

Cyclic nucleotide phosphodiesterase of rat pancreatic islets

Effects of Ca^{2+} , calmodulin and trifluoperazine

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(Received 12 March 1981/Accepted 20 March 1981)

Cyclic nucleotide phosphodiesterase activity towards cyclic AMP and cyclic GMP was studied in extracts of rat islets of Langerhans. Biphasic Eadie plots [Eadie (1942) *J. Biol. Chem.* **146**, 85–93] were obtained with either substrate suggesting the presence of both 'high' and 'low'- K_m components. The apparent K_m values were 6.2 ± 0.5 ($n = 8$) μM and 103.4 ± 13.5 (6) μM for cyclic AMP and 3.6 ± 0.3 (12) μM and 61.4 ± 7.5 (13) μM for cyclic GMP. With cyclic AMP as substrate, phosphodiesterase activity was increased by calmodulin and Ca^{2+} and decreased by trifluoperazine, a specific inhibitor of calmodulin. With cyclic GMP as substrate, phosphodiesterase activity was decreased by omission of Ca^{2+} or addition of trifluoperazine. Addition of exogenous calmodulin had no effect on activity. The data suggest that Ca^{2+} may influence the islet content of cyclic AMP and cyclic GMP via effects on calmodulin-dependent cyclic nucleotide phosphodiesterase(s).

A rise in the intracellular concentration of free Ca^{2+} is believed to trigger insulin release by the pancreatic β -cell. The mediator of the regulatory effects of Ca^{2+} on insulin secretion may be the Ca^{2+} -dependent regulator calmodulin. Thus glucose-stimulated insulin release is inhibited by trifluoperazine, a specific inhibitor of calmodulin function. This inhibition is not due to impairment of islet viability or glucose utilization (Sugden *et al.*, 1979; Gagliardino *et al.*, 1980).

The role of calmodulin in insulin secretion is not known. Calmodulin has been implicated in the regulation of brain adenylate cyclase (Brostrom *et al.*, 1975) and phosphodiesterase (Cheung, 1970; Kakiuchi *et al.*, 1970) and one possible site of action for calmodulin in insulin secretion might therefore be regulation of islet cyclic nucleotide concentrations. It has recently been reported that islet adenylate cyclase is activated by calmodulin (Valverde *et al.*, 1979). The present experiments demonstrate that islet cyclic AMP phosphodiesterase is also stimulated by calmodulin. This stimulation is not observed in the absence of Ca^{2+} . Such activation may terminate the effects of secretagogues that act by stimulation of adenylate cyclase (for review, see Sharp, 1979) and may explain the transient nature of the increase in cyclic AMP observed when islets are exposed to glucose (Zawalich *et al.*, 1975). We also

report the existence of cyclic GMP phosphodiesterase activity in rat pancreatic islets. Kinetic constants for these activities were determined. Cyclic GMP phosphodiesterase activity was inhibited by trifluoperazine (a specific inhibitor of calmodulin; Levin & Weiss, 1977) or omission of Ca^{2+} , but was not activated by addition of exogenous calmodulin.

Experimental

Materials

Bovine albumin (fraction V), 5'-nucleotidase and collagenase (type I) were from Sigma (London) Chemical Co., Poole, Dorset, U.K. Cyclic AMP and cyclic GMP were from BCL, Lewes, East Sussex, U.K. All radioisotopes were obtained from The Radiochemical Centre, Amersham, Bucks., U.K. QAE-Sephadex was from Pharmacia (GB), London, U.K. Trifluoperazine (Stelazine) was a gift from Smith, Kline and French Laboratories. Other reagents, of the purest available grade, were from BDH Chemicals, Poole, Dorset, U.K. Calmodulin purified from skeletal-muscle phosphorylase kinase according to Cohen *et al.* (1978) was a gift from Dr. P. Cohen, Department of Biochemistry, University of Dundee, Dundee, Scotland, U.K. The ability of this preparation of calmodulin to activate brain

phosphodiesterase has been described in detail previously (Sugden *et al.*, 1979).

Preparation of islet extracts

Islets were obtained by collagenase digestion (Coll-Garcia & Gill, 1969) from the pancreases of 300 g male albino Wistar rats fed *ad libitum*. Islets (300) were disrupted in 300 μ l of 10 mM-Tris/HCl, pH 8, by sonication (3 \times 5 s on Soniprobe Dawe Instruments, point 1) and the extract was kept at 4°C until use.

