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Activation of Human Blood Platelets by Arginine-Vasopressin

Role of Bivalent Cations

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SUMMARY

Arginine-vasopressin caused platelet activation, i.e., a shape change reaction and a rise in intracellular free Ca^{2+} ($[Ca^{2+}]_i$) only in the presence of certain bivalent cations. The EC_{50} of arginine-vasopressin (concentration causing half-maximal shape change) decreased with rising concentrations of Mn^{2+}, Mg^{2+}, or Ca^{2+} in the medium, but was at least an order higher with Ca^{2+} than with Mn^{2+} or Mg^{2+}. The EC_{50} of the active bivalent cations (concentrations enabling 100 nM arginine-vasopressin to exert half-maximal shape change and rise in $[Ca^{2+}]_i$) varied with the individual cations, being by far the highest for Ca^{2+}. The K_D of [^{3}H]arginine-vasopressin binding to platelet membranes and intact platelets markedly decreased when extracellular Mg^{2+} or Mn^{2+} were present, and the K_D values were inversely related to the concentration of the cations. Ca^{2+} also lowered the K_D values; however, the effect was less marked than that of Mg^{2+} or Mn^{2+} and, in physiological conditions, significant only in intact platelets. Vasopressin-1 antagonists counteracted arginine-vasopressin binding and the shape change reaction and $[Ca^{2+}]_i$ rise induced by arginine-vasopressin. In the presence of Mn^{2+} in the medium, administration of arginine-vasopressin led to quenching of the intracellular fluorescence of 2-methyl-6-methoxy-8-nitroquinoline-loaded platelets, possibly due to influx of Mn^{2+}. In conclusion, the dependency of the arginine-vasopressin-induced platelet activation on bivalent cations is at least partly due to an enhancement by these cations of the affinity of the vasopressin-1 receptor for arginine-vasopressin. Thereby, under physiological conditions, Mg^{2+} seems to be of primary importance. Other mechanisms may be involved, too, e.g., an enhancement by arginine-vasopressin of the influx of bivalent cations into the platelets.

INTRODUCTION

In blood platelets of humans which exhibit V_1-receptors,[1] (1, 2), AVP, a V_1-receptor agonist, causes a shape change reaction (transition of the normally occurring discoid form into a spheroid shape with extrusion of pseudopods) and aggregation, together with a rise in $[Ca^{2+}]_i$. For these effects, extracellular Ca^{2+} was thought to be essential and Mg^{2+} seemed also to be involved (3, 4). However, in recent experiments it was found that AVP even caused a shape change reaction and a $[Ca^{2+}]_i$ rise when Mg^{2+} was the only bivalent cation in the extracellular medium. In the presence of the Mg^{2+} concentration occurring physiologically in human blood plasma, AVP showed higher potency and efficacy in inducing the shape change reaction than with physiological levels of Ca^{2+} (5). These and other observations led to the conclusion that under physiological conditions extracellular Mg^{2+} was more important than Ca^{2+} for the AVP-induced platelet activation and that this activation depended at least in part on calcium released from intracellular stores. However, the mode of action on Mg^{2+} in the AVP-induced platelet activation has not been clarified.

In the present work we show that, in addition to Ca^{2+} and Mg^{2+}, other bivalent cations enable AVP to cause platelet activation, i.e., a shape change reaction and a rise in $[Ca^{2+}]_i$. Evidence is presented that this activation is partly due to an enhancement by the cations of the affinity of AVP for its membrane receptor. In addition, AVP may increase the influx of bivalent cations into the platelets.

[1] The abbreviations used are: V_1-receptor, vasopressin receptor of the V_1-subtype; AVP, arginine-vasopressin; $[Ca^{2+}]_i$, intracellular free calcium; quin-2, 2-methyl-6-methoxy-8-nitroquinoline; PRP, platelet-rich plasma; EDTA, ethylenediaminetetraacetate; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N′,N′*,-tetraacetic acid; Hepes, 4-(2-hydroxyethyl-1-piperazineethanesulfonic acid; BSA, bovine serum albumin; Gpp(NH)p, guanylylimidodiphosphate; EC_{50}, concentrations of AVP causing half-maximal shape change reaction or concentrations of cations at which 100 mM AVP induces half-maximal shape change reaction and $[Ca^{2+}]_i$ rise; IC_{50}, molar concentrations at which V_1-antagonists cause 50% of their maximal effects.

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EXPERIMENTAL PROCEDURES

Materials. AVP, $d(CH_2)_5$-Tyr(Me)AVP (V_1-antagonist), and $d(CH_2)_5$-D-Tyr(Et)AVP (mixed V_1/V_2-antagonist) were kindly provided by Prof. K. Hofbauer, Ciba-Geigy Ltd. (Basel, Switzerland), and quin-2 as its tetraacetoxymethylester was provided by Sandoz Ltd. (Basel, Switzerland) and Ciba-Geigy Ltd. [^{3}H]AVP (40 Ci/mmol) was purchased from New England Nuclear (Boston, MA). All other substances were obtained from commercial sources.

