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Influence of the Red Blood Cell Ca^{2+}-Ion Concentration on the Erythrocyte Aggregation in Stasis

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Four essential components influence the blood circulation through the vessels. These are (a) the hematocrit, (b) the plasma viscosity, (c) erythrocyte aggregation, and (d) the erythrocyte deformability. In different diseases increased red blood cell aggregation is assumed to be caused by the plasma fibrinogen and other macromolecules (1,2). Hemodilution and defibrinogenation give only restricted possibilities to regulate the passage of the erythrocytes through microcirculation where the aim is the reduction of the peripheral resistance in the microvessels.

The erythrocyte aggregation in stasis (MEA) is the result of macromolecular bridging; electrostatic repulsive forces of the cell surface charges; cell geometry; membrane binding forces; and the mechanical shear stress leading to an equilibrium between aggregation and disaggregation (1,2). For example perturbance of the equilibrium is known to be related to increased fibrinogen concentration in the case of infectious diseases (3). But there are many diseases where the erythrocyte aggregation is increased irrespective of normal fibrinogen concentration (4).

Until recently only alterations of the extracellular compartment were regarded when determining erythrocyte aggregation. It is well known that augmentation of the intracellular Ca^{2+}-ion concentration leads to dramatic alterations of the cells, i.e., disturbances of membrane structure and function (5). An increase of the intracellular Ca^{2+}-ion concentration can be achieved by ionophore A 23187. A 23187 is an electrically charged ion carrier of low molecular weight (523 Da) producing an electric neutral zwitter ion after complexing the Ca^{2+} ion. This ion carrier is able to bind ions in aqueous solution. The protonized ionophore diffuses inside the membrane to one side where the proton is released; the ionophore anion then complexes with the cation resolving it from its hydration sphere. The ion selectivity is a function of the solvation energy and the complex binding energy. The zwitter ions cause a concentration gradient resulting in a membrane diffusion process. When the complex has passed through the membrane the charged free ionophore returns complexing a new cation and the cycle repeats

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(6,7). It also seems reasonable to examine the erythrocyte interactions as a function of intracellular (cytosolic) ion conditions, especially the influence of altered cytosolic Ca^{2+}-ion concentration.

MATERIALS AND METHODS

Materials

Blood samples, obtained from 75 healthy donors (50 males, 25 females, age 18–45 years) of the University of Göttingen, were anticoagulated by addition of heparin (7 IU/ml blood) and centrifuged 10 min at 3000 U/min at 4°C. Other complications like infectious diseases which are known to influence erythrocyte aggregation were excluded by laboratory screening tests. After the plasma and buffy coat were carefully aspirated, the erythrocyte suspension was washed three times in a 310 mosm phosphate buffer (pH 7.4). Composition of the phosphate buffer was 0.133 mole/liter $Na_2HPO_4 * 2H_2O$, 0.155 mole/liter $NaH_2PO_4 * H_2O$.

Aggregation Measurement

Hematocrit was adjusted to 45% by addition of autologous plasma or a 6% albumin solution (Ca^{2+}, Na^+, and K^+ ions are added in physiological concentrations), respectively. Fifty microliters of the suspension was used for the aggregation measurement. Determination of the mean evaluation of the erythrocyte aggregation in stasis (MEA) was realized in a counter-rotating chamber according to Schmid-Schönbein *et al.* (8).

The rheophotometer consists of a transparent cone–plate chamber in which the erythrocyte suspensions are filled. The suspended red cells are dispersed by the rotation of the cone. A counter-rotation of the chamber allows microscopic observation of the erythrocytes. The orientation of the erythrocytes in the suspension, i.e., the aggregation or dispersion of the red cells, can be recorded photometrically by the fact that the more the erythrocytes are dispersed the less translucent the suspensions becomes. When the rotation of the cone is stopped the red cell aggregate and the suspension becomes more permeable to the light source of the microscope. The intensity of the light is measured by a photocell added to the tube of the microscope coupled with a multiplier and a recorder. The velocity of the aggregate formation in stasis as well as the final size of the aggregates can be recorded as a function of time. The MEA in stasis is defined as the integral of the light intensity during a period of 10 sec after the onset of stasis expressed in millimeters squared. All measurements were carried out in duplicate within 4 hr after blood collection at 25°C.

Fibrinogen concentration was determined by means of the thrombin time after collection in citrate plasma (9). Fibrinogen obtained from Sigma was added to the plasma and the albumin solution, respectively, to the final desired concentration. α_2-macroglobulin and ionophore A23187 were also obtained from Sigma and added to the solutions to the final desired concentrations.