Assay of cyclic nucleotide phosphodiesterase

The method for the measurement of cyclic nucleotide phosphodiesterase activity with cyclic AMP as substrate has been described in detail (Sugden *et al.*, 1979). The reaction mixture, final volume 100 μ l, contained 50 mM-Tris/HCl (pH 8), 3 mM-MgCl₂, 0.1 mg of albumin/ml, and cyclic [³H]AMP (1.3 μ M–0.2 mM; 5 μ Ci/ml). Ca²⁺, EGTA, trifluoperazine or calmodulin, where present, were at concentrations given in the text or Tables. Reaction was initiated by addition of an amount of islet extract corresponding to 15–20 islets and allowed to proceed for 45–60 min at 30°C. Reaction rates were linear for this period and were proportional to the amount of homogenate added. The reaction was terminated by boiling, and the [³H]AMP formed was converted into [³H]adenosine by 5'-nucleotidase. [³H]Adenosine was then separated from unchanged cyclic [³H]AMP by chromatography on columns (0.5 cm \times 1 cm) of QAE-Sephadex (formate form) and quantified by liquid-scintillation spectrometry as previously described (Sugden *et al.*, 1979). The procedure for measuring cyclic GMP phosphodiesterase activity was as described above but using cyclic [³H]GMP as substrate.

Results

Kinetic analysis of rat islet cyclic nucleotide phosphodiesterase activities

Previous studies have demonstrated that mouse (Ashcroft *et al.*, 1972) and guinea-pig (Sams & Montague, 1972) islets contain at least two forms of cyclic AMP phosphodiesterase. Kinetic analysis of the activity in rat islet homogenates measured in the present experiments also implies the existence of two enzyme forms. Initial rates of hydrolysis (v) of various concentrations of cyclic AMP ([S]) were plotted in the form v against $v/[S]$. Such plots were curvilinear, suggesting the presence in homogenates of two forms of phosphodiesterase with different K_m values for cyclic AMP (Fig. 1). Estimates of apparent K_m yielded mean (\pm S.E.M.) values of 6.2 ± 0.5 ($n = 8$) μ M and 103.4 ± 13.5 (6) μ M. Previous studies have reported K_m values for cyclic AMP phosphodiesterase ranging from 1 to 10 μ M

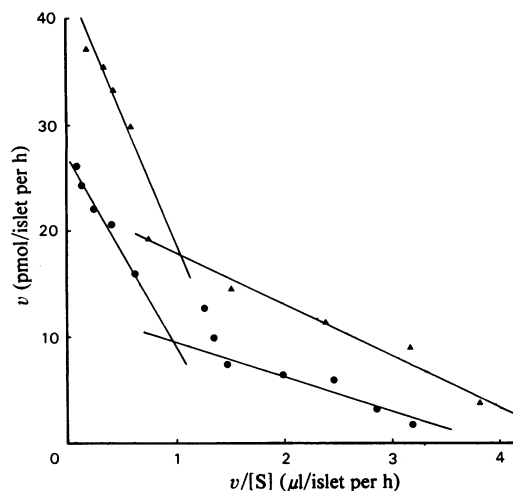


Fig. 1. Eadie plot for islet phosphodiesterase at various substrate concentrations

Islet cyclic nucleotide phosphodiesterase activity in the presence of 50 μ M-Ca²⁺ was determined as described in the Experimental section with various concentrations of cyclic AMP (\blacktriangle) or cyclic GMP (\bullet). The data are from a typical experiment. The mean K_m and V_{max} values were calculated from the intercepts of 8–13 such experiments.

and 20 to 500 μ M for low- and high- K_m activities respectively (Sams & Montague, 1972; Ashcroft *et al.*, 1972; Capito & Hedekov, 1974; Schubart *et al.*, 1974). Mean (\pm S.E.M.) values of V_{max} for the two phosphodiesterase activities were 29.3 ± 1.9 ($n = 8$) pmol/h per islet and 37.9 ± 3.2 (6) pmol/h per islet.

Analogous plots were drawn for rat islet phosphodiesterase activity with cyclic GMP as substrate (Fig. 1). Again curvilinear plots were obtained. The K_m value for the low- K_m form was 3.6 ± 0.3 (12) μ M [$V_{max} = 17.3 \pm 1.3$ (13) pmol/h per islet]. The K_m value of the high- K_m form was 61.4 ± 7.5 (13) μ M ($V_{max} = 29.6 \pm 4.7$ pmol/h per islet).

