# PROTEIN CARBOXYMETHYLATION IN RAT ISLETS OF LANGERHANS

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## 1. Introduction

Reversible methylation is a post-translocational modification of proteins that plays an important role in regulatory processes in both prokaryotes and eukaryotes [1,2]. The free carboxyl groups of methyl acceptor proteins (MAPs) can be esterified by methyl groups derived from *S*-adenosylmethionine (SAM) in a reaction catalysed by protein carboxymethylase (PCM).

In eukaryotes, protein carboxymethylation has been implicated in sperm mobility [3], leucocyte chemotaxis [4] and neural function [5]. Several studies have also suggested that protein carboxymethylation may be involved in stimulus–secretion coupling. Secretory tissues for which support for this hypothesis has been obtained include adrenal medulla [6], parotid [3] and exocrine pancreas [7] (but see also [8]). In addition it has been shown that calmodulin is a notably good substrate for PCM and that methylation of calmodulin diminishes its ability to activate cyclic nucleotide phosphodiesterase [9]. Since there is a growing body of evidence to implicate calmodulin in $Ca^{2+}$-dependent stimulus–secretion coupling [10–12], PCM could also be linked to secretion via calmodulin methylation.

There is no information as to whether these ideas may be applicable to insulin secretion. Here, we demonstrate the presence of PCM and endogenous MAPs in rat islets of Langerhans and present data consistent with a role for protein carboxymethylation in the regulation of islet function.

## 2. Materials and methods

### 2.1. *Reagents*

Collagenase (type 1), swine skin gelatin (type 1), 3-isobutyl-1-methylxanthine (IBMX) and bovine albumin were from Sigma (London) Chemical Co. (Poole, Dorset). *S*-Adenosyl-L-[*methyl*-$^3$H]methionine (72 Ci/mmol) and L-[*methyl*-$^3$H]methionine (93 Ci/mmol) were from the Radiochemical Centre (Amersham, Bucks). Other reagents, of the purest grade available, were from British Drug Houses Chemicals (Poole, Dorset). Calmodulin, prepared from bovine brain, was a gift from Dr M. P. Esnouf of this department.

### 2.2. *Isolation of islets*

Islets of Langerhans were isolated from pancreases of fed male Wistar rats by a collagenase method [13].

### 2.3. *Assay of PCM*

PCM activity in pancreatic islet homogenates was assayed using [*methyl*-$^3$H]SAM as the methyl donor and pig skin gelatin as MAP as in [14] with minor modifications. Islets were homogenised by sonication (1 min at position 1 on a Soniprobe, Dawe Instr.) in ice-cold 0.2 mol/l sodium acetate buffer (pH 6.0). The reaction mixture (final vol. 40 μl) contained 0.2 mol/l sodium acetate buffer (pH 6.0); 0.556 μmol/l [$^3$H]SAM; 0.4 mmol/l EDTA-$Na_2$; 0.75 mmol/l 2-mercaptoethanol, 12.5 mg/ml pig skin gelatin and islet homogenate corresponding to ~15 islets. In experiments to study methylation of endogenous islet proteins, the gelatin was omitted; and in some experiments gelatin was replaced by exogenous calmodulin (250 μg/ml). Following incubation at 37°C for the times indicated in the text or tables, the reaction was terminated by the addition of 1 ml trichloroacetic acid 10% (w/v). After centrifugation the precipitated protein was taken up in 0.4 ml 1 mol/l borate buffer (pH 11.0) containing 0.7% methanol (v/v). After 15 min at room temperature to permit hydrolysis of the protein methylesters, 0.1 ml aliquots were transferred to 1 ml plastic tubes and then placed in stop-

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pered scintillation vials containing 1 ml methanol. The radioactive methanol in the tubes was selectively recovered in the outer vials by allowing the vials to equilibrate at 37°C overnight. The radioactive methanol thus recovered was counted by liquid scintillation spectrometry using an external standard method to correct for quenching. Control experiments showed that the recovery in the outer vials of radioactive methanol originally present in the inner tube was >90%.

