Interaction amongst calcineurin subunits

Stimulatory and inhibitory effects of subunit B on calmodulin stimulation of subunit A phosphatase activity depend on Mn^{2+} exposure of the holoenzyme prior to its dissociation by urea

Ramesh C. Gupta†, Ramji L. Khandelwal* and Prakash V. Sulakhe+

Departments of Physiology and **Biochemistry, College of Medicine, University of Saskatchewan, Saskatoon, Saskatchewan S7N OWO, Canada*

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Calcineurin was dissociated into subunits A and B by 6 M urea in the presence (method A) and absence (method B) of $MnCl_2$ and dissociated subunits were isolated by gel filtration in urea in the absence (method B) or presence (method A) of $MnCl_2$. Phosphatase activity was associated with the A subunit isolated by either method. The phosphatase activity (nmol/mg) of subunit A isolated by method A was greater (2–5-fold) than by method B. Mn^{2+} increased subunit A phosphatase and calmodulin further increased the enzyme activity. Subunit B isolated by method A or B increased Mn^{2+} + calmodulin stimulated subunit A phosphatase prepared by method B but interestingly and unexpectedly inhibited such stimulated activity of the subunit A prepared by method A. These results imply the tightly bound cation (in our case, most likely Mn^{2+}) with subunit A dramatically and differentially influences the effects of two Ca^{2+}-binding proteins, calmodulin and subunit B, on the subunit A phosphatase.

Calcineurin *Calcineurin subunit* *Protein phosphatase* *Calmodulin* *Divalent cation*

1. INTRODUCTION

Bovine brain calcineurin is now established to be a phosphatase capable of dephosphorylating a wide variety of protein and non-protein substrates (review [1]). The holoenzyme is composed of two dissimilar subunits, A (M_r 61 000) and B (M_r 15 000). Subunit A contains catalytic (phosphatase) activity [2,3] and domains for interaction with calmodulin [4,5], subunit B [6,7] and divalent metals like Mn^{2+} and Ni^{2+} [1,6,7]. The smaller subunit B binds 4 mol Ca^{2+} [8]. Recently, Merat et al. [6] resolved subunit A by urea/gel filtration and showed the stimulation of subunit A phosphatase activity by Mn^{2+}, calmodulin and subunit B; the latter two, under defined assay conditions, stimulated enzyme in synergistic fashion. Our recent work described the resolution of calcineurin A subunit by SDS-polyacrylamide gel electrophoresis [3] or SDS gel filtration [7] in the presence of $MnCl_2$. Our results also showed the stimulation of subunit A phosphatase activity by Mn^{2+}, calmodulin and subunit B [7].

Here, we report an interesting, albeit unexpected, observation regarding the dissimilar influence of subunit B on subunit A phosphatase isolated by two methods – gel filtration in urea in the absence (method B) or presence (method A) of $MnCl_2$. While the Mn^{2+} plus calmodulin-dependent phosphatase of subunit A isolated by method B was stimulated markedly by subunit B, a finding in

+ To whom correspondence should be addressed
† Present address: Department of Pharmacology and Cell Biophysics, University of Cincinnati Medical Center, Cincinnati, OH 45267, USA

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agreement with that in [6], the phosphatase of subunit A isolated by method A was in fact inhibited by subunit B. And yet Mn^{2+} ± calmodulin increased the phosphatase activity of subunit A isolated by either method. These results reveal critical influence of tightly bound metal (in this instance, perhaps Mn^{2+}) with subunit A in determining the nature of influence of subunit B on enzymatic expression of subunit A. The significance of this finding is briefly presented.

2. EXPERIMENTAL

2.1. *Materials*

Calmodulin and calcineurin were prepared from bovine brain as in [3,7]. Myelin basic protein (kindly provided by Eli Lilly) was phosphorylated as described [9] and was used as substrate for dephosphorylation by calcineurin or its subunit A. Sources for other chemicals and reagents have been described in earlier publications from our laboratory [3,7,9].

2.2. *Methods*

2.2.1. Dissociation and separation of calcineurin subunits by urea/Sephadex G-100 chromatography

Method A: lyophilized calcineurin (1.6 mg) was dissolved in 0.4 ml buffer A (50 mM Tris-Cl, pH 7.5, 5 mM DTT, 1 mM $MnCl_2$, 6 M urea, 1 mM EGTA) and then applied on a Sephadex G-100 column (0.8 × 40 cm). The column was preequilibrated with buffer A, which was also the elution buffer. 1-ml fractions were collected. An aliquot (20 μl) of each fraction (diluted 4 times) was assayed for phosphatase activity (see below) and subjected to electrophoresis in SDS on polyacrylamide gels (see below).

