Investigation of the role of Ca^{2+} and calmodulin in the regulation of platelet guanylate cyclase activity

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Both soluble and particulate forms of human platelet guanylate cyclase were found to be sensitive to sub-micromolar concentrations of free Ca^{2+} ; soluble enzyme activity increased as Ca^{2+} was increased from 10 nM to 1 μM ; particulate enzyme activity showed a biphasic response to Ca^{2+} , with maximal enzyme activity between 1 and 10 nM free Ca^{2+} and inhibition occurring at higher Ca^{2+} concentrations. Neither Ca^{2+} -sensitivity appeared to be calmodulin-dependent.

INTRODUCTION

Despite the presence in the cell of enzymes for the synthesis, degradation and biological activity of cyclic GMP, there is still for most tissues no clearly defined function for this nucleotide. Agents which cause an increase in cyclic GMP formation in intact cells do not seem to activate either particulate or soluble forms of guanylate cyclase directly in broken-cell preparations (Goldberg & Haddox, 1977). This and other lines of evidence suggest that an intermediate step for guanylate cyclase activation may exist *in vitro*. Thus agents that increase cyclic GMP formation in intact cells often require extracellular Ca^{2+} for their action (Hardman *et al.*, 1974).

Ca^{2+} is a possible candidate for indirect regulation of guanylate cyclase. Ca^{2+} mediates many of its intracellular effects by the activation of calmodulin, and acting through calmodulin it regulates enzymes governing cyclic AMP synthesis (adenylate cyclase) and cyclic AMP and cyclic GMP hydrolysis (cyclic AMP/cyclic GMP phosphodiesterase). A role for Ca^{2+} and calmodulin in the regulation of protozoan guanylate cyclase has also been reported for *Tetrahymena* and *Paramecium* (Nagao *et al.*, 1979; Klumpp *et al.*, 1983). There are no reports of Ca^{2+} /calmodulin regulation of mammalian guanylate cyclase.

In the present paper the Ca^{2+} /calmodulin-sensitivity of human platelet guanylate cyclase was examined. The human platelet possesses a high guanylate cyclase activity (Adams & Haslam, 1978), and manipulation of platelet cyclic GMP concentrations can affect 5-hydroxytryptamine secretion in the platelet (Haslam & Davidson, 1985). This latter effect is Ca^{2+} -sensitive. Further, the platelet has been reported to possess a Ca^{2+} /calmodulin-dependent form of phosphodiesterase and a Ca^{2+} /calmodulin-dependent adenylylase also (Resink *et al.*, 1986).

MATERIALS AND METHODS

Chemicals

$[\alpha\text{-}^{32}\text{P}]\text{GTP}$ (10 Ci/mmol) and cyclic $[8\text{-}^3\text{H}]\text{GMP}$ (20–30 Ci/mmol) were obtained from Amersham International, Amersham, Bucks., U.K. Cyclic GMP as the crystallized free acid, the sodium salt of GTP, phosphocreatine and creatine kinase and calmodulin-deficient ox heart phosphodiesterase were obtained from Boehringer-

Mannheim. King-cobra (*Ophiophagus hannah*) venom, Dowex 1 (X8; 200–400 mesh) anion-exchange resin, Coomassie Blue, prostaglandin E_1 , LaCl_3 , bovine serum albumin (fraction V), isobutylmethylxanthine and theophylline were obtained from Sigma, Poole, Dorset, U.K.

Phenyl-Sepharose 6B was from Pharmacia, Hounslow, Middx., U.K. *N*-(6-Aminoethyl)-5-chloro-1-naphthalenesulphonamide (W7) and *N*-(6-amino-octyl)-5-iodo-1-naphthalenesulphonamide (5-iodo- C_8 W7) were prepared by Dr. G. M. Blackburn of the University of Sheffield. Details relating to the specificity of these drugs are as given in Mac Neil *et al.* (1986). All other chemicals were of analytical grade.

Preparation of calmodulin

Pig brain calmodulin was prepared by the method of Kakiuchi *et al.* (1981). Preparations were over 95% pure as determined by SDS/polyacrylamide-gel electrophoresis (Laemmli, 1970).