2.4. *Effect of incubation of islets on PCM activity*

In these experiments, islets were incubated under various conditions before homogenisation and assay as above. The incubations were carried out at 37°C for 95 min Krebs–Henseleit bicarbonate medium [15] containing 2 mg/ml albumin and continually gassed with $O_2$:$CO_2$ (95:5). The medium contained either 3.3 or 20.0 mmol/l glucose with or without 1 mmol/l IBMX. After incubation, the medium was removed and the islets washed twice in sodium acetate buffer, gently centrifuged (1 min at position 1 on an MSE bench centrifuge), disrupted by sonication in 25–50 μl sodium acetate buffer and then assayed for PCM activity as above.

2.5. *Protein carboxymethylation in intact islets*

Islets were incubated as above but in the presence of 10 mmol/l L-[*methyl*-$^3$H]methionine. After incubation, the islets were washed twice in albumin-free incubation medium and then sonicated in the same medium. Proteins were precipitated by the addition of 1 ml trichloroacetic acid. The precipitate was taken up in 0.4 ml borate buffer containing methanol and an aliquot counted to determine total incorporation of [$^3$H]methionine into islet proteins. The protein carboxymethyl esters were hydrolysed and quantified as in section 2.3.

2.6. *Analysis of results*

The statistical significance of data obtained was assessed by Student's *t*-test for unpaired data.

## 3. Results

When islet homogenates were incubated with [*methyl*-$^3$H]SAM and gelatin, label was incorporated into trichloroacetic acid-precipitable material. That the incorporation was into carboxyl groups of gelatin was shown by the solubilization of the radioactivity in the trichloroacetic acid-precipitated material by mild alkaline treatment and its subsequent recovery as [$^3$H]methanol. The isotopic equilibration method introduced here to recover the [$^3$H]methanol is similar in principle to a technique previously successfully used to separate and assay $^3H_2O$ from mixtures of non-volatile compounds [16]. The recovery of [$^3$H]-methanol was >90%.

Carboxymethylation of gelatin catalysed by islet PCM was linear for 1 h (fig.1) and proportional to the amount of islet tissue used (table 1). In the absence of exogenous substrate, carboxymethylation of endogenous protein substrate(s) could be demonstrated (table 1) although rates were low compared to that seen in the presence of a saturating concentration of gelatin. Addition of calmodulin also enhanced protein carboxymethylation (table 1). The increased incorporation was presumably into calmodulin since under the conditions used (absence of $Ca^{2+}$ and presence of

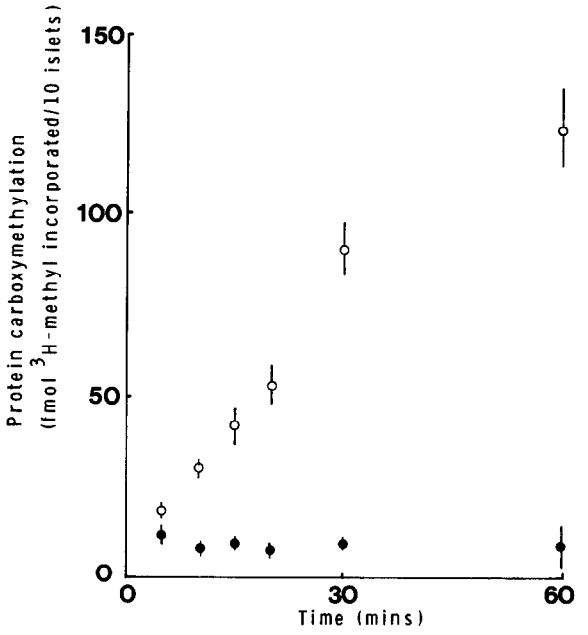


Fig.1. PCM activity in islet homogenates: time course of methylation of gelatin. Aliquots of islet homogenate were incubated with [*methyl*-$^3$H]SAM and gelatin for the times in section 2. The gelatin was precipitated by trichloroacetic acid and the radioactive carboxymethyl groups after hydrolysis by alkali were recovered as [$^3$H]methanol and counted by liquid scintillation spectrometry. Results are given as mean ± SEM for 5 obs. The open symbols (○) represent the experimental points with islet homogenate and the closed symbols (●) are control incubations without homogenate.