It is not known whether a single cyclic nucleotide phosphodiesterase catalyses the hydrolysis of both cyclic AMP and cyclic GMP. Attempts to separate the activities by DEAE-cellulose chromatography were not successful. Hydrolysis of [³H]cyclic AMP was, however, greatly inhibited by addition of unlabelled cyclic GMP, and conversely hydrolysis of [³H]cyclic GMP was inhibited by unlabelled cyclic AMP (results not shown). This suggests either that the cyclic nucleotide phosphodiesterases have a relatively broad substrate specificity or that the same enzymes may be responsible for the hydrolysis of both cyclic AMP and cyclic GMP. That the former

may be the case is suggested by the observations (see below) that exogenous calmodulin stimulated hydrolysis of cyclic AMP but not of cyclic GMP.

Effects of calmodulin on rat islet cyclic AMP phosphodiesterase activities

The effects of Ca^{2+} , trifluoperazine and exogenous calmodulin on islet cyclic AMP phosphodiesterase activity measured at $100\mu\text{M}$ -cyclic AMP are shown in Table 1. Addition of Ca^{2+} did not significantly affect islet cyclic AMP phosphodiesterase activity. Addition of trifluoperazine decreased islet cyclic AMP phosphodiesterase activity both in the presence and absence of added Ca^{2+} (by 22% and 11% respectively). It should be noted that the inhibitory action of trifluoperazine was greater in the presence of Ca^{2+} , consistent with inhibition of calmodulin function. The reason for inhibition of cyclic AMP phosphodiesterase activity by trifluoperazine in the absence of Ca^{2+} is not known. Cyclic GMP phosphodiesterase was not affected by trifluoperazine in the absence of Ca^{2+} (see below). Addition of exogenous calmodulin stimulated islet cyclic AMP phosphodiesterase activity in the presence of Ca^{2+} but not in the absence of Ca^{2+} .

The results indicate that rat islets contain calmodulin-stimulated cyclic AMP phosphodiesterase activity. In addition to this calmodulin-stimulated activity, there is significant basal activity that does not require calmodulin (activity observed in the presence of trifluoperazine). This might be explained if the preparation contained calmodulin-insensitive phosphodiesterase isoenzyme(s) contributing a substantial portion of the stimulated activity. Therefore kinetic studies were performed either without

added calmodulin or at calmodulin concentrations that 'saturated' the enzyme in the sense that further addition of calmodulin did not further increase the activity. The results shown in Fig. 2 represent the mean values obtained for four preparations of islets. Addition of exogenous calmodulin increased activity at all concentrations of cyclic AMP ($1\text{--}200\mu\text{M}$). Trifluoperazine addition decreased activity at all cyclic AMP concentrations.

Effects of calmodulin on rat islet cyclic GMP phosphodiesterase activities

In contrast with rat islet cyclic AMP phosphodiesterase activity, rat islet cyclic GMP phosphodiesterase activity was inhibited by omission of Ca^{2+} (and addition of EGTA). Trifluoperazine addition also decreased islet cyclic GMP phosphodiesterase activity (Fig. 3). The decrease was similar in magnitude to that produced by omission of Ca^{2+} (Fig. 3). In the presence of trifluoperazine, omission of Ca^{2+} did not elicit a further decrease in islet GMP phosphodiesterase activity (Fig. 3). These observations strongly suggest that trifluoperazine addition and Ca^{2+} omission inhibit cyclic GMP phosphodiesterase by a common mechanism, i.e. inhibition of calmodulin function. However, in the presence of Ca^{2+} , the addition of exogenous calmodulin did not increase the rate of cyclic GMP hydrolysis (Fig. 4).

Discussion

The important role ascribed to Ca^{2+} in the regulation of insulin release and the hypothesis that effects of Ca^{2+} may be mediated by calmodulin

Table 1. *Effects of Ca^{2+} , trifluoperazine and calmodulin on islet cyclic AMP phosphodiesterase activity*

The phosphodiesterase activity of rat islet extracts was measured as described in the Experimental section with $100\mu\text{M}$ -cyclic AMP as substrate in the absence of Ca^{2+} and presence of 1mM -EGTA ($-\text{Ca}^{2+}$) or in the presence of $50\mu\text{M}$ - Ca^{2+} ($+\text{Ca}^{2+}$). Trifluoperazine ($60\mu\text{M}$) or calmodulin ($2\mu\text{g/ml}$) were present where indicated. Results are means \pm S.E.M. for five separate preparations and the probability of no real difference between the rates under the condition indicated was assessed by the paired Student's *t* test. Abbreviation used: NS, not significant.