Platelet soluble and particulate preparations

Human platelet preparations were obtained by mixing fresh human blood with 0.1 vol. of 3.8% (w/v) sodium citrate/100 mM- Na_4EDTA . Blood was centrifuged at 200 *g* (r_{av} 20.8 cm) in a Sorvall RC-3B centrifuge for 10 min at 4 °C, and the platelet-rich supernatant removed and centrifuged at 600 *g* (r_{av} 20.8 cm) for 10 min at 4 °C. The pellet was resuspended in 15 mM-Tris/HCl (pH 7.4)/1 mM- Na_4EDTA /140 mM-NaCl and washed twice by centrifugation at 600 *g* (r_{av} 20.8 cm) for 10 min at 4 °C. The final pellet was resuspended in 50 mM-Tris/HCl (pH 7.4 at 4 °C)/250 mM-sucrose, and the platelets were lysed by rapid freezing in liquid N_2 followed by thawing at 37 °C (twice).

Soluble preparations were prepared by centrifugation of this lysate at 11950 *g* (r_{av} 10.69 cm) in a Sorvall RC-5B centrifuge for 15 min at 4 °C. Particulate preparations were washed twice with Tris/sucrose (as described above), and each time centrifuged at 11950 *g* as described above and finally resuspended in the appropriate assay buffer. Both preparations were used immediately in the assay of guanylate cyclase activity.

Measurement of guanylate cyclase activity

Guanylate cyclase activity was measured by the conversion of $[\alpha\text{-}^{32}\text{P}]\text{GTP}$ into cyclic $[\alpha\text{-}^{32}\text{P}]\text{GMP}$ as described in detail by Amirrasooli (1981).

Reaction mixtures (100 μ l) contained 65 mM-Tris/HCl (pH 7.6 at 37 °C), 1 mM-[α - 32 P]GTP (approx. 10^6 c.p.m./incubation), 4 mM-cyclic GMP, 1 mg of bovine serum albumin/ml, 5 mM-phosphocreatine, 20 units of creatine kinase/ml and MnCl_2 or MgSO_4 as detailed in the Results section.

Incubations at 37 °C were started by the addition of particulate preparations diluted into 150 mM-Tris/HCl (pH 7.4 at 4 °C), and usually contained 20–30 μ g of protein per incubation. Incubations were terminated at 15 min by the addition of 100 μ l of 1 M-HCl and heating to 95 °C for 2 min. Cyclic [32 P]GMP was isolated by chromatography on Dowex and alumina; details as described by Amirasooli (1981). Assays were performed in triplicate and results expressed as means $\pm$ S.E.M. of the triplicate determinations.

Use of phenyl-Sepharose 6B to remove calmodulin from platelet supernatant

Details of this were as described by Walker *et al.* (1984) with fluphenazine-Sepharose 6B. Material not binding to the column was assayed for protein and calmodulin activity.

Measurement of calmodulin activity

Calmodulin activity in particulate and supernatant preparations was determined as described previously (Mac Neil *et al.*, 1984), with a calmodulin-dependent ox heart phosphodiesterase.

Ca^{2+} determination

Ca^{2+} present in buffers, particulate and supernatant preparations was determined by the method of Gitelman (1967).

Free Ca^{2+} calculation

The dissociation constants of Caldwell (1970) for Ca^{2+} , Mg^{2+} and EGTA at pH 7.4 were used to calculate free Ca^{2+} in the presence of EGTA.

Protein

Protein was determined by the method of Bradford (1976) with bovine serum albumin (fraction V) as standard.

Use of LaCl_3 to remove endogenous calmodulin from particulate preparations

Preparations were incubated for 15 min with 200 μ M- LaCl_3 in 50 mM-Tris/HCl (pH 7.4 at 4 °C), followed by two washes with La^{3+} -free buffer. Control preparations were treated similarly but in La^{3+} -free buffer. Details of this procedure are as given in Mac Neil *et al.* (1984) and Lakey *et al.* (1985).

Statistics

The difference between means was assessed for significance by Student's *t* test. Values of $P < 0.05$ were taken as statistically significant.