Shape change reaction. The shape change reaction was measured, as previously described (6), by light absorbance of suspensions of human platelets. In brief, PRP, obtained by centrifugation of blood drawn from the cubital vein of healthy individuals and containing 13 mM trisodium citrate as anticoagulant, was put on a discontinuous dextran gradient (consisting of two layers of 10 and 20% dextran) (6). Thereafter, centrifugation for 10 min at 6000 × *g* and 4° was performed. The platelets which assembled between the two layers were removed, counted in a Thrombocounter, and diluted to 10^8 platelets/ml with artificial buffer, pH 7.4, containing 140 mM NaCl, 7.6 mM KCl, 5.6 mM D-glucose, 7.7 mM Tris, 13 mM trisodium citrate, and, in some instances, 2 mM EDTA or 0.5 mM EGTA. In most experiments the buffer was also supplemented with other cations which alone had no effect on the platelet shape. For the measurements, 100 μl of the platelet suspension (10^8 platelets/ml) were diluted with 450 μl of buffer devoid of citrate so that the final concentration of the latter was 2.4 mM. The platelets were preincubated for 10 min at 37° before addition of AVP. In some experiments V_1-antagonists were added to the medium 1 min before AVP. The incubation temperature was 37°. The shape change reaction was measured in an Elvi aggregometer at 37° while stirring the platelets at 1000 rpm.

Determination of $[Ca^{2+}]_i$. For the $[Ca^{2+}]_i$ measurements PRP was incubated with the fluorescent probe quin-2-tetraacetoxymethylester (15 μM) for 10 min at 37°. The platelets were isolated by gel filtration using a 10 mM Hepes buffer, pH 7.4, containing 140 mM NaCl, 5 mM KCl, 0.5 mM Na_2HPO_4, 6 mM glucose, and 2.4 mM trisodium citrate. The platelets were preincubated up to 5 min at 37° in buffer supplemented with or without additional cations which alone had no effect on $[Ca^{2+}]_i$. In some experiments V_1-antagonists were also present. Then AVP was added and the incubation continued for 1–5 min (for details see Refs. 7 and 8). Measurements of fluorescence were performed on a Perkin-Elmer LS-5 fluorimeter to subtract autofluorescence of bivalent cations. Comparisons with platelet suspensions containing 0.1% BSA had shown that the absence of BSA in the present experiments did not influence the results.

[^{3}H]AVP binding. Platelet membranes were prepared as previously described (9) and suspended in buffer to a concentration of 10 mg of protein/ml (stock solution). The buffer was composed of Tris (50 mM), NaCl (130 mM), KCl (5 mM), and EGTA (0.5 mM), pH 7.4. Aliquots of 25 μl of membrane stock suspension were diluted to 200 μl with buffer devoid of EGTA (final EGTA concentration 63 μM), containing 0.5% BSA. For the equilibrium experiments the buffer also contained various concentrations of [^{3}H]AVP and bivalent cations and, in some experiments, 10 μM Gpp(NH)p. Unlabeled AVP (1 μM; for the determination of nonspecific binding), 2 mM EDTA, or V_1-antagonists were also added to some samples. After incubation for various times (time course) or for 30 min (K_D, B_{max}) at 25°, the binding reaction was stopped by addition of 3 ml of ice-cold buffer, containing 0.5% BSA, immediately filtered under vacuum over Whatman GF/C glass fiber filters. The latter were rinsed twice with 3 ml of ice-cold buffer, containing 0.1% BSA, and transferred to a plastic vial. The radioactivity on the filter was extracted with 10 ml of Quickszint (Zinsser Analytic, Frankfurt, FRG) and counted by liquid scintillation spectrometry.

For the dissociation experiments membrane stock solution (diluted to 3 ml as indicated above) was incubated with 2 nM [^{3}H]AVP plus 1 mM Mg^{2+} for 30 min at 25°. Thereafter, unlabeled AVP (10 μM), EDTA (2 mM) with or without unlabeled AVP (1 mM), or Ca^{2+} (5, 10, or 50 mM) plus AVP (1 μM; all final concentrations) was added and the incubation was continued for a further 60 min. At various time intervals, samples of 200 μl were taken from the incubation mixture, diluted with 3 ml of ice-cold buffer containing 0.1% BSA, and processed as indicated above.

For the experiments with intact platelets, PRP was prepared as previously described (6). After centrifugation at 600 × *g* for 30 min, the pellet was resuspended in the buffer used for the membrane experiments but without EGTA to give a stock suspension of 5×10^9 platelets/ml. The binding experiments were carried out as described for membranes, in samples containing 25 μl of stock suspension plus 175 μl of EGTA-free buffer used in the membrane experiments.

An incubation temperature of 25° was optimal for the binding experiments and 37° was optimal for the shape change and $[Ca^{2+}]_i$ experiments. Control experiments showed that the results of the binding and shape change studies were not influenced by the moderate differences in the compositions of the buffer systems used for the two types of experiments. The presence of citrate in the shape change and $[Ca^{2+}]_i$ experiments was allowed for in the calculations.