Loading of the Erythrocyte with Ca^{2+} Ions

An increase of the intracellular Ca^{2+}-ion concentration was achieved by ionophore A 23187 (25 μmole/liter). After the erythrocyte was loaded with Ca^{2+} ions the

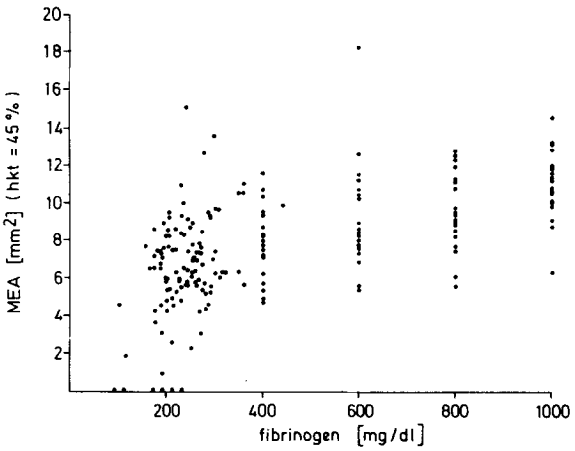

FIG. 1. Aggregation of the erythrocytes as a function of fibrinogen concentration in autologous plasma (normal value 200–350 mg/dl). The aggregation values at the fibrinogen concentrations of 400, 600, 800, and 1000 mg/dl are realized by the addition of fibrinogen to autologous plasma in the final desired concentration.

ionophore could be removed by washing it three times with a phosphate/albumin buffer solution (10).

Determination of the Ca^{2+}-Ion concentration in the Red Blood Cell

The erythrocyte suspension (3 ml) was buffered in a solution containing 5 ml Hepes (2-(4-(2-hydroxyethyl)-1-piperazinyl)-ethanesulfonic acid), 100 mmole $MgCl_2$, 50 mmole sucrose, and 50 mmole KCl (37°C, pH 7.4). Determination of the Ca^{2+}-ion concentration was performed with an ORION 93-20-01 Ca^{2+}-ion electrode connected to an ORION 811 pH/mv voltmeter. The electric potentials were measured before and after hemolysis of the red blood cells with 10 mmole digitonin. The potential difference is directly proportional to the intracellular Ca^{2+}-ion concentration. Before each measurement the electrode has to be calibrated (11). The normal intraerythrocytic Ca^{2+}-ion concentration is given by 0.2 ± 0.04 μmole/liter (11).

Statistical Analyses

All results are expressed as means and standard error of the means. Significance levels were determined by the Wilcoxon rank-sum test.

RESULTS

Figure 1 shows the aggregation of erythrocytes correlated to fibrinogen concentrations in autologous plasma from healthy donors. The values in the lower concentration range (80–150 mg/dl), autologous plasma) are obtained from blood samples of newborn infants (13). Aggregation values obtained at fibrinogen concentrations of 400, 600, 800, and 1000 mg/dl are realized by addition of fibrinogen to the autologous plasma. The exponential part of the curve in the lower range is similar to that described by other authors (14), while a linear fit appears in

TABLE 1
Aggregation of the Erythrocytes in Plasma and a 6% Albumin Solution, Respectively, as a Function of Fibrinogen Concentration[a]

Fibrinogen in plasma (mg/dl)	200–350 (normal range)	400	600	800	1000
MEA $(mm)^2$	7.2 ± 1.9	7.7 ± 1.9	8.7 ± 1.9	9.6 ± 2.1	11.1 ± 2.0
Range	3.4–15.0	4.6–11.5	5.3–12.5	5.5–12.7	6.2–14.4
n	75	20	20	20	20
$P <$		0.001	0.001	0.001	0.001
Fibrinogen in albumin (mg/dl)	**200**	**400**	**600**	**800**	**1000**
MEA $(mm)^2$	0	0	0	2.5 ± 0.6	3.7 ± 1.1
Range				1.0–4.2	1.6–6.4
n	20	20	20	20	20
$P <$	n.s.	n.s.		0.001	0.001

[a] The aggregation values at the fibrinogen concentrations of 400, 600, 800 and 1000 mg/dl are realized by the addition of fibrinogen to autologous plasma and albumin, respectively, in the final desired concentration. There are significant differences between the aggregation values ($P < 0.001$, n = number of donors).

the upper concentration range. The normal range of erythrocyte aggregation in stasis in our case at fibrinogen concentrations between 200 and 350 mg/dl is in good agreement with the reference values from Jung *et al.* (12). Table 1 compares the erythrocyte aggregation measured in autologous plasma with that in a 6% albumin solution including physiologic concentration of Ca^{2+}, Na^+, and K^+ ions when increasing fibrinogen concentration. Red cell aggregation increases when fibrinogen was increased in the albumin solution, but normal values as demonstrated in the plasma solution could not be achieved even at fibrinogen concentration up to 1000 mg/dl. There are significant differences between the normal fibrinogen concentrations from 200 to 350 mg/dl and higher fibrinogen concentrations ($P < 0.001$).

