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The effect of Mg^{2+} and calmidazolium on the calmodulin binding to inside-out vesicles of the human red cell membrane

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Calmodulin binds to inside-out vesicles of the human red cell membrane in a Ca^{2+}-dependent manner. Mg^{2+} was found to be essential to this binding, half maximum binding occurring at decreasing Ca^{2+} concentration with increasing Mg^{2+} concentration. The calmodulin antagonist calmidazolium strongly reduced the Ca^{2+}-dependent binding of calmodulin to the inside-out vesicles.

An increase in intracellular calcium and consequent binding of Ca^{2+} to calmodulin result in an activated calmodulin which binds to the target protein [1]. Much evidence has accumulated verifying the role of calmodulin in stimulating the human red cell membrane ($Ca^{2+} + Mg^{2+}$)-ATPase and active translocation of Ca^{2+} across the membrane [2–5]. The activation of the ($Ca^{2+} + Mg^{2+}$)-ATPase in erythrocytes and ghosts caused by a rise in Ca^{2+} concentration is antagonized by increasing intracellular Mg^{2+}, indicating a competition between Ca^{2+} and Mg^{2+}. The Ca^{2+}-Mg^{2+} antagonism is suggested to be due to Mg^{2+} competing with Ca^{2+} for the binding sites at the ($Ca^{2+} + Mg^{2+}$)-ATPase molecule rather than for the binding sites at the calmodulin molecule [6]. Evidence has also been presented which supports the contention that erythrocyte inside-out vesicles contain a Ca^{2+}-dependent K^+ channel which requires calmodulin for activation, and previous studies have shown that calmodulin binds to human erythrocyte ghosts and inside-out vesicles in a Ca^{2+}-dependent manner [7–9].

The purpose of the present study was to determine the influence of Mg^{2+} on the Ca^{2+}-dependent binding of calmodulin to inside-out vesicles of the human red cell membrane. The calmodulin antagonist calmidazolium was tested in order to investigate whether the inhibitory effect is due to binding of the drug to calmodulin, thereby preventing interaction between calmodulin and the membrane [10]. The binding of [^{3}H]-calmodulin to inside-out vesicles was studied by varying the Mg^{2+} concentration at different Ca^{2+} concentrations. The Ca^{2+} uptake was concurrently studied under the same conditions using ^{45}Ca as tracer.

Materials and methods. Inside-out vesicles were prepared from human red cells, 2/3 of the vesicles normally being inside-out as determined by measuring acetylcholine esterase activity [11]. The assay medium for binding and transport studies consisted of 2 mM NaCl, 5 mM KCl, 0.5 mM EGTA, 0.1 mM ouabain, 1 mM ATP, 1 μg [^{3}H]calmodulin/ml, 2 mM Tris (pH 7.4) and the appropriate amounts of $MgCl_2$ and $CaCl_2$ required to produce the final concentration for each divalent cation, determined by calculation [12,13]. The concentration of calmidazolium in the calmodulin antagonist experiments was 5 μM. To measure binding samples containing 100 μg inside-out vesicle protein were incubated for 30 min at 37°C in 0.5 ml assay medium. After centrifugation the pellets were resuspended and washed twice in 1 ml

of ice cold incubation medium with calmodulin omitted, and transferred to Opti-Fluor™, Packard, for counting in a Liquid Scintillation spectrometer. Only traces of radioactivity were found in the supernatant after the second wash. The Ca^{2+} flux was determined according to a previous work using a filter technique [7].

Results. Fig. 1 shows the amount of [^{3}H]-calmodulin bound to inside-out vesicles at increasing amounts of tagged calmodulin added to the assay medium containing 1 mM free Mg^{2+} and 1 mM free Ca^{2+} or devoid of divalent cations. It demonstrates that Ca^{2+} enhances the binding of calmodulin to inside-out vesicles (Ca^{2+}-dependent binding), and that binding increases linearly in the absence of Mg^{2+} and Ca^{2+} (non-specific binding) [9].