High pressure liquid chromatographic analysis of the supernatants of membrane and platelet suspensions showed that less than 5% of the [^{3}H]AVP was degraded during the incubation procedure.

Data analysis. Although the dissociation experiments indicated the presence of two classes of binding sites, the saturation binding experiments were analyzed according to a one-site model by a computerized, weighted nonlinear regression procedure as described elsewhere (10). Since we were unable to resolve the two affinity populations from saturation experiments (for reasons, see Ref. 10), it was felt that a one-site model was more appropriate than speculative numbers from a two-site fit. Furthermore, because in membranes in the absence of Mg^{2+} or Mn^{2+} the affinity of [^{3}H]AVP was low, only a small range of the saturation curve could be covered and the determination of K_D and B_{max} values was not possible.

The constants for dissociation kinetics were calculated according to either a single ($B(t) = B_1 \cdot e^{-k_{-1} \cdot t}$ + NSB) or a double ($Bt) = B_1 \cdot e^{-k_{-1} \cdot t} + B_2 \cdot e^{-k_{-2} \cdot t}$ + NSB) exponential model, using weighted, nonlinear regression. $B(t)$ is the binding as a function of time, t, B_1 and B_2 are the sizes of the fast and slow dissociating components, k_{-1} and k_{-2} are the corresponding off-rate constants, and *NSB* represents the nonspecific binding.

Calculations. The concentrations of $[Ca^{2+}]_i$ were calculated as previously indicated (11). Each EC_{50} value was determined on the basis of five to six different concentrations of AVP or cations in the medium. Statistical analysis was carried out using the Wilcoxon test for unpaired data. EC_{50} of AVP is the molar concentration of AVP which caused half-maximal shape change reaction. EC_{50} of bivalent cations is the molar concentration of the cations at which 100 nM AVP induced half-maximal shape change reaction or $[Ca^{2+}]_i$ rise. IC_{50} indicates the molar concentrations at which V_1-antagonists caused 50% of their maximal effects.

In the shape change and $[Ca^{2+}]_i$ experiments, citrate, which complexes bivalent cations, was used. For the calculation of the free concentration of the cations, the mass action law was applied for a single ligand-complexer model using the constants of Sillén and Martell (12). The low concentrations of EGTA (63 μM) used in the binding experiments were neglected since they do not have much complexing capacity, especially in the physiologically interesting concentration ranges of bivalent cations such as Ca^{2+} and Mg^{2+}.

RESULTS

Shape change reaction and $[Ca^{2+}]_i$ rise. In the absence of bivalent cations in the incubation medium, AVP did not generally cause a shape change reaction. Occasional shape change reactions seen under these conditions were likely to be due to insufficient removal of bivalent cations, probably Mg^{2+}, from the platelet membranes, since these shape change reactions were suppressed by addition of EDTA (0.5 mM) but not of EGTA (2 mM) to the

buffer. However, in the presence of certain bivalent cations in the medium (Table 1), AVP always caused a shape change reaction and (when measurable) a $[Ca^{2+}]_i$ rise. Typical recordings for Mg^{2+} and Ca^{2+} were given in a previous paper (5).

In platelets loaded with quin-2 at the relatively low concentrations used for the $[Ca^{2+}]_i$ experiments, the ED_{50} value of AVP in the shape change reaction was not different from that in unloaded platelets. In contrast, the amplitude of the shape change was slightly reduced (by about 30%).

Potency of bivalent cations. Only bivalent cations with an ionic radius between 0.6 and 1.0×10^{-10} m enabled AVP to induce a shape change reaction and a $[Ca^{2+}]_i$ rise. The EC_{50} values of the cations varied, Ca^{2+} being least potent (Table 1). Li^+, Al^{3+}, and Fe^{3+} were ineffective in both the shape change and the $[Ca^{2+}]_i$ experiments. The maximal amplitudes of the shape change reactions (i.e., the amplitudes in the presence of supramaximal concentrations of the active bivalent cations and AVP) were not markedly different (Table 1).

Potency of AVP. The EC_{50} of AVP in the shape change reaction decreased with increasing concentrations of Mg^{2+}, Ca^{2+}, or Mn^{2+} in the incubation medium. At equimolar concentrations of the cations, AVP was at least an order of magnitude more potent in the presence of Mg^{2+} than in the presence of Ca^{2+}, and in the presence of Mn^{2+} the peptide was even more potent than with Mg^{2+} (Fig. 1).

Specific binding of [^{3}H]AVP. In platelet membranes and intact platelets, [^{3}H]AVP (2 nM) in the presence of bivalent cations showed specific binding, the equilibrium being established after 30 min (Fig. 2; results for membranes not shown) (13). In membranes suspended in buffer devoid of bivalent cations or containing only Ca^{2+}, the affinity was too low for calculating K_D and B_{max} (see "Calculations" under "Experimental Procedures"). Mg^{2+} and Mn^{2+} in the medium markedly enhanced the binding affinity of [^{3}H]AVP (Fig. 3). It increased with rising concentrations of the cations (Fig. 4). The K_D value in the presence of Mn^{2+} was smaller than that in presence of Mg^{2+}, and the B_{max} value was higher with Mn^{2+} than with Mg^{2+} (Table 2). The GTP analogue Gpp(NH)p (10 μM) did not affect the K_D value of [^{3}H]AVP in the presence of 0.5 mM Mg^{2+}.