RESULTS

Distribution and cofactor preference of soluble and particulate guanylate cyclase activity

The apparent distribution of guanylate cyclase activity in the platelet was dependent on the choice of substrate cofactor used (see Table 1). In the presence of Mn^{2+} ,

Table 1. Distribution, substrate-cofactor preference and sensitivity to La^{3+} and calmodulin antagonists of human platelet soluble and particulate guanylate cyclase activity

Particulate and soluble guanylate cyclase preparations from freshly isolated human platelets (as described in the Materials and methods section) were incubated for 15 min at 37 °C with 1 mM-GTP substrate and Mn^{2+} , Mg^{2+} or Ca^{2+} as substrate cofactor as indicated, except for data showing the sensitivity of the enzymes to La^{3+} and calmodulin antagonists, where Mn^{2+} (4 mM) was used as cofactor. (Similar results were also obtained here with 4 mM- Mg^{2+} .) For enzyme distribution the amount of soluble or particulate activity per 10^8 platelets was calculated. Results shown are means $\pm$ S.E.M. for three separate experiments with different platelet donors. Statistical significance between soluble and particulate preparations was assessed by Student's paired *t* test; N.S., not significant.

	(a) Soluble preparation	(b) Particulate preparation	(a) versus (b)
Enzyme distribution (nmol of cyclic GMP/min per 10^8 platelets)			
Mn^{2+}	71.3 ± 15	6.4 ± 2.7	$P < 0.05$
Mg^{2+}	10.9 ± 3.1	6.2 ± 4.0	N.S.
Ca^{2+}	2.6 ± 0.5	0.1 ± 0.1	$P < 0.05$
Specific activity (nmol of cyclic GMP/min per mg of protein)			
Mn^{2+}	1.63 ± 0.46	0.79 ± 0.33	$P < 0.05$
Mg^{2+}	0.25 ± 0.10	0.53 ± 0.41	N.S.
Ca^{2+}	0.06 ± 0.01	0.09 ± 0.03	N.S.
Cofactor sensitivity [concn. of cation (μ M) for half-maximal activity]			
Mn^{2+}	0.8 ± 0.08	0.17 ± 0.09	$P < 0.005$
Mg^{2+}	1.9 ± 0.5	1.9 ± 1.9	N.S.
Ca^{2+}	—	—	—
Sensitivity to inhibition by La^{3+} and calmodulin antagonists [concn. (μ M) for half-maximal inhibition]			
La^{3+}	361 ± 10	350 ± 62	N.S.
W7	550 ± 75	570 ± 30	N.S.
5-iodo- C_8 W7	> 250	> 250	N.S.

significantly greater enzyme activity was always associated with the soluble preparation. Particulate and soluble preparations also differed significantly in their sensitivity to Mn^{2+} , the soluble form requiring a 4-fold greater concentration of Mn^{2+} for half-maximal activation of enzyme activity.

However, in the presence of Mg^{2+} as a cofactor, there was no consistent difference between the amount of enzyme activity associated with the soluble and particulate preparations, and both showed similar sensitivity to Mg^{2+} .

Neither enzyme preparation was able to use Ca^{2+} at millimolar concentrations as an effective substrate cofactor, either alone (Table 1) or in combination with sub-saturating (2.5 mM) concentrations of Mn^{2+} or Mg^{2+} (results not shown).

Ca^{2+} -sensitivity of soluble and particulate guanylate cyclase activity

As particulate and soluble enzyme preparations differed in their response to Mn^{2+} (but not Mg^{2+}), it was

Fig. 1. Soluble and particulate forms of platelet guanylate cyclase: (a) comparison of their Ca²⁺-sensitivity and (b) effect of calmodulin depletion on the Ca²⁺-sensitivity of the particulate enzyme

Soluble and particulate platelet preparations (details as in the Materials and methods section) were assayed over a wide range of calculated free Ca²⁺ concentrations. The EGTA concentrations added to the assay to give the free Ca²⁺ concentrations shown are indicated on the lower abscissa (assay buffers were found to contain 20 μM-Ca²⁺). (a) Combined results (means ± S.E.M.) of three experiments with soluble preparations (●) and four experiments with particulate preparations (○). Enzyme activity at a calculated free [Ca²⁺] of 0.2 μM (chosen to approximate to resting intracellular free [Ca²⁺]) has been designated 100% for ease of comparison of data. (b) Results (means ± S.E.M.) of a single representative experiment in which the control particulate preparation (●) contained 0.27 μg of calmodulin/mg of particulate protein and the La³⁺-treated preparation (○) contained 0.07 μg of calmodulin/mg of particulate protein.

decided to study the possible Ca²⁺/calmodulin-sensitivity of each preparation separately.