Line	Conditions	Phosphodiesterase activity (pmol/islet per h)	Significance
1	$-\text{Ca}^{2+}$	58.1 ± 2.4	—
2	$+\text{Ca}^{2+}$	61.2 ± 1.9	versus 1, NS
3	$-\text{Ca}^{2+}$, +trifluoperazine	51.7 ± 2.6	versus 1, $P \leq 0.05$
4	$+\text{Ca}^{2+}$, +trifluoperazine	44.5 ± 0.4	versus 1, $P \leq 0.01$ versus 2, $P \leq 0.01$ versus 3, $P \leq 0.05$
5	$-\text{Ca}^{2+}$, +calmodulin	58.1 ± 3.6	versus 1, NS
6	$+\text{Ca}^{2+}$, +calmodulin	69.5 ± 3.3	versus 1, $P \leq 0.01$ versus 2, $P \leq 0.001$ versus 5, $P \leq 0.001$
7	$-\text{Ca}^{2+}$, +calmodulin, +trifluoperazine	52.5 ± 3.2	versus 5, $P \leq 0.01$

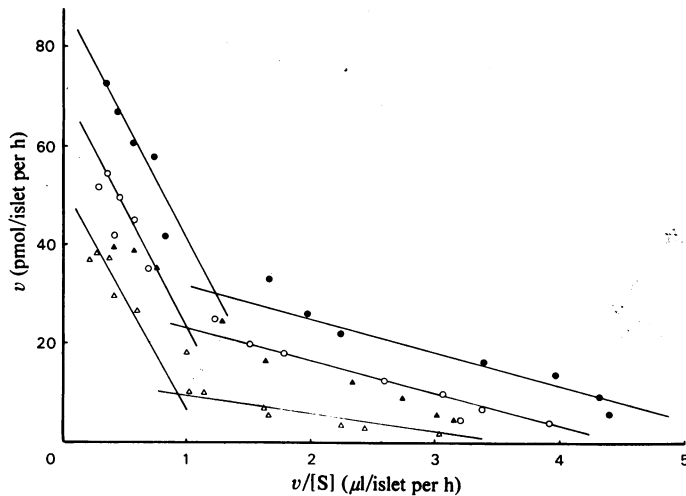


Fig. 2. *Effects of calmodulin plus Ca^{2+} and of trifluoperazine on islet cyclic AMP phosphodiesterase*
Islet cyclic AMP phosphodiesterase activity was determined at various concentrations of cyclic AMP in the absence (○ and △) or presence (● and ▲) of excess added calmodulin plus $50\ \mu\text{M-Ca}^{2+}$ and in the absence (○ and ●) or presence (△ and ▲) of $60\ \mu\text{M-trifluoperazine}$. Where Ca^{2+} was absent, the reaction mixture contained 1 mM-EGTA.

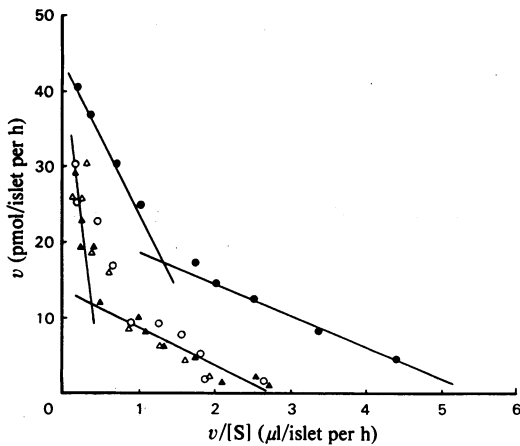


Fig. 3. *Effects of Ca^{2+} and trifluoperazine on islet cyclic GMP phosphodiesterase*

Islet cyclic GMP phosphodiesterase activity was determined at various concentrations of cyclic GMP in the absence (○ and ●) or presence (△ and ▲) of $60\ \mu\text{M-trifluoperazine}$ either in the presence (● and ▲) of $50\ \mu\text{M-Ca}^{2+}$ or in the absence of Ca^{2+} and presence of 1 mM-EGTA (○ and △).

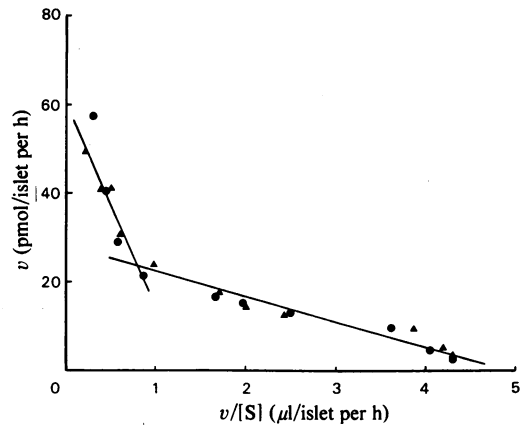


Fig. 4. *Effect of exogenous calmodulin on islet cyclic GMP phosphodiesterase*

Islet cyclic GMP phosphodiesterase activity was determined at various concentrations of cyclic GMP in the absence (●) or presence (▲) of calmodulin ($2\ \mu\text{g/ml}$).