In intact platelets Ca^{2+} moderately but significantly ($p < 0.01$) lowered the K_D of [^{3}H]AVP, and Mg^{2+} and Mn^{2+} decreased the K_D by more than an order of magnitude, to even smaller values than in membranes. The B_{max} values were not significantly different, whether the binding experiments had been performed in the presence or the absence of bivalent cations ($p < 0.05$) (Fig. 3, Table 2).

Dissociation kinetics of [^{3}H]AVP in platelet membranes. In medium containing Mg^{2+} the off-rate of [^{3}H]AVP in the presence of excess amounts of unlabeled AVP was gradually enhanced by addition of increasing amounts of Ca^{2+} (5, 10, and 50 mM). EDTA also caused a marked enhancement of the dissociation of [^{3}H]AVP which was equal in the presence (results not shown) and in the absence of unlabeled AVP and was even more marked than that induced by 50 mM Ca^{2+} (Fig. 5). Computerized analysis of the dissociation time course experiments suggests that there are two populations of [^{3}H]AVP-binding sites: A fast dissociating component with an average off-rate constant of 1.41 min^{-1} and a slow dissociating component with an off-rate constant of 0.014 min^{-1}. When the dissociation was initiated by the addition of 10^{-6} M AVP, the data were compatible with the presence of 79% slow dissociating component. The simultaneous addition of 50 mM Ca^{2+} decreased the proportion of slow dissociation component to 69%. EDTA (2 mM) caused a more marked shift toward the fast dissociating component, leaving only about 30% of the specifically bound [^{3}H]AVP in the high affinity state. These data correspond to a model with two interconvertible [^{3}H]AVP-binding sites, where Mg^{2+} (and probably Mn^{2+}) facilitates the conversion of the low into the

TABLE 1

Shape change and $[Ca^{2+}]_i$ rise induced in human blood platelets by 100 nM AVP in the presence and absence of various bivalent cations

Averages with SE of 3–10 experiments are given. In the presence of Mn^{2+} and Cd^{2+}, $[Ca^{2+}]_i$ was not measurable due to quenching and autofluorescence, respectively. The basal levels of $[Ca^{2+}]_i$ showed an average concentration of 100 nM; the values in the presence of the various cations alone (without AVP) did not differ significantly from the basal level.

Ion	Radius in 10^{-10} m	Shape change		$[Ca^{2+}]_i$ rise	
		EC_{50}[a]	Maximal amplitude	EC_{50}[a]	Maximum rise
		μM	*cm*	*μM*	*nM*
Be^{2+}	0.35	2000 (NE[b])		2000 (NE)	
Mg^{2+}	0.66	15 ± 4	9.7 ± 1.1	16 ± 4	390 ± 45
Ni^{2+}	0.69	0.2 ± 0.1	12.4 ± 0.9	0.2 ± 0.1	401 ± 68
Co^{2+}	0.72	0.4 ± 0.3	11.6 ± 1.6	0.5 ± 0.2	378 ± 57
Zn^{2+}	0.74	0.3 ± 0.05	15.0 ± 1.3	0.4 ± 0.1	470 ± 55
Fe^{2+}	0.74	1.7 ± 0.6	11.7 ± 1.1	2.1 ± 0.9	428 ± 71
Mn^{2+}	0.80	1.2 ± 0.4	11.0 ± 1.6		
Cd^{2+}	0.97	2.6 ± 1.0	14.5 ± 1.5		
Ca^{2+}	0.99	1250 ± 29	9.4 ± 1.2	1082 ± 34	542 ± 88
Sr^{2+}	1.12	1150 (NE)		1130 (NE)	
Ba^{2+}	1.34	450 (NE)		450 (NE)	

[a] Concentration of free cations enabling 100 nM AVP to induce half-maximal shape change and $[Ca^{2+}]_i$ rise.
[b] NE, no effect up to concentrations indicated.