Fig. 1 illustrates that the soluble preparation showed a slight but significant [$+35.2 \pm 9.5\%$, mean ± S.E.M. ($n = 4$ expts.), $P < 0.025$ by paired t test] increase in enzyme activity as the calculated free Ca²⁺ in the assay was increased from 10 nM to 10 μM (this range was chosen to represent the widest range of free Ca²⁺ that

might be encountered in the platelet). Similar results were also obtained if Mg²⁺ was used as cofactor. Conversely, over this same concentration range, particulate guanylate cyclase activity was significantly decreased [by $-40.0 \pm 9\%$, mean ± S.E.M. ($n = 5$ expts.), $P < 0.05$ by paired t test]. In these experiments, enzyme activity at 0.2 μM (chosen to represent intracellular free Ca²⁺ in the resting cell) was arbitrarily designated as 100% for comparison of the data.

With the particulate enzyme there was also evidence of a biphasic response to Ca²⁺; low concentrations of free Ca²⁺ increased enzyme activity, with maximum enzyme activity occurring between 1 and 10 nM calculated free Ca²⁺; higher concentrations of Ca²⁺ were progressively inhibitory (Fig. 1).

Effect of calmodulin antagonists and of LaCl₃ on platelet guanylate cyclase activity

A possible involvement of calmodulin in the regulation of platelet guanylate cyclase activity was first investigated indirectly by examining enzyme sensitivity to calmodulin antagonists and to La³⁺. The latter has been found to be particularly inhibitory to calmodulin-dependent enzymes (Klumpp *et al.*, 1983; Lakey *et al.*, 1985).

Two calmodulin antagonists were examined, *N*-(6-aminoethyl)-5-chloro-1-naphthalenesulphonamide (W7) and an analogue of W7 (6-iodo-C₈ W7), which is more potent and more specific for calmodulin-dependent enzymes than W7 (Mac Neil *et al.*, 1986). These drugs inhibit calmodulin-stimulated bovine heart phosphodiesterase activity, concentrations giving 50% inhibition (IC₅₀) being 29 ± 2 and 3 ± 0.6 μM respectively (means ± S.E.M. for four expts.) (Mac Neil *et al.*, 1986). Both were relatively ineffective in inhibiting soluble and particulate guanylate cyclase activity (see Table 1). Similar results were obtained whether Mn²⁺ or Mg²⁺ was used as substrate cofactor, and preincubation of enzyme preparations with the drugs did not improve the inhibition (results not shown).

Similarly, La³⁺ had a much less potent effect (IC₅₀ approx. 350 μM) on guanylate cyclase activity, irrespective of whether this was soluble or particulate and of whether Mn²⁺ or Mg²⁺ was used as substrate cofactor (Table 1), than it has been found to have against calmodulin-dependent enzymes, where IC₅₀ values of around 5 μM have been found (Lakey *et al.*, 1985).

Effect of addition of exogenous calmodulin and removal of endogenous calmodulin on guanylate cyclase activity

Guanylate cyclase activity in soluble and particulate platelet preparations was unaffected by the addition of exogenous calmodulin (up to 10 μg/ml at the highest concentration tested).

For the soluble guanylate cyclase enzyme it has not so far proved possible to lower calmodulin concentration significantly, by using phenyl-Sepharose affinity chromatography for calmodulin, without major protein loss. Accordingly, in this paper it is not possible to comment on the consequences of calmodulin depletion on the activity or Ca²⁺-sensitivity of the soluble guanylate cyclase enzyme.

For the particulate preparation, LaCl₃ treatment of membranes successfully decreased endogenous calmodulin (from 0.27 to 0.07 μg of calmodulin activity/mg of protein for the example shown in Fig. 1b), but guanylate cyclase activity was not significantly affected by this

treatment. (Adenylate cyclase activity in the same particulate preparations after calmodulin depletion showed a significant loss of enzyme activity at all assay Ca^{2+} concentrations; results not shown.) Similarly, addition of further exogenous calmodulin (up to $10 \mu\text{g/ml}$) to calmodulin-depleted membranes had no effect on particulate guanylate cyclase activity.

