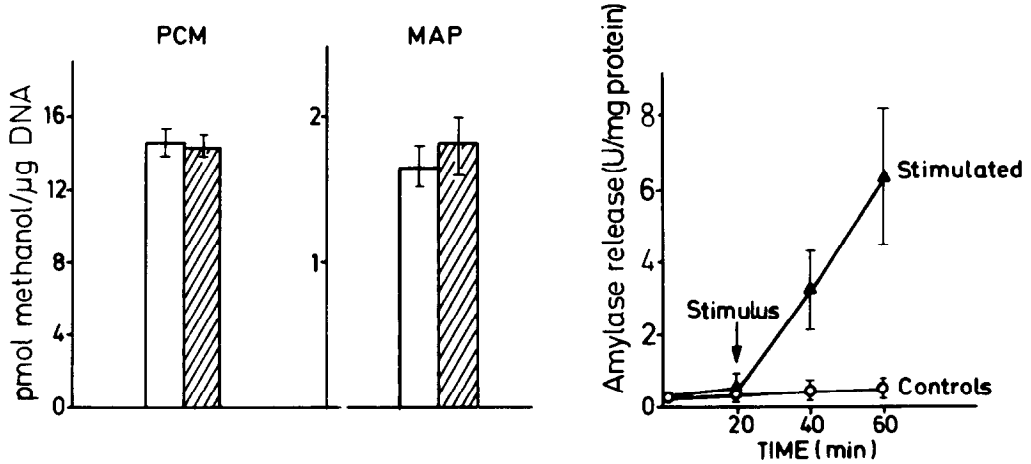

Fig.3. Effects of stimulation of isolated pancreatic lobules from overnight-fasted rats by 6×10^{-5} M carbamylcholine on *S*-adenosylmethionine–protein carboxylmethyltransferase activity (PCM), methyl-group accepting activity of protein carboxy groups (MAP), and release of α-amylase. Preparation and incubation of isolated pancreatic lobules was done as in section 2. The incubation was stopped 40 min after the addition of carbamylcholine: open columns, controls; hatched columns, stimulation by carbamylcholine; data represent mean values ± SD; $n = 3$ for each condition.

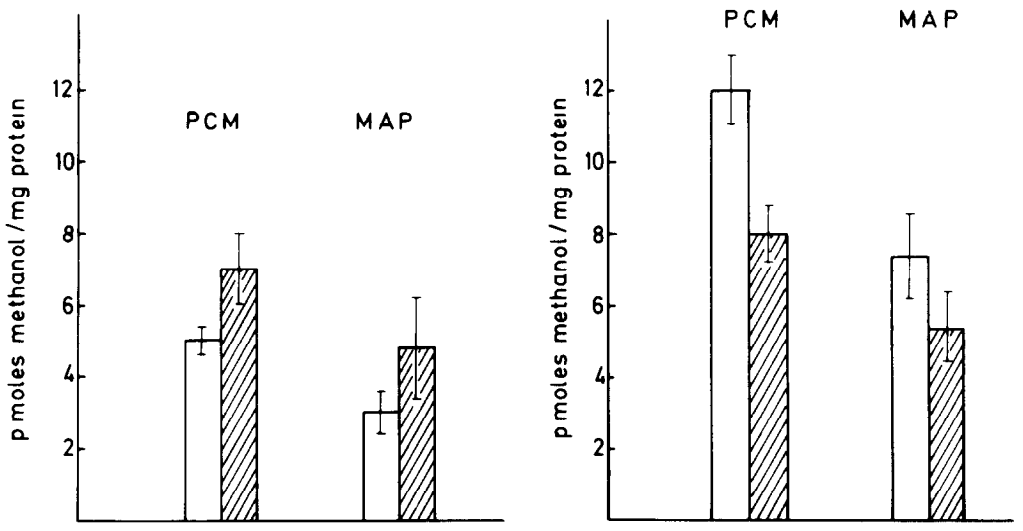

Fig.4. Effects of in vivo treatment of overnight-fasted rats with 8 mg isoproterenol/kg i.p. or 0.16 M saline i.p. on *S*-adenosylmethionine–protein carboxylmethyltransferase activity (PMC) and methyl-group accepting activity of protein carboxy groups (MAP) in rat parotid glands. The animals were anesthetized with 50 mg pentobarbital/kg i.p. prior to stimulation. 30 min after injection of isoproterenol (or saline) the parotid glands were removed and prepared for the determination of PCM, MAP, protein, and DNA. PCM and MAP were related either to protein (left-side) or to DNA (right-side): open columns, saline-treated animals; hatched columns, isoproterenol-treated animals; data represent mean values ± SD; $n = 6$ for each group.

the activities to total protein. If the activities are expressed on the basis of DNA measurements it turns out that stimulation by isoproterenol rather decreased PCM and MAP activities.

This discrepancy is easily explained: In our experiments the amylase content of the parotid glands from the isoproterenol-treated rats was considerably lower than in the control animals, indicating that the parotid glands had lost substantial amounts of protein after stimulation; the protein/DNA ratio decreased from 238 in the control animals to 112 in the isoproterenol-treated rats, which means that isoproterenol had led to a loss of almost 50% of the total parotid gland protein. Under these circumstances it is not valid to relate PCM or MAP activities to protein as in [5]. These results contradict the hypothesis [7,14] that changes in protein carboxymethylation are directly involved in stimulus–secretion coupling.

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