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INTERACTION OF TERBIUM WITH HUMAN PLATELETS

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The effect of terbium on platelets has been studied by aggregation experiments and by fluorescence measurements. $TbCl_3$ does not substitute for $CaCl_2$ in the aggregation of platelets induced by ADP, but it may even inhibit, probably by a competition mechanism. It was impossible to observe a sensitized emission of Tb^{3+} in the presence of platelets. Instead the lanthanide, like Ca^{2+}, significantly increases the aggregation of platelets induced by A23187. The fluorescence yield of this compound is greater in the presence of platelets than in buffer alone. Energy transfer appears to take place from the aromatic amino acids of the platelet membrane to the bound ionophore.

The role of Ca^{2+} in the regulation of many cellular processes has been demonstrated (1). In particular extracellular Ca^{2+} can modulate the aggregation (2) and secretion (3) of platelets and their interaction with clotting factors (4). Direct information about binding of calcium to molecular and cellular systems is however difficult to obtain since this metal is silent toward most spectroscopic techniques. In the last years several reports showed that Tb^{3+} can substitute Ca^{2+} in various biological systems (5, 6). Since Tb^{3+} is luminescent, particularly when bound in the proximity of suitable energy donors (5) we tried to investigate a possible interaction of this lanthanide with human platelets by fluorescence spectroscopy. The effect of Tb^{3+} on the aggregation of platelets was also studied.

Materials and Methods

Platelets were obtained from blood samples collected from healthy volunteer donors who had not taken any drugs for at

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least 10 days. Platelet rich plasma (PRP) was obtained by centrifugation (100 g x 10 min) of blood samples collected with 3.8% sodium citrate or with heparin (5 U). Washed platelets were prepared by centrifugation of PRP at 1200 g x 10 min. The platelets were washed three times in Dulbecco's Ca- and Mg-free phosphate buffered saline, pH 7.4, containing 5 mM EDTA and resuspended in the same buffer. For ADP-induced aggregation, fibrinogen (2 mg/ml) was added to the platelet suspension.

The aggregation was followed by the turbidimetric method of Born (7). Briefly 0.4 ml of PRP or washed platelet suspension containing fibrinogen (50 µl) were placed in a plastic cuvette of an ELVI aggregometer (Mod 840 dual channel, ELVI Logos-Milan Italy) at 37°C under magnetic stirring at 1000 rpm with a teflon coated stir bar. The change in light transmission at 650 nm was recorded. The aggregating agents used were either ADP or the ionophore A23187.

Fluorescence spectra were taken at room temperature either with the usual 90° geometry using a FICA 55 L spectrofluorimeter, which gives spectra corrected for the fluctuation of the lamp and for the response of the phototube, or with the front-face geometry using an Aminco Bowman spectrofluorimeter equipped with a C-73-62140 Solid Sample Accessory.

Results

Effect of $TbCl_3$ on aggregation

As reported previously, the presence of Ca^{2+} is needed for the aggregation of PRP. In fact the aggregation triggered by ADP is completely abolished in the presence of 5 mM EDTA (fig. 1 A, b) and restored by the addition of 5 mM $CaCl_2$ (fig. 1, c). Other divalent cations, namely Mg^{2+} and Mn^{2+} are able to substitute for Ca^{2+} either in the presence and in the absence of EDTA (fig. 1, D, a, b, c). The effectiveness of these cations in the ADP-induced aggregation was in the order $Mn > Mg \geq Ca$. Thus about ten times less Mn^{2+} was needed to obtain the same aggregation observed in the presence of Ca^{2+}. Higher concentrations of Mn^{2+} caused the lysis of platelets. Tb^{3+} is unable to restore the aggregation of platelets inhibited by EDTA (Fig. 1 A, d). Furthermore in the absence of EDTA, the aggregation induced by ADP was inhibited by the addition of 1 mM $TbCl_3$ (Fig. 1 B, b).

A different result was obtained when the aggregation was triggered by the ionophore A23187 which does not require the presence of extracellular Ca^{2+}. The addition of 1 mM $CaCl_2$

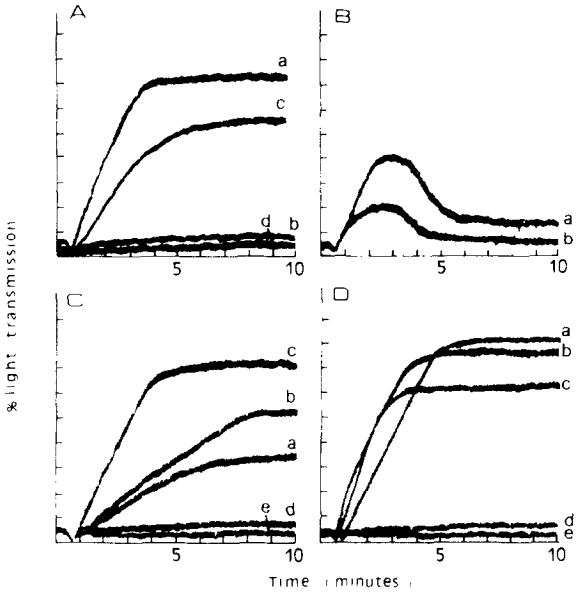

Fig. 1: Effect of metals on the aggregation of platelets.
A) Aggregation of platelets in PRP triggered by ADP (2 μM) a) ADP alone; b) ADP + 5 mM EDTA; c) ADP + 5 mM EDTA + 5 mM $CaCl_2$; d) ADP + 5 mM EDTA + 5 mM $TbCl_3$.