Table 1
PCM activity of islet homogenates

| Substrate | No. of islets | Incubation time (min) | Protein carboxymethylation (fmol) | Protein carboxymethylation (fmol . islet$^{-1}$ . min$^{-1}$ − blank) |
|---|---|---|---|---|
| Gelatin (12.5 mg/ml) | 0 | 15 | 9.3 ± 1.3 (12) | – |
| Gelatin (12.5 mg/ml) | 2.5 | 15 | 17.4 ± 1.6 (6) | 0.22 ± 0.04 (6) |
| Gelatin (12.5 mg/ml) | 5 | 15 | 39.0 ± 7.0 (6) | 0.34 ± 0.09 (6) |
| Gelatin (12.5 mg/ml) | 10 | 15 | 95.2 ± 10.9 (6) | 0.57 ± 0.07 (6) |
| Gelatin (12.5 mg/ml) | 20 | 15 | 153.2 ± 23.6 (10) | 0.48 ± 0.08 (10) |
| Gelatin (12.5 mg/ml) | 40 | 15 | 238.3 ± 24.4 (10) | 0.38 ± 0.04 (10) |
| Gelatin (12.5 mg/ml) | 0 | 30 | 9.4 ± 0.9 (3) | – |
| Gelatin (12.5 mg/ml) | 30 | 30 | 519.0 ± 22.0 (4) | 0.57 ± 0.02 (4) |
| Endogenous only | 0 | 30 | 8.9 ± 0.2 (3) | – |
| | 30 | 30 | 18.5 ± 1.3 (7) | 0.01 ± 0.001 (7) |
| Calmodulin (15 μM) | 0 | 30 | 8.4 ± 0.3 (3) | – |
| | 30 | 30 | 82.7 ± 5.7 (3) | 0.08 ± 0.006 (3) |

Aliquots of islet homogenate corresponding to the number of islets given were incubated at 37°C for 15 or 30 min as indicated, with [*methyl*-$^3$H]SAM in acetate buffer (pH 6). The extent of protein carboxymethylation was determined using either endogenous substrate only or exogenous gelatin or calmodulin. Incorporated [$^3$H]methyl was liberated as [$^3$H]methanol by alkaline hydrolysis and collected and counted as in section 2. Data are given as mean ± SEM for the number of observations in parentheses

EDTA) a stimulatory effect of calmodulin on PCM seems unlikely.

Two approaches to assess the relevance of protein carboxymethylation to insulin release were tried. In the first of these the amount of extractable PCM activity was compared in islets incubated under basal or stimulated conditions. The PCM activity in islets incubated with 20 mmol/l glucose (0.65 ± 0.1 fmol . islet$^{-1}$ . min$^{-1}$, $n = 8$) was not significantly different from that in islets incubated with 3.3 mmol/l glucose (0.60 ± 0.01 fmol . islet$^{-1}$ . min$^{-1}$, $n = 8$). The addition of IBMX (1 mmol/l) to the incubation medium was without effect on extractable PCM activity at either high (0.46 ± 0.09 fmol . islet$^{-1}$ . min$^{-1}$, $n = 8$) or low (0.51 ± 0.05 fmol . islet$^{-1}$ . min$^{-1}$, $n = 8$) glucose concentration. The possibility that change may be obscured by reversal during extraction of islets could not be excluded.