Method B: same as above except $MnCl_2$ was omitted from buffer A, the Sephadex G-100 column was 0.5 × 115 cm and 0.8 ml/fraction was collected.

2.2.2. Phosphatase assay

This has been described in [3,7,9]. Standard incubation (33°C, 5 or 10 min) was carried out in assay mixture containing 50 mM Tris-Cl, pH 7.5, 1 mg/ml BSA, 100 mM NaCl, 0.5 mM DTT, 50 or 100 pmol ^{32}P-myelin basic protein and calcineurin or its subunits (see figure legends). Other additions to the assay (i.e. divalent cations, calmodulin) are specified in figure legends. One unit of enzyme is defined as the amount that dephosphorylated ^{32}P-labelled myelin basic protein at a rate of 1 nmol ^{32}P/min.

2.2.3. Electrophoresis

Gel electrophoresis in the presence of 0.1% SDS was carried out on mini-slab containing 12% acrylamide according to Laemmli [10]. Proteins were stained with Coomassie blue and destained by acetic acid.

Protein was determined by the Bio-Rad microassay procedures.

3. RESULTS

3.1. *Separation of calcineurin subunits by gel filtration in the presence of 6 M urea and 1 mM $MnCl_2$*

Fig.1 summarizes the results on elution of protein, phosphatase activity and electrophoretic profile of eluted column fractions (no 8–24). Subunit A was primarily present in fractions 10–12 and subunit B, free from subunit A, was eluted in fractions 17–19. Fractions 13–16 contained decreasing amounts of subunit A with increasing amounts of subunit B. Thus it was not surprising that fractions 9–16 showed the presence of phosphatase activity, although when expressed as enzyme units/fraction, the peak was seen in fraction 13 when assay contained Mn^{2+} plus calmodulin (● in fig.1). The most likely explanation for activity peak in fraction 13 is the recently noted synergistic stimulatory effect of calmodulin (added to assay) and subunit B (present in fraction 13) on subunit A phosphatase [6,7]. Thus it is not surprising that the protein elution peak in fraction 11, reflecting the elution of subunit A, did not correspond to the activity peak. On the other hand, phosphatase assayed with Mn^{2+} (calmodulin absent) or without any divalent metal (□ in fig.1) showed the main activity peak in fractions 11–12 and the secondary peak in fractions 15–16. The latter can be explained by the weaker stimulatory action of subunit B on Mn^{2+}-stimulated subunit A phosphatase [7] as well as by decreased amount of subunit A in these latter fractions. The fact that fractions devoid of subunit A but containing sub-

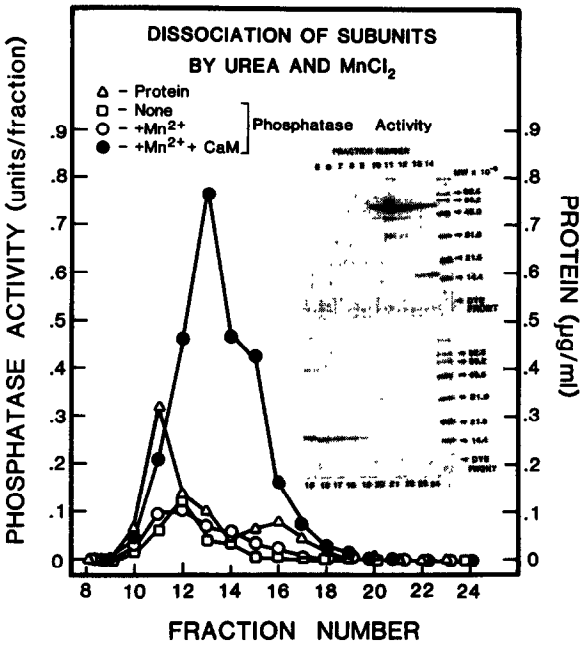

Fig.1. Elution of calcineurin subunits by gel filtration in the presence of 6 M urea and 1 mM $MnCl_2$. Dissociation and elution of calcineurin was carried out by method A (see section 2). Appropriately diluted fractions were used in the standard phosphatase assay containing no metal (□), 2.2 mM $MnCl_2$ (○) and $MnCl_2$ plus 1 μM calmodulin (●). Aliquots of fractions were subjected to SDS-polyacrylamide gel electrophoresis (mini-slab); mobility of M_r markers is shown. Protein concentration in eluted fraction was determined (Δ).