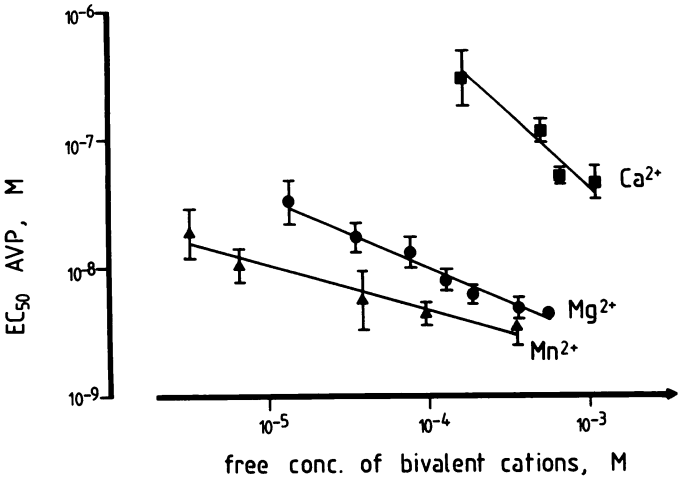

FIG. 1. *Shape change reaction of human platelets: dependence of the EC_{50} values of AVP (concentrations of AVP causing half-maximal shape change reaction) on the free concentration of bivalent cations in the buffer*

Each point is an average with SE of 3–4 experiments, each carried out with 4–5 different concentrations of AVP.

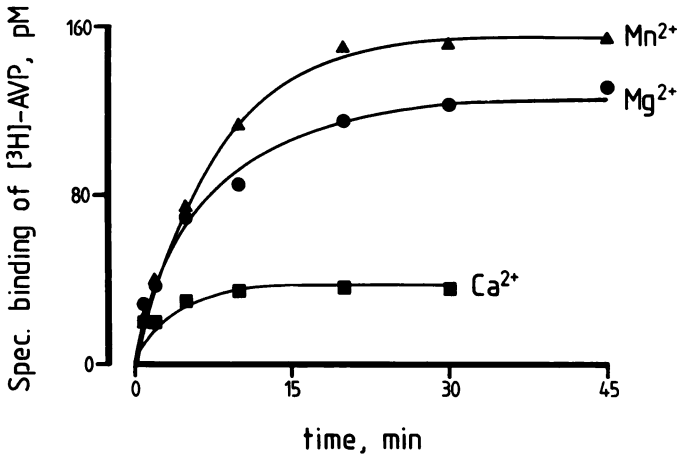

FIG. 2. *Time course of the specific binding of 2 nM [^{3}H]AVP to human platelets in the presence of 1.5 mM Ca^{2+}, 0.5 mM Mg^{2+}, or 0.5 mM Mn^{2+}*

high affinity [^{3}H]AVP-binding state, and where Ca^{2+} also has a moderate effect.

AVP antagonists. In the presence of physiological concentrations of Mg^{2+} (0.5 mM), rising concentrations of the V_1-antagonists $d(CH_2)_5$-Tyr(Me)AVP and $d(CH_2)_5$-D-Tyr(Et)AVP gradually reduced the AVP-induced shape change reaction and $[Ca^{2+}]_i$ rise in platelets and the specific binding of [^{3}H]AVP to platelet membranes. The EC_{50} values of the antagonists were of the same order in the three types of experiments (Table 3).

Quenching of quin-2-Ca fluorescence. In aqueous quin-2-Ca solutions, Mn^{2+} causes a marked quenching of the fluorescence, whereas the other bivalent cations in the concentrations studied did not markedly interfere with the fluorescence. In quin-2-loaded platelets suspended in buffer containing Mn^{2+}, addition of 100 nM AVP induced a fast initial rise of the intracellular fluorescence, which was inversely related to the Mn^{2+} concentration and was probably due to release of intracellular calcium. This

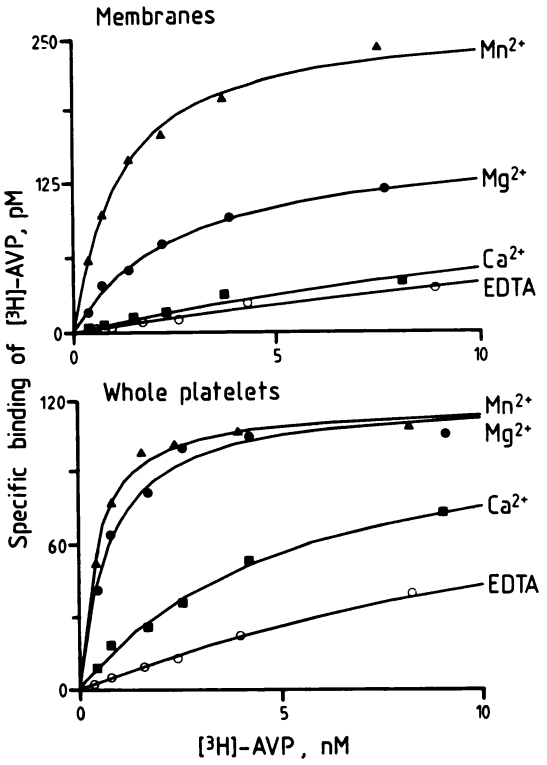

FIG. 3. *Specific binding of [^{3}H]AVP to membranes of blood platelets and whole platelets of humans in the presence and absence of bivalent cations in the medium*

The incubation time was 30 min. Concentrations of bivalent cations were: Ca^{2+}, 1.5 mM; Mg^{2+}, 0.5 mM; Mn^{2+}, 0.5 mM. The values for nonspecific [^{3}H]AVP binding (in percent of total binding) were: membranes with EDTA or Ca^{2+}, 70–75; with Mg^{2+} or Mn^{2+}, 15–50; whole platelets with EDTA, 30; with Ca^{2+}, 15–25; with Mg^{2+} or Mn^{2+}, 4–15.