B) Aggregation of platelets in PRP triggered by ADP (1 μM). a) ADP alone; b) ADP + 1 mM $TbCl_3$.

C) Aggregation of washed platelets triggered by A23187 (20 μM). a) A23187 alone; b) A23187 + 1 mM $CaCl_2$; c) A23187 + 1 mM $TbCl_3$; d) as b but in the presence of 100 μM verapamil; e) as c but in the presence of 100 μM verapamil. The platelets were $3x10^8$/ml suspended in Ca-free medium. For details see Materials and Methods.

D) Aggregation of washed platelets triggered by ADP (3 μM). a) ADP + 1 mM $MgCl_2$; b) ADP + 1 mM $CaCl_2$; c) ADP + 50 μM $MgCl_2$; d) ADP + 1 or 10 mM $TbCl_3$; e) ADP alone.

significantly increased the extent of aggregation and even more so the addition of 1 mM $TbCl_3$ (Fig. 1, C). The presence of verapamil completely abolished the aggregation induced by A23187 (Fig. 1 C, d & e) in the presence and absence of added cations. A23187 also induces the secretion of various intraplatelet components. Table I shows that the secretion of ATP stimulated by A23187 is severely reduced by the addition of Ca^{2+} and to a greater extent by Tb^{3+}.

Effect of $TbCl_3^{3+}$ on the fluorescence of platelets and A23187

A possible binding of Tb^{3+} to platelets was investigated by

TABLE I

ATP release from platelets after stimulation with A23187

Metal added	ATP released by 10 μM A23187 ($nmol/10^9$ platelets)		
Experiment N°	1	2	3
none	30	35	25
1 mM $CaCl_2$	10	15	5
1 mM $TbCl_3$	12	20	5

fluorescence spectroscopy taking advantage of the emissive properties of this lanthanide (5). No enhancement of Tb^{3+} emission was observed upon addition of 1 mM $TbCl_3$ to a platelet suspension, nor any effect of this metal on the intrinsic emission of the proteins of platelet membrane. Instead the addition of A23187 quenched the platelet fluorescence while the fluorescence of the ionophore was considerably higher than in buffer alone (Fig. 2, lower frame). The excitation spectrum of the A23187 fluorescence at 440 nm was identical, up to 300 nm, to that of intrinsic platelet emission, suggesting the presence of energy transfer from platelets to the ionophore. The fluorescence yield increased almost linearly with the concentration of A23187 added to a platelet suspension ($3x10^8$ platelets/ml) up to 100 μM (Fig. 2, upper frame). The addition of 1 mM $CaCl_2$ or $TbCl_3$ increased by a 30% the fluorescence of A23187 without affecting the intrinsic platelet fluorescence. This effect of metals on fluorescence was not observed when A23187 was dissolved in buffer alone or in buffer:p-dioxane 1:1 where the fluorescence of the ionophore is considerably higher.

Neither metal affected the shape of the fluorescence titration curve (Fig. 2).

Discussion

The above reported data show that Mg^{2+} and Mn^{2+}, but not Tb^{3+} can substitute Ca^{2+} in the aggregation stimulated by ADP.