A second approach was tried in which islets were incubated with [*methyl*-$^3$H]methionine to label intracellular SAM under basal and stimulatory conditions (table 2). The radioactivity incorporated into protein comprised methionine incorporated during de novo protein synthesis plus radioactivity incorporated into

Table 2
Effects of glucose and IBMX on protein carboxymethylation in intact islets

| Incubation conditions | | Incorporation of radioactivity from [*methyl*-$^3$H]methionine (fmol . 50 islets$^{-1}$ . 1.5 h$^{-1}$) | |
|---|---|---|---|
| Glucose (mmol/l) | IBMX (1 mmol/l) | Total incorp. | Protein carboxymethylation |
| 3.3 | – | 50.0 ± 7.8 (5) | 4.0 ± 0.6 (16) |
| 3.3 | + | 70.8 ± 11.3 (5) | 5.1 ± 1.0 (14) |
| 20.0 | – | 98.6 ± 13.1 (5)[a] | 4.4 ± 0.6 (16) |
| 20.0 | + | 209.8 ± 29.4 (5)[b] | 9.2 ± 1.4 (14)[b] |

[a] Significantly >3.3 mmol/l glucose; $P \leq 0.01$
[b] Significantly >3.3 mmol/l glucose + 1 mmol/l IBMX ($P \leq 0.01$) and 20 mmol/l glucose ($P \leq 0.01$)

Batches of 50 islets were incubated in Krebs bicarbonate medium containing L-[*methyl*-$^3$H]methionine and glucose or IBMX as stated for 1.5 h at 37°C. The total incorporation of radioactivity into protein was determined after precipitation of islet protein by trichloroacetic acid: the extent of protein carboxymethylation that had occurred was determined by alkaline treatment of total protein followed by recovery and counting of [$^3$H]-methanol liberated as in section 2. Data are given as mean + SEM for the number of observations in parentheses

endogenous MAPs by PCM. The total incorporation of label into protein was stimulated by high glucose as expected from [17] whereas the incorporation corresponding to carboxymethylation (~10% of total incorporation) was not enhanced by high glucose. IBMX affected neither protein synthesis nor protein carboxymethylation in the presence of 3.3 mmol/l glucose. However IBMX with 20 mmol/l glucose further enhanced incorporation into total protein and doubled the rate of protein carboxymethylation. The mechanisms involved are unclear: neither glucose nor IBMX affect PCM activity in homogenates of islets and cyclic AMP was also without effect (not shown).

## 4. Discussion

This study demonstrates that rat islets of Langerhans contain PCM activity and endogenous substrates for such activity. Using gelatin as a substrate, islet homogenate PCM activity had a mean value of 0.37 ± 0.02 fmol . islet$^{-1}$ . min$^{-1}$ ($n$ = 63). The mean islet protein content in these experiments was 0.2 μg/islet. Hence the PCM was 1.8 fmol . min$^{-1}$ . μg protein$^{-1}$. This may be compared with reported values for adrenal medulla (3 fmol . min$^{-1}$ . μg protein$^{-1}$) [6], pituitary (4.5 fmol . min$^{-1}$ . μg protein$^{-1}$) [14] and for a supernatant fraction of rat pancreas (1.5 fmol . min$^{-1}$ . μg protein$^{-1}$) [7].

Rates of carboxymethylation of endogenous proteins were some 2% of the gelatin-assayed PCM activity under the conditions used.

Exogenous calmodulin could be carboxymethylated by islet extracts in agreement with studies on other tissues [9]. Calmodulin has been implicated in the regulation of insulin secretion [10,18], and the activity of calmodulin has been shown to be modulated by carboxymethylation [9]. It is therefore conceivable that carboxymethylation of calmodulin could be involved in regulation of secretion. Protein carboxymethylation occurred in the intact islets and was increased by the combination of high glucose and IBMX.

These data raise the possibility that protein carboxymethylation may play a role in islet stimulus–secretion coupling. The nature of the endogenous MAPs in islets of Langerhans requires investigation.

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