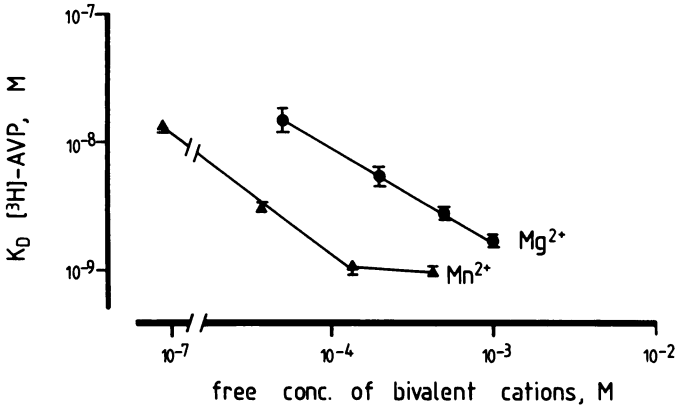

FIG. 4. *Dependence of the K_D for [^{3}H]AVP binding in platelet membranes on the free concentration of bivalent cations*

Each point is an average with SE of 3–4 experiments, each carried out with 4–5 different concentrations of [^{3}H]AVP.

fluorescence then rapidly decreased to values considerably below basic levels. With Mg^{2+}, which, at the concentrations used, does not markedly quench quin-2-Ca fluorescence, the rise in fluorescence was not followed by a decrease below basal levels. However, when Mg^{2+} was combined with Mn^{2+}, the secondary fluorescence drop below control levels was seen (Fig. 6). High pressure liquid chromatographic analysis showed that AVP in the

TABLE 2

K_D and B_{max} of [^{3}H]AVP binding in platelet membranes and whole platelets in the presence and absence of various bivalent cations

The incubation time was 30 min. Each value is an average with SE of 3–9 experiments. The K_D values of the experiments with bivalent cations are all significantly different from the corresponding control values (without bivalent cations + EDTA), $p < 0.01$. Furthermore, K_D values in membranes are significantly different from those in platelets ($p < 0.01$), and in membranes the B_{max} value with Mn^{2+} was significantly different from that with Mg^{2+} ($p < 0.05$).

Cation	Membranes		Platelets	
	K_D	B_{max}	K_D	B_{max}
	nM	*fmol/mg protein*	*nM*	*sites/cell*
None (EDTA, 2 mM)			24 ± 3.1	156 ± 34
Ca^{2+} (1.5 mM)			6.8 ± 0.8	117 ± 15
Mg^{2+} (0.5 mM)	2.7 ± 0.2	148 ± 10	0.74 ± 0.02	124 ± 20
Mn^{2+} (0.5 mM)	0.95 ± 0.05	176 ± 18	0.43 ± 0.02	134 ± 12

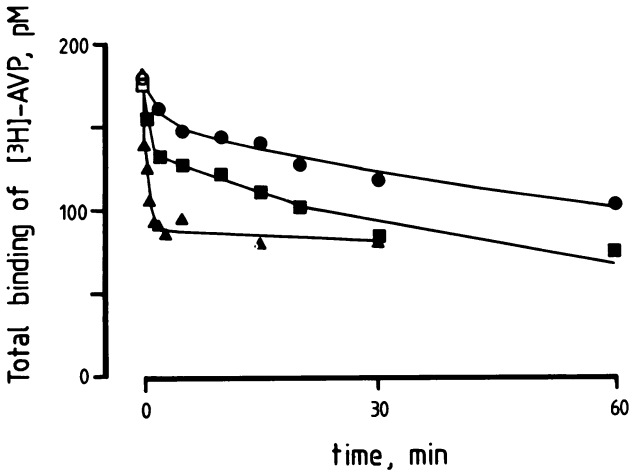

FIG. 5. *Dissociation kinetics of [^{3}H]AVP totally bound to membranes of human platelets*

After preincubation of the membranes for 30 min at 25° with 2×10^{-9} M [^{3}H]AVP in the presence of 10^{-3} M Mg^{2+}, 10^{-6} M unlabeled AVP (●), 10^{-6} M AVP plus 50×10^{-3} M Ca^{2+} (■), or 2×10^{-3} M EDTA (▲) were added at time zero and the incubation was continued for another 60 min. Typical curves are shown.

TABLE 3

Interference by d(CH$_2$)$_5$-Tyr(Me)AVP (V_1-antagonist) and d(CH$_2$)$_5$-D-Tyr(Et)AVP (mixed V_1/V_2-antagonist) with the shape change reaction and $[Ca^{2+}]_i$ rise induced by 100 nM AVP in human platelets and with the specific binding of 2 nM [^{3}H]AVP in human platelet membranes

The buffer contained 0.5 mmol/liter Mg^{2+}. IC_{50} values of the antagonists are given. Each value is an average with SE of 3 experiments.