The addition of α_2-macroglobuline to the albumin solutions shows significant differences in aggregation between the value without α_2-macroglobulin content and increasing α_2-macroglobulin concentrations ($P < 0.01$, Table 2). Figure 2 demonstrates the Ca^{2+}-ion uptake in the erythrocyte as a function of increased ionophore A 23187 concentration. The values at ionophore concentration of 0 μmole/liter and 25 μmole/liter are the means ± SD of 20 different healthy donors (11). Ca^{2+}-ion values rise up to 0.6 μmole (normal value 0.20 μmole/liter ± 0.04) when the ionophore concentration is 50 μmole/liter. In our experiments an ionophore concentration of 25 μmole/liter was used and an Ca^{2+}-ion uptake of 0.40 ± 0.08 μmole/liter could be achieved.

Table 3 shows the results when the intracellular Ca^{2+}-ion concentration was increased. Erythrocyte aggregation in autologous plasma could be significantly

TABLE 2
Aggregation of the Erythrocytes as a Function of α_2-Macroglobulin Concentration (normal value 1.8 g/dl) in a 6% Albumin/1000 mg/dl Fibrinogen Solution[a]

α_2-Macroglobulin (g/liter)	0	0.9	1.8	2.7	3.6	4.5
MEA (mm^2)	3.7 ± 1.0	4.9 ± 1.5	5.4 ± 1.7	6.0 ± 1.7	6.4 ± 1.8	6.7 ± 1.7
Range	2.0–5.3	2.5–6.6	2.6–7.2	2.8–8.0	3.0–8.7	3.6–9.4
n	10	10	10	10	10	10
$P <$		0.01	0.01	0.01	0.01	0.01

[a] There are significant differences between the aggregation values ($P < 0.01$).

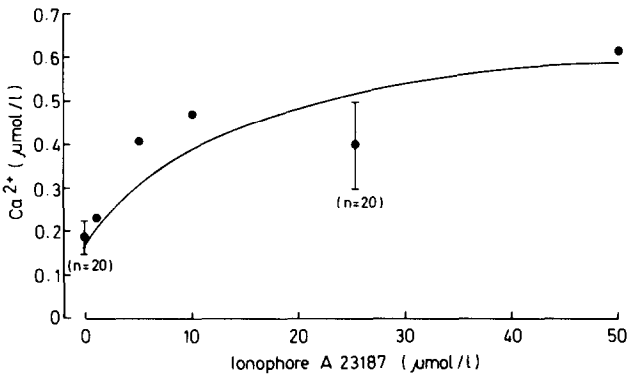

FIG. 2. Loading of the erythrocytes with Ca^{2+} ions by means of the ion carrier ionophore A23187. For our experiments the ionophore concentration was 25 µmole/liter which realizes a calcium uptake of 0.4 ± 0.08 µmole/liter (normal value 0.20 ± 0.04 µmole/liter). Each value is the result of two determinations with a standard deviation of SD = ±0.03 µmole/liter. The standard deviations given in the figure at ionophore concentrations of 0 and 25 µmole/liter are the result of the determination of 20 healthy donors.

TABLE 3
Erythrocyte Aggregation as a Function of Intraerythrocytic Ca^{2+}-Ion Concentration in Plasma (Fibrinogen 200–350 mg/dl)[a]

Ca^{2+} (µmole/liter)	0.2 ± 0.04	0.4 ± 0.08
MEA (mm^2)	7.2 ± 1.9	11.5 ± 3.7
Range	3.4–15.0	7.9–21.2
n	75	12
$P <$		0.001

[a] There is a significant difference between the two values ($P < 0.001$).

TABLE 4

Erythrocyte Aggregation as a Function of the Combination of Fibrinogen (400 mg/dl, A,B,C,D; and 1000 mg/dl, A*,B*,C*,D*), α_2-Macroglobulin, and Intraerythrocytic Ca^{2+}-Ion Concentration in a 6% Albumin Solution[a]

	A	B	C	D
MEA (mm^2)	0.7 ± 0.8	1.0 ± 0.6	2.5 ± 0.8	3.0 ± 0.9
Range	0–1.6	0–1.6	1.7–4.1	1.6–4.2
n	6	5	6	5
$P <$		n.s.	0.05	0.01
			n.s.	
	A*	B*	C*	D*
MEA (mm^2)	3.9 ± 1.2	5.8 ± 1.7	7.7 ± 2.1	9.3 ± 4.6
Range	2.4–6.7	2.6–8.8	4.9–11.9	5.2–19.7
n	39	21	23	8
$P <$		0.02	0.001	0.001
			n.s.	