DISCUSSION

The purpose of this study was to examine whether Ca^{2+} and calmodulin have any role to play in the activation of platelet guanylate cyclase. Several lines of evidence suggest that Ca^{2+} and calmodulin should be investigated in this respect: the requirement for some agents which increase cellular cyclic GMP formation for extracellular Ca^{2+} in the medium (Hardman *et al.*, 1974); the involvement of Ca^{2+} and calmodulin in the regulation of other enzymes of cyclic nucleotide synthesis (Mac Neil *et al.*, 1985) and hydrolysis (Walker *et al.*, 1984); and the regulation of protozoan guanylate cyclase activity by Ca^{2+} and calmodulin (Nagao *et al.*, 1979; Klumpp *et al.*, 1983).

In the present study, a series of criteria which have previously proved to be useful in the investigation of calmodulin-dependent adenylate cyclase (Mac Neil *et al.*, 1984; Lakey *et al.*, 1985) and phosphodiesterase (Walker *et al.*, 1984) were applied to platelet guanylate cyclase activity.

The results show that both forms of platelet guanylate cyclase activity are Ca^{2+} -sensitive, but the soluble form is slightly increased by increasing free Ca^{2+} , whereas the particulate form is inhibited.

This sensitivity to small changes in free Ca^{2+} does not appear to be related to the enzymes' cofactor requirements. Soluble and particulate forms share a similar sensitivity and responsiveness to Mg^{2+} as a substrate cofactor, but have very different responses to Mn^{2+} . Neither is able to use Ca^{2+} at high (millimolar) concentrations as a substrate cofactor, and no synergy between Ca^{2+} (low-millimolar concentrations) and either Mn^{2+} or Mg^{2+} at sub-saturating concentrations was observed, as has been reported previously for guanylate cyclase in other tissues (Goldberg & Haddox, 1977). The difference in cofactor preference between soluble and particulate forms of guanylate cyclase confirms the results of several other workers (as reviewed by Goldberg & Haddox, 1977), some of whom have speculated that, despite the greater sensitivity and responsiveness to Mn^{2+} , the concentrations of Mn^{2+} (sub-micromolar) and of Mg^{2+} (25 mM) present in the cell may dictate that the cofactor actually used by the enzyme forms is Mg^{2+} rather than Mn^{2+} . If so, this could make the contribution of the particulate enzyme more important than it seems *in vitro*.

It is not clear which enzyme form is activated by those agents which increase intracellular cyclic GMP. The location of enzyme activity in the membrane might permit direct interaction of physiological agents with the enzyme, as for other receptor-coupled enzymes. In this respect it was interesting that the particulate guanylate cyclase had a similar Ca^{2+} -sensitivity to that of particulate adenylate cyclase. Both enzymes exhibit maximum activity at sub-micromolar free Ca^{2+} concen-

trations and are significantly inhibited by micromolar free Ca^{2+} . This biphasic response to Ca^{2+} has been reported for several calmodulin-dependent adenylate cyclase enzymes, including that of platelets (Resink *et al.*, 1986).

However, unlike particulate adenylate cyclase activity, neither particulate nor soluble guanylate cyclase showed any evidence of calmodulin regulation. Particulate and soluble guanylate cyclase activities were inhibited by LaCl_3 and calmodulin antagonists only at very high concentrations. Particulate guanylate cyclase was not affected by the removal of endogenous calmodulin, and in neither intact preparations nor calmodulin-depleted membranes could guanylate cyclase activity be stimulated by the addition of calmodulin over a wide range of concentrations.

In summary, both species of guanylate cyclase activity in human platelet have the ability to respond to small changes in free Ca^{2+} with a significant change in activity. The soluble enzyme is increased by rising $[\text{Ca}^{2+}]$; the particulate form is inhibited. The significance of this remains to be established. For neither enzyme form was there any evidence to suggest that this Ca^{2+} -sensitivity is mediated by calmodulin. The Ca^{2+} -sensitivity of both enzyme forms, however, may provide a means of indirectly regulating guanylate cyclase activity in the cell.

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