(Sugden *et al.*, 1979; Gagliardino *et al.*, 1980; Ashcroft, 1980) have prompted a search for Ca^{2+} /calmodulin-dependent activities in pancreatic islets. Evidence has been presented for Ca^{2+} /calmodulin-

dependent protein phosphorylation (Gagliardino *et al.*, 1980) and adenylate cyclase activity (Valverde *et al.*, 1979). Since calmodulin was originally discovered as an activator of phosphodiesterase (Cheung, 1970), the present study was concerned with assessing the possible dependence of islet cyclic nucleotide phosphodiesterase on Ca^{2+} and calmo-

dulin. The studies are complicated by the obvious heterogeneity of such activity in islets. Kinetic evidence from this and previous studies (Ashcroft *et al.*, 1972) indicates at least two forms with differing affinities for cyclic AMP. Moreover there may be distinct species responsible for the hydrolysis of cyclic AMP and cyclic GMP. The present studies also suggest that sensitivity to Ca^{2+} (via calmodulin) may be a property of only a fraction of the total activity.

Nevertheless the results described here show that hydrolysis of both cyclic AMP and cyclic GMP catalysed by islet phosphodiesterase activities is capable of being modulated by Ca^{2+} /calmodulin. Thus with cyclic AMP as substrate, the addition of calmodulin in the presence of Ca^{2+} elicits an increase in phosphodiesterase activity that is blocked by trifluoperazine. However, the stimulation is only some 10–20%, with an amount of calmodulin that gives some 6-fold stimulation of calmodulin-free brain phosphodiesterase (Sugden *et al.*, 1979). This suggests that either the islet phosphodiesterase in the extracts is partially activated by endogenous calmodulin or the major portion of the activity is not sensitive to Ca^{2+} /calmodulin. That the latter is the correct explanation is suggested by the lack of effect of Ca^{2+} omission on the cyclic AMP phosphodiesterase activity in the absence of added calmodulin; and also by the fact that substantial activity remains in the presence of trifluoperazine.

With cyclic GMP as substrate, however, somewhat different findings were observed. Hydrolysis of cyclic GMP was not augmented by exogenous calmodulin. This might imply that the cyclic GMP phosphodiesterase activities are entirely Ca^{2+} /calmodulin-independent species. This does not seem to be the case, however, since cyclic GMP hydrolysis was clearly inhibited, although not abolished, in the absence of Ca^{2+} . That this partial dependence on Ca^{2+} may be mediated by calmodulin present in the extract is suggested by the finding that trifluoperazine inhibited cyclic GMP hydrolysis to the same extent as did the omission of Ca^{2+} .

These data thus support the view that Ca^{2+} , via calmodulin, may play a role in the regulation of the islet content of both cyclic AMP and cyclic GMP. A modulatory role for cyclic AMP in insulin release is well documented (for review see Sharp, 1979). When the present findings are considered together with the reported activation of adenylate cyclase by Ca^{2+} /calmodulin (Valverde *et al.*, 1979) one may speculate that such a dual action of Ca^{2+} could underlie the kinetics of changes in islet cyclic AMP content in response to glucose, i.e. an initial rise followed by a decline (Zawalich *et al.*, 1975).

There is evidence that cyclic GMP may affect insulin biosynthesis (Lin *et al.*, 1979; Howell & Montague, 1974). However, there is no evidence that

changes in islet cyclic GMP concentration modulate glucose-induced insulin release; thus in the presence of stimulatory concentration of glucose (Howell & Montague, 1974; Gagerman & Hellman, 1977) or glucose plus arginine (Charles *et al.*, 1976) islet content of cyclic GMP was unchanged. An increase in cyclic GMP content of guinea-pig islets stimulated by acetylcholine has been reported (Howell & Montague, 1974) but, in mouse islets, although acetylcholine elicited insulin release and membrane electrical activity no change in cyclic GMP could be detected (Gagerman *et al.*, 1978).

A role for Ca^{2+} in activation of islet guanylate cyclase is suggested by the observation that removal of extracellular Ca^{2+} reduced the cyclic GMP content of islets from *ob/ob* mice (Gagerman & Hellman, 1977) and by direct measurement of islet guanylate cyclase activity (Howell & Montague, 1974). The present study indicates that Ca^{2+} could also regulate the rate of degradation of cyclic GMP.

These studies were supported by grants from the Medical Research Council, The British Diabetes Association and the British Insulin Manufacturers.

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