[a] There is no significant difference between C,C* and D,D* while in the other cases differences are significant. A,A*, 400/1000 mg/dl fibrinogen/albumin solution; B,B*, + 1,8 mg/dl α_2-macroglobulin; C,C*, + 25 μmole/liter ionophore; D,D*, + 1,8 mg/dl α_2-macroglobulin + 25 μmole/liter ionophore.

enhanced ($P < 0.001$). Both in plasma and in the albumin solution, no increase in the aggregation tendency could be observed when the extracellular Ca^{2+}-ion concentration was increased (not shown).

Addition of α_2-macroglobulin to the albumin/400 mg/dl fibrinogen solution leads to a small but not significant increase in aggregation, while at 1000 mg/dl fibrinogen a significant difference could be demonstrated (Table 4). When adding ionophore, i.e., calcium, to the albumin/fibrinogen (400 mg/dl, A,B,C,D; 1000 mg/dl, A*,B*,C*,D*) solutions the aggregation further increases, while the addition of α_2-macroglobulin does not lead to a further significant increase in aggregation. The main aggregation effect is obtained by increasing the Ca^{2+}-ion concentration in the red blood cell.

DISCUSSION

It is well known that the erythrocyte aggregation in stasis is controlled by the increase of the "acute phase proteins" especially fibrinogen. The precise interacting mechanism between cell and protein and cell/cell interactions are still object of investigations (1,2).

We showed that the interaction between fibrinogen, α_2-macroglobulin, and ionized Ca^{2+} ion in the red blood cell leads to an increased aggregation in stasis. Increase of the fibrinogen concentration alone in a 6% albumin solution containing Na^+, K^+, and Ca^{2+} ions in physiological concentrations resulted only in a small increase of aggregation. Other substances in plasma and the cell seem to be necessary for determining the tendency of red blood cell aggregation together with fibrinogen.

The intracellular Ca^{2+}-ion content of human erythrocytes is normally about

0.20 ± 0.04 μmole/liter (11) and thus 5000–10000 times lower than the plasma Ca^{2+}-ion values. Despite the low intracellular Ca^{2+}-ion values they seem to control many cellular functions. Fluctuations of the Ca^{2+}-ion concentration can be important for the regulation of biological reactions such as muscle contractions and cell divisions (15). Changes of the cytoplasmatic Ca^{2+}-ion concentration are associated with deviations of the membrane permeability and flexibility. Abnormal cell function and even cell death can be induced if the intracellular Ca^{2+}-ion concentration surpasses a critical level (16). The influx of Ca^{2+} ions proceeds through the lipid bilayer of the plasmalemma and the cytoskeleton of the erythrocyte membrane (17). It is important to emphasize that the Ca^{2+} ion might activate some inner membrane contractile proteins of the red cell leading to an alteration of cell shape and deformability. Ca^{2+} ions seem to be responsible for the active and passive contractions in the cell membrane (18,19).

There are many diseases where erythrocyte aggregation is increased irrespective of normal fibrinogen concentration (diabetes mellitus, hereditary spherocytosis, sickle cell anemia, and other hemolytic anemias) (4). Our measurements allow us to speculate that when an increase of the cytoplasmatic Ca^{2+} ion changes the physical and/or biochemical conditions of the aggregation receptors on the membrane surface, cell–protein interactions are regulated by alteration of the intracellular Ca^{2+}-ion concentration. At least a part of the receptor molecule must be on the cytoplasmatic side of the membrane as alterations of the extracellular Ca^{2+}-ion concentration do not provoke an increased aggregation tendency. Ca^{2+} ions might induce a complex inside of the membrane which recognizes fibrinogen binding outside of the membrane as already could be shown in the case of platelets (20). Recently the erythrocyte-binding site in the fibrinogen molecule has been described (21).

Further investigations about the nature of the membrane-bound complex as well as the receptor are necessary to elucidate the mechanism of red cell aggregation. Probably Ca^{2+}-ion-binding inside the membrane leads to a conformational rearrangement of a part of the receptors outside the membrane, thus leading to an increased binding capacity.

SUMMARY

Ca^{2+} ions were transported into the cell by incubation of the erythrocyte suspension with ionophore A23187, a lipophil electric neutral ion complexing substance. Erythrocyte aggregation could be increased twice, when doubling the intracellular Ca^{2+}-ion concentration. Our measurements lead to the suggestion that an increase of the cytoplasmatic Ca^{2+} ion changes the physical and/or biochemical properties of the aggregation receptors on the membrane surface, i.e., cell–protein interactions are regulated by alteration of the intracellular Ca^{2+}-ion concentration.

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