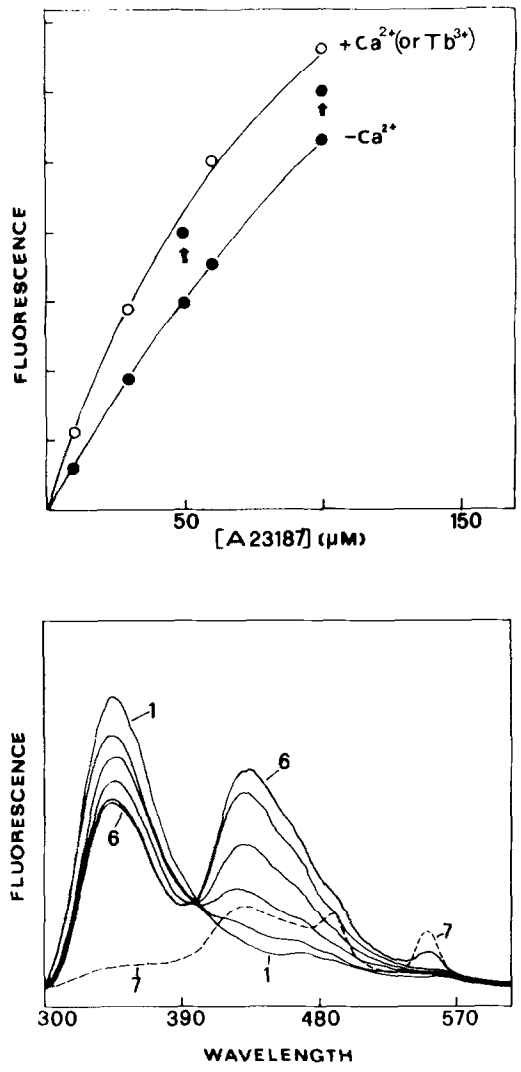

Fig. 2: Fluorescence of A23187 in the presence of platelets. Bottom: a suspension containing 1 x 10^9 platelets/ml in PBS pH 7.4 showed a fluorescence emission when excited at 290 nm (curve 1). To this suspension increasing amounts of A23187 were added (final concentrations): 10 μM (curve 2); 30 μM (curve 3); 60 μM (curve 4); 100 μM (curve 5). Curve 6 is the same of curve 5 after the addition of 1 mM TbCl. Curve 7 was obtained from a solution containing 100 μM A23187 and 1 mM TbCl in PBS pH 7.4.

Top: ^{3}Titrations of platelet suspensions (1 x 10^9 platelets/ml in PBS pH 7.4) in the absence (full symbols) and presence (empty symbols) of 1 mM $CaCl_2$ or $TbCl_3$. The arrows indicate the addition of 1 mM $CaCl_2$ or $TbCl_3$ to a platelet suspension titrated with A23187 in the absence of these ions. The excitation maximum was 290 nm and the emission maximum was 440 nm. All these fluorescence measurements were done at room temperature using a front-face geometry. The suspensions were not stirred.

This finding might indicate that an ADP-Me^{2+} complex can be responsible for the aggregation of platelets, by analogy with most biochemical systems where ADP or ATP are involved (8). Such hypothesis seems to be supported by the effectual concentrations of the different metals. In fact Mn^{2+} shows its maximum effect at a one tenth the concentration needed for Mg^{2+} and Ca^{2+} in keeping with their respective affinity constants for the binding to ADP, i.e. $K_{Mn}=1.15\text{-}3 \times 10^{4} M^{-1}$, $K_{Mg}=0.85\text{-}2 \times 10^{3} M^{-1}$, $K_{Ca}=0.63\text{-}0.8 \times 10^{3} M^{-1}$ (9,10). According to this hypothesis Tb^{3+} might be ineffective in promoting the aggregation of platelets and even inhibitory due to a lower ability of forming complexes with ADP or most probably due to the formation of a different type of complex (11, 12). In any case the net charge of a ADP-Tb complex would be different from those of ADP with Mn, Mg or Ca. Different explanations on the role of metals other than Ca^{2+} toward the aggregation have been proposed (13, 14).

Instead Tb^{3+} behaves similarly to Ca^{2+} when the aggregation or secretion of platelets is triggered by A23187. Both ions seem to act through the "activation" of a Ca^{2+} flux as their effect is abolished by verapamil (15).

A spectroscopic evidence of interaction between A23187 and platelets was obtained. In fact the fluorescence yield of A23187 increased upon addition to platelets as if it was experiencing a more hydrophobic environment (16). Furthermore an energy transfer from platelets to the ionophore was apparent. It was impossible however to determine by fluorescence the affinity or the number of the binding sites for A23187 present in platelets. The fluorescence experiments also demonstrated that Ca^{2+} and Tb^{3+} affect the fluorescence of platelet-bound A23187. This effect is not due to an increased binding of the ionophore to platelets, that would have produced a further quenching of platelet intrinsic fluorescence. A higher fluorescence of the Ca- or Tb-complex of A23187 with respect to the free ionophore might be postulated. However a similar effect of Ca^{2+} on the fluorescence of platelet-bound fluorescent probe

1-anilinonaphtalene 8-sulfonate that presumably does not bind the metal has been reported (17). In the present investigation we did not observe a direct binding of Tb^{3+} to platelets or to A23187. Such binding might have been inferred if a sensitized Tb emission were apparent. These findings were at variance with those reported for erythrocytes where a binding of lanthanides to the membranes was apparent (6). Furthermore a binding of Ca^{2+} to platelet membrane has been demonstrated, probably associated with the surface glycoproteins IIb and IIIa (18). Our data indicate that if it is the case, the binding sites are far from the aromatic amino acid residues of these proteins (5).

The above reported data seem to indicate that Tb^{3+} can replace Ca^{2+} when the aggregation is triggered by A23187 but not when is triggered by ADP. The aggregation is thought to be always primed by an increase of free intracellular Ca^{2+} above a threshold value which may be different from that needed for shape change or secretion (19). Though the aggregation induced by A23187 has been challenged (20), certainly it is significantly enhanced by Ca^{2+} and Tb^{3+} and the A23187-induced ATP secretion is strongly inhibited by the metal addition. It has been reported that platelets are impermeable to Ca^{2+} complexes of A23187 (21). Therefore it appears that Ca^{2+} and Tb^{3+} should affect the level of intracellular Ca^{2+} from the exterior. It is in fact known that this level could vary independently from the presence of extracellular Ca^{2+} (19). More work is needed to ascertain whether the effect of Ca^{2+} and Tb^{3+} on the fluorescence of platelet-bound A23187 and the redistribution of intracellular calcium are interdependent phenomena.

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