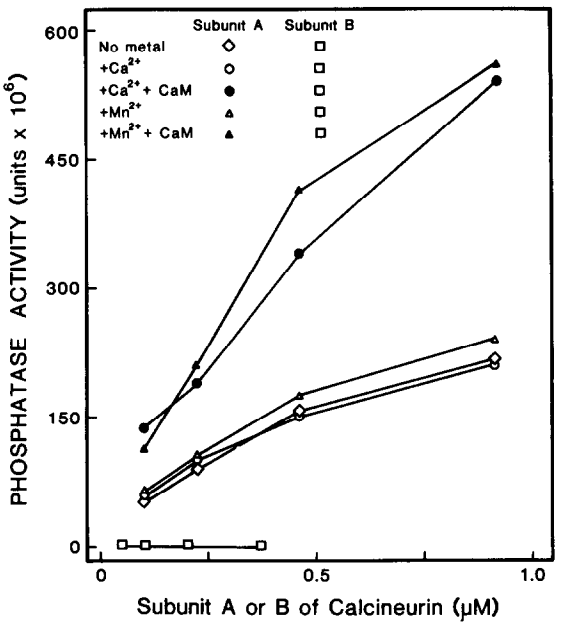

Fig.2. Phosphatase activity of dialyzed A and B subunits. Fractions 10 (subunit A) and 17 (subunit B) (see fig.1) were dialyzed overnight and assayed for the phosphatase activity in the standard assay mixture containing 2.2 mM $CaCl_2$ or 2.2 mM $MnCl_2$ ± 1 μM calmodulin, as indicated. Note under all conditions subunit B failed to show any detectable enzyme activity (□).

unit B showed no phosphatase activity supports the previous contention [2,3] that catalytic site resides in subunit A. It is also implicit that the somewhat diffuse profile of phosphatase activity in eluted fractions results from two main factors: the amount of subunit A phosphatase/fraction and the marked stimulatory effect of subunit B especially evident when assay contained Mn^{2+} and calmodulin.

3.2. *Phosphatase activity is associated with subunit A*

To understand more clearly the influence of subunit B on subunit A, fractions 11 (that contained only subunit A) and 17 (that contained only subunit B) were dialyzed prior to assay. The phosphatase activity (assayed under various conditions) was detected only in subunit A (fig.2). It was evident that calmodulin increased subunit A phosphatase in the presence of Ca^{2+} or Mn^{2+}. Mn^{2+} by itself increased the phosphatase only modestly. It is noteworthy to state here that calcineurin was exposed to $MnCl_2$ throughout the isolation procedure. Thus, although we have dialyzed out free Mn^{2+}, the firmly bound Mn^{2+} (vide infra) may be still present. Elsewhere, we have reported (Gupta et al., submitted) that prior exposure to Mn^{2+} leads to the attainment of the 'high activity, long-lived state' of calcineurin. The phosphatase activity of such 'preactivated' holoenzyme was increased by calmodulin with Ca^{2+} or Mn^{2+} present. The results of this study suggest this may also occur in the case of isolated subunit A.

3.3. *Inhibitory influence of subunit B*

From the results shown in fig.1, the preceding description and recent observations by Merat et al. [6] and Gupta et al. [7], it was anticipated that subunit B increased the subunit A phosphatase ac-

Table 1

Inhibitory effect of varying concentrations of subunit B on Mn^{2+}-calmodulin-dependent phosphatase of subunit A

Subunit B (nM)	Phosphatase activity (%)
0	100
25	100
50	92
100	86
150	83
200	72
350	61

Subunits were isolated (method A) and dialyzed overnight prior to assay. The assay mixture (80 μl) contained 100 nM subunit A, 1 μM calmodulin, 2.2 mM $MnCl_2$, 1 mM EGTA, 50 mM Tris-Cl, pH 7.5, 5 mM DTT, 1 mg/ml BSA and 100 pmol ^{32}P-myelin basic protein. Subunit A phosphatase activity (units/mg) in the absence of subunit B was 0.36 and considered as 100%

tivity. The results in table 1 shows this was not the case. In fact, addition of dialyzed B subunit caused inhibition of the subunit A phosphatase assayed in the presence of Mn^{2+} plus calmodulin. This unexpected finding may indicate several possibilities. For example, dialysis per se may have removed factor(s) that aid or are necessary for the stimulatory (in fact, synergistic type) effect of the subunit B on calmodulin stimulation of subunit A phosphatase. The Mn^{2+}-preactivated state of subunit A responds differently to subunit B than the non-activated state. The possibility exists that Mn^{2+} may be tightly bound to subunit B. If so, this may impart the noted inhibitory effect as well. To gain further insight into these questions, we separated calcineurin subunits by gel filtration in urea in the absence of $MnCl_2$.