	IC_{50}	
	d(CH$_2$)$_5$-Tyr(Me)AVP	d(CH$_2$)$_5$-D-Tyr(Et)AVP
	nM	
Shape change	31 ± 3	24 ± 4
$[Ca^{2+}]_i$	18 ± 3	30 ± 5
Specific binding	17 ± 5	13 ± 3

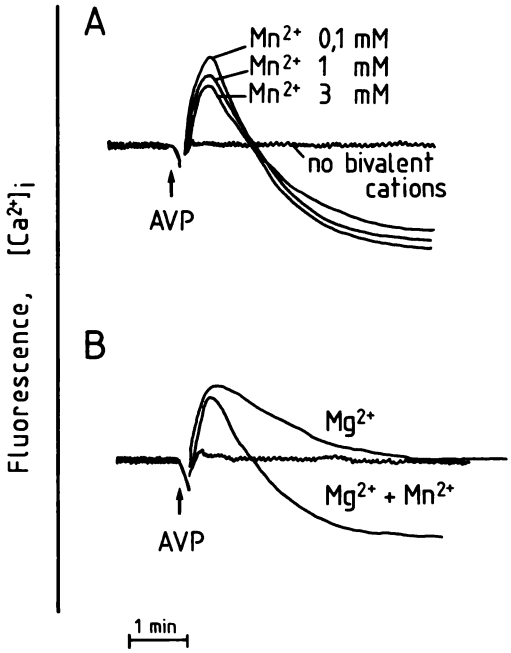

FIG. 6. *Influence of 100 nM AVP in the presence of Mn^{2+} on the intracellular quin-2-Ca fluorescence in suspensions of quin-2-loaded human platelets*

A, suspensions containing various concentrations of Mn^{2+} as the only bivalent cation; B, suspensions containing either 0.3 mM Mg^{2+} alone or 0.3 mM Mg^{2+} plus 0.1 mM Mn^{2+}. Mn^{2+} or Mg^{2+} added alone (without AVP) did not influence the intracellular quin-2-Ca fluorescence.

presence of Mn^{2+} did not increase the quin-2 concentration in the supernatant of suspensions of quin-2-loaded, washed platelets. Therefore, the quenching of the fluorescence was not caused by leakage of quin-2 from the platelets into the medium.

DISCUSSION

The present investigation confirms that AVP in the absence of bivalent cations, especially when EDTA is present in the incubation medium, does not cause platelet activation, i.e., a shape change reaction and a $[Ca^{2+}]_i$ rise (5). In addition, it shows that not only Mg^{2+} and Ca^{2+}, but also other bivalent cations are active. Their action seems to depend on the presence of a double charge and to a certain extent also on the ionic radius, although under the present experimental conditions no strict correlation between the latter and the biological activity could be detected. A shape change reaction was only seen in combination with raised $[Ca^{2+}]_i$ levels. Also, the EC_{50} values of the cations were of similar order in the shape change as in the $[Ca^{2+}]_i$ experiments. Furthermore, in higher concentrations, quin-2 (which complexes intracellular Ca^{2+}) markedly reduced the AVP-induced shape change (IC_{50} of quin-2 about 1 mM (11)). Finally, the IC_{50} values of the V_1-antagonists d(CH$_2$)$_5$-Tyr(Me) AVP and d(CH$_2$)$_5$-D-Tyr(Et)AVP in the shape change experiments were similar to those in the $[Ca^{2+}]_i$ experiments. These findings are compatible with the view that both the AVP-induced shape change reaction and $[Ca^{2+}]_i$ rise are due to stimulation of V_1-receptors and that the AVP-induced shape change reaction is dependent on $[Ca^{2+}]_i$.

The binding experiments indicate that bivalent cations enhance the affinity of AVP for its platelet receptor. In fact, our results with whole platelets show that Mg^{2+} and Mn^{2+} decreased the K_D for [^{3}H]AVP binding and that Ca^{2+} had a similar although less marked effect, whereby the B_{max} values were not significantly changed. The K_D of [^{3}H]AVP which we found in platelets in the presence of Mg^{2+} was lower than that reported previously (13), but this may be due to the use of ligands with different labels (^{3}H in our experiments, ^{125}I in earlier ones).

In platelet membranes K_D and B_{max} could only be determined in the presence of Mg^{2+} or Mn^{2+}. However, the decrease of the K_D values for [^{3}H]AVP in the presence of these cations as well as the dissociation experiments confirm that bivalent cations enhance the affinity of AVP for its receptor. The K_D values were somewhat higher in membranes than in platelets. The presence of GTP in whole platelets but not in membranes cannot be the reason for this difference, since Gpp(NH)p did not affect the K_D of [^{3}H]AVP in platelet membranes. Furthermore, in membranes, B_{max} in the presence of Mn^{2+} was somewhat higher than with Mg^{2+} in the incubation medium. This difference cannot be explained. Since it was not to be seen in whole platelets, it may be due to an artifact, e.g., to different accessibility of membrane receptors as a consequence of differences in the degree of membrane aggregation.