Binding of calmodulin to inside-out vesicles was studied as a function of free Mg^{2+} concentration at varying free Ca^{2+} concentrations. Fig. 2 shows that Mg^{2+} has a stimulating effect on calmodulin binding to inside-out vesicles. If Mg^{2+} is omitted from the assay medium an increase in Ca^{2+} in the range 10^{-8}–10^{-4} M has hardly any effect on calmodulin binding. With increasing Mg^{2+} concentration binding is enhanced, most pronouncedly at high Ca^{2+} concentrations (10^{-4} M). At 1 mM Ca^{2+} it seems as if Ca^{2+} is partly able to substitute for Mg^{2+}, as only a minor rise in Mg^{2+} concentration results in almost maximum binding.

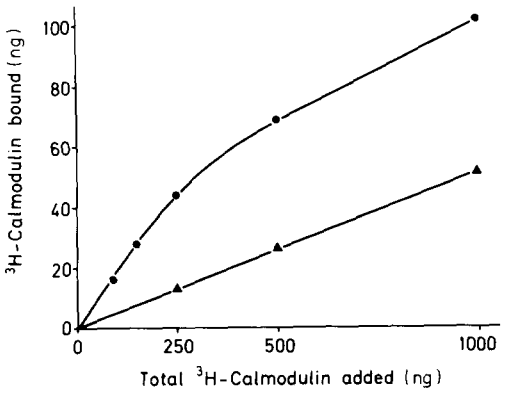

Fig. 1. [^{3}H]Calmodulin binding to inside-out vesicles. ●, Total [^{3}H]calmodulin (ng) bound to 100 μg inside-out vesicle protein in 0.5 ml incubation medium containing 1 mM free Ca^{2+} and 1 mM free Mg^{2+}. ▲, [^{3}H]Calmodulin (ng) bound to 100 μg inside-out vesicle protein in 0.5 ml incubation medium devoid of divalent cations. Abscissa: [^{3}H]calmodulin added (ng).

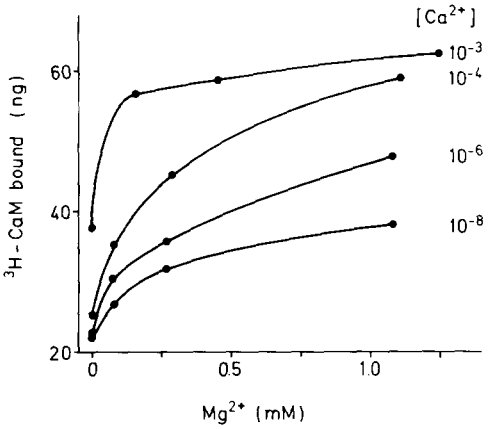

Fig. 2. [^{3}H]Calmodulin binding to inside-out vesicles. [^{3}H]-Calmodulin (ng) bound to 100 μg inside-out vesicle protein as a function of free Mg^{2+} concentration at varying free Ca^{2+} concentrations (M).

Binding experiments were carried out at fixed high (1.2 mM) and low (0.1 mM) free Mg^{2+} with varying Ca^{2+} concentrations, see Figs. 3A and B. The figures show that half maximum binding is achieved at a lower Ca^{2+} concentration at the high free Mg^{2+} concentration. At the low Mg^{2+} concentration binding is almost equivalent to the non-specific binding until 1 mM Ca^{2+}, where binding increases significantly. Mg^{2+} is known to affect Ca^{2+} binding to calmodulin competitively, presumably by modifying Ca^{2+} binding to the different sites on the molecule [14]. Upon Ca^{2+} binding, calmodulin exhibits domains that interact with the target protein. The results shown here suggest that Mg^{2+} facilitates the unmasking of these domains. Preincubation of calmodulin with the calmodulin antagonist calmidazolium for 30 min before addition of inside-out vesicles strongly reduced the Ca^{2+}-dependent binding to the level of non-specific binding, which remained unaltered. It is suggested that the inhibition is due to calmidazolium occupying the site on the calmodulin molecule responsible for membrane binding [15]. The inhibition by calmidazolium on the binding is diminished by high concentrations of Mg^{2+} and Ca^{2+}.