3.4. *Separation of calcineurin subunits by urea/gel filtration in the absence of $MnCl_2$*

The eluted fractions 34–40 (Sephadex column, 0.8× 115 cm) contained subunit A and fractions 47–51 contained subunit B. Fractions 41–46, as expected, showed the presence of both subunits with decreasing amounts of A and increasing amounts of subunit B.

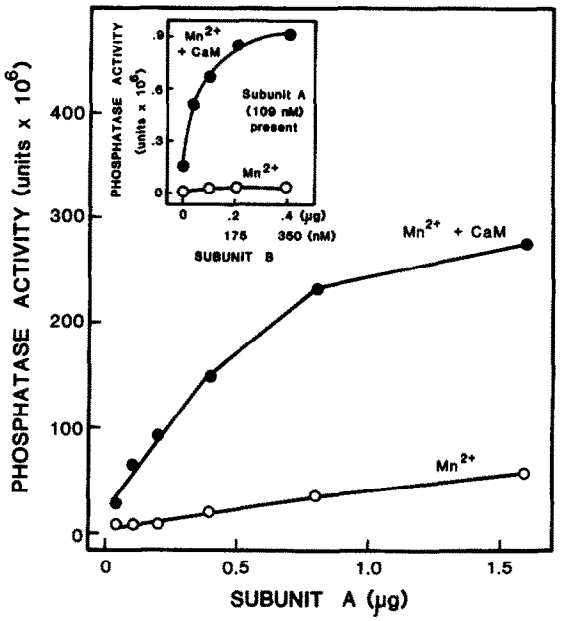

Fig.3. Stimulatory effect of subunit B on subunit A phosphatase. Subunits were isolated by method B, dialyzed and used in phosphatase assay. Main figure shows stimulation of subunit A phosphatase by 2.2 mM $MnCl_2$ or $MnCl_2$ plus 1 μM calmodulin. The inset shows the stimulatory effect of subunit B on Mn^{2+} plus calmodulin-dependent subunit A phosphatase.

Fractions containing A and B subunits were dialyzed and then tested for ascertaining the influence of A-B interaction on the phosphatase activity. As shown by the results in fig.3, Mn^{2+} increased subunit A phosphatase activity, which was further increased by calmodulin. Subunit B markedly increased Mn^{2+} + calmodulin-dependent phosphatase (inset). This finding agrees with the recent documentation by us [7] and Merat et al. [6] concerning the synergistic stimulatory effect of calmodulin and subunit B on subunit A phosphatase activity. The results are also consistent with the presence of distinct domains on subunit A for interaction with two calcium-binding proteins – calmodulin [11] and subunit B [8]. The results of fig.3 and table 1, nonetheless, document a differential influence that the Mn^{2+}-activated state, possibly that of subunit A, has on the nature of influence of subunit B on calmodulin-stimulated subunit A phosphatase.

4. DISCUSSION

The role of subunit B in the regulation of calcineurin phosphatase by divalent metals and calmodulin has remained enigmatic. Recent findings by Merat et al. [6] using urea-dissociated subunits and the results of fig.3 in this study document the stimulatory effect of subunit B on Mn^{2+} plus calmodulin-dependent subunit A phosphatase. Calmodulin also stimulates Mn^{2+}-dependent subunit A phosphatase activity ([6]; and this study). Despite this, it is puzzling, albeit interesting, that the two calcium-binding proteins with considerable homology (i.e. calmodulin and subunit B) exert a stimulatory effect on subunit A, whereas only calmodulin exerts such a stimulatory effect on the holoenzyme [6]. Thus, whether in the cell calmodulin or subunit B or both regulate (stimulate) calcineurin phosphatase and if so whether either protein could do so under the prevailing in vivo conditions (i.e. with micromolar Ca^{2+}) remain challenging areas for further work. The unexpected observation that subunit B inhibits the Mn^{2+} plus calmodulin-dependent phosphatase of subunit A isolated in the presence of $MnCl_2$ provides an interesting clue in this context. It seems that Mn^{2+}-preactivated subunit A discriminates between calmodulin and subunit B. The implication of this is that if tightly bound Mn^{2+} were to be required for the catalytic expression, as has been suggested earlier by us [9] and others [12], the holoenzyme in the cell is of the low activity state owing to the inhibitory effect of subunit B. This could prevent unwanted dephosphorylation of substrates. In the stimulated neuron, Ca^{2+} influx (transient) via calmodulin then conceivably activates calcineurin phosphatase in a reversible manner.

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