Therefore, the activation of platelets induced by AVP in the presence of bivalent cations might be related to an enhancement by the cations of the AVP affinity. This view is supported by additional findings. Thus, there is a correlation between the K_D of [^{3}H]AVP binding in membranes and the EC_{50} values of AVP for platelet activation in the presence of different concentrations of Mg^{2+}, Mn^{2+} (compare Figs. 1 and 4). This correlation was also seen when the binding experiments had been performed in whole platelets (results not shown). Furthermore, we have found that the potencies of the V_1-antagonists $d(CH_2)_5$-Tyr(Me)AVP and $d(CH_2)_5$-D-Tyr(Et)AVP in the [^{3}H]AVP-binding experiments with platelet membranes were of the same order as those in the shape change and $[Ca^{2+}]_i$ experiments.

Bivalent cations seem to affect the shape change reaction due to AVP rather specifically. In fact, neither the 5-hydroxytryptamine- nor the ADP-induced shape changes were markedly influenced by the presence of EDTA (2–4 mM) (11).[2] Further evidence for the dependence of the shape change reaction on AVP binding is provided by recent experiments from our laboratory. In rabbit platelets, which show at the best only a minimal AVP-induced shape change, no specific binding sites for AVP in the presence of Mg^{2+} could be detected.

Two mechanisms of action of bivalent cations in the AVP-induced platelet activation have to be considered. First, an enhancement by the bivalent cations of AVP binding to its V_1-receptor could be responsible for triggering enzymatic membrane processes, e.g., activation of phospholipase C leading to an accumulation of inositol triphosphate and, through elevation of diacylglycerol levels, to stimulation of protein kinase C. Especially the rise in inositol triphosphate, which by liberation of intracellular calcium causes an elevation of $[Ca^{2+}]_i$, might be causally connected with the shape change reaction. Experiments in our laboratory have indeed indicated that AVP in the presence of low concentrations of Mg^{2+} and Mn^{2+} and of higher concentrations of Ca^{2+}, but not in the absence of bivalent cations, enhances phosphatidylinositol turnover in human platelets.[3]

Second, AVP seems to promote the influx of bivalent cations into the platelets. This has been suggested before for Ca^{2+}, since AVP caused a more marked rise of $[Ca^{2+}]_i$ (about 1 μM) in the presence of extracellular Mg^{2+} plus Ca^{2+} than with either Mg^{2+} or Ca^{2+} alone (5, 14). An AVP-induced enhancement of the entry of bivalent cations is also indicated by our experiments with Mn^{2+}. In fact, when AVP was added to platelet suspensions containing Mn^{2+} (which quenches the fluorescence of the quin-2-Ca complex), the intracellular quin-2 fluorescence disappeared virtually completely, and this disappearance was not due to leakage of the dye from the cells. Recently, other platelet-activating substances, i.e., thrombin, platelet-activating factor, and ADP have also been shown to decrease intracellular quin-2 fluorescence in human platelets in the presence of extracellular Mn^{2+}. Therefore, these substances were thought to act through receptor-operated Ca^{2+} channels (15). AVP may also cause an opening of some kind of membrane channel for bivalent cations. The possibility has to be considered that entry of bivalent cations (Ca^{2+} and others), leading to changes in their intracellular content and distribution, might have additional effects on platelet function.

There is no evidence for an involvement of the cyclooxygenase pathway since, as previously shown (5), neither aspirin nor indomethacin counteracted the AVP-induced shape change reaction and $[Ca^{2+}]_i$ rise in human platelets. Furthermore, as previously indicated (5), no evidence was obtained for an involvement of classical slow calcium channels, since the calcium entry blockers diltiazem, verapamil, and $LaCl_3$ influenced neither the shape change reaction nor the $[Ca^{2+}]_i$ rise.

Under physiological conditions Mg^{2+} seems to play a prominent role in the AVP-induced platelet activation. Thus, the EC_{50} of this cation (14.5 μM) was considerably below the free concentrations (approximately 500 μM) occurring in plasma, and, according to present and previous (5) investigations, AVP was markedly less potent in the presence of Ca^{2+} than of Mg^{2+}. However, under physiological conditions external Ca^{2+} may have an additional role for the action of AVP, due to influx of the cation into the platelets leading to a more marked rise of $[Ca^{2+}]_i$ than in the presence of Mg^{2+} alone. Whether other bivalent cations can exert an effect under physiological and pathophysiological conditions remains to be elucidated. Bivalent cations may also have an important physiological role in the activation of V_1-receptors of other tissues. Indeed, it has been shown that the specific binding of [^{3}H]AVP was enhanced by Ni^{2+} and Co^{2+} in brain membranes of rats and by Mg^{2+} in membranes of bovine adrenal medulla (16, 17).

[2] A. Pletscher, unpublished results.

[3] I. Roos, F. Ferracin, and A. Pletscher, submitted for publication.

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