To test the intactness of the inside-out vesicle preparation the Ca^{2+} uptake rate was measured as

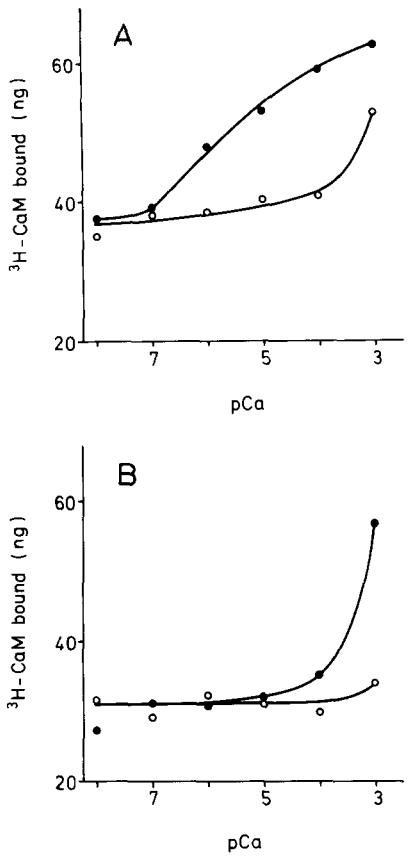

Fig. 3. [^{3}H]Calmodulin binding to inside-out vesicles. ●, [^{3}H]Calmodulin (ng) bound to 100 μg inside-out vesicle protein as a function of Ca^{2+} concentration at 1.2 mM (A) and 0.1 mM (B) free Mg^{2+}. ○, 5 μM calmidazolium added to the incubation media (A and B).

a function of Ca^{2+} concentration at two different Mg^{2+} concentrations, 1.2 mM and 0.1 mM free Mg^{2+}. From the results obtained it was confirmed that Mg^{2+} exerts a modulatory role on the Ca^{2+} sensitivity of the Ca^{2+} pump. Half maximum stimulation occurred at approx. 10^{-6} M Ca^{2+} at the high Mg^{2+} concentration. At decreasing free Mg^{2+} (0.1 mM) half maximum stimulation occurred at a lower Ca^{2+} concentration (approx. $7 \cdot 10^{-7}$ M). Also the Ca^{2+} concentration for optimal activation was decreased as the Mg^{2+} concentration decreased. These findings are in agreement with the results of other authors, who find that the Ca^{2+} concentration for half maximal as well as for optimal activation decreases at decreasing Mg^{2+} concentration [4,6,16].

Discussion. The results presented here suggest that Mg^{2+} is essential to the Ca^{2+}-dependent binding of calmodulin to inside-out vesicles of the red cell membrane. The free Mg^{2+} concentration in the intact, aerobic erythrocyte is believed to be approx. 0.4 mM [17]. At free Ca^{2+} concentrations below 10^{-7} M calmodulin binds Mg^{2+} but no Ca^{2+}. In the activated cell Ca^{2+} concentration is increased and calmodulin exchanges most Mg^{2+} with Ca^{2+}, but still retains some Mg^{2+} [18]. The experiments here show that binding of calmodulin to the membrane is very sensitive within the range of free Mg^{2+} in the intact cell. A slight increase in Ca^{2+} concentration causes an immediate increase in calmodulin binding to the membrane and consequent stimulation of the Ca^{2+} pump. Calmidazolium is a very potent calmodulin antagonist with a high affinity to calmodulin [15]. When bound to calmodulin the Ca^{2+}-dependent binding of calmodulin to the membrane is inhibited, and calmodulin is thereby being prevented from exhibiting its regulatory function.

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