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# $Ca^{2+}$ IONOPHORES AND THE ACTIVATION OF HUMAN BLOOD PLATELETS

## THE EFFECTS OF IONOMYCIN, BEAUVERICIN, LYSOCELLIN, VIRGINIAMYCIN S, LASALOCID-DERIVATIVES AND McN 4308

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## Summary

Platelet activation is linked to an increase in the cytoplasmic $Ca^{2+}$ concentration and consequently can also be induced by ionophores which mobilize $Ca^{2+}$ from intracellular storage sites or transport it through the plasma membrane. The ionophores mostly used in studies on platelet activation are A 23187 and lasalocid (X-537A). The effects of eight compounds with known $Ca^{2+}$-ionophoric activity in synthetic or natural membrane systems were studied in order to investigate the relationship between transport of $Ca^{2+}$ and activation of platelets.

Ionomycin acts as a true $Ca^{2+}$ ionophore: it elicits rapid shape change, aggregation, the release reaction (secretion) and clot retraction (contraction). Beauvericin activates platelets too, but probably not by increasing the cytoplasmic $Ca^{2+}$ concentration. Lysocellin does not activate platelets but induces a passive loss of serotonin. Virginiamycin S has no effect on platelets. Bromolasalocid and one epimer of dihydrolasalocid, like lasalocid, activate platelets by increasing the cytoplasmic $Ca^{2+}$ concentration, and also induce a passive loss of serotonin. McN 4308 does not activate platelets but induces a slow uptake of $^{45}Ca^{2+}$.

## Introduction

Many cells can be stimulated to display a variety of responses. Among these responses are secretion, contraction and activation of phospholipases, processes

Abbreviations: EGTA, ethyleneglycol bis(β-aminoethyl-ether)-*N*,*N*'-tetraacetic acid; Tes, *N*-tris[hydroxymethyl]methyl-2-aminoethanesulphonic acid.

which are generally believed to be mediated by an increase in the cytoplasmic $Ca^{2+}$ concentration. In the last few years evidence has accumulated that $Ca^{2+}$ plays a similar role in the activation of platelets. This evidence is based mainly on the finding that $Ca^{2+}$ ionophores elicit the whole range of activation responses of this cell, such as rapid shape change [1], aggregation [2,3], release reaction (secretion) [2,4] and clot retraction (contraction) [2], while at the same time inducing uptake of external $Ca^{2+}$.

These investigations were carried out mainly with two substances, viz., A 23187 (first described by Reed and Lardy [5]) and lasalocid (X-537 A, first described by Pressman [6]). In addition to these compounds, cardiolipin and phosphatidic acid have been shown to induce $Ca^{2+}$ uptake and at least partial activation of platelets [7].

Recently, many compounds have been described which have ionophoric activity towards divalent cations in artificial as well as in natural systems. We have investigated the action of some of these substances on human blood platelets, in order to increase knowledge of the properties of these substances, and moreover in order to test and expand the hypothesis of the role of $Ca^{2+}$ in the activation of platelets.

The following substances were investigated.

Ionomycin is an antibiotic which transports $Ca^{2+}$ through a layer of chloroform [8] and increases the $Ca^{2+}$-permeability of the membrane of rabbit sarcoplasmic reticulum vesicles and of liposomes [9].

Beauvericin is a cyclic hexadepsipeptide of the enniatin family capable of mediating the transport of $Ca^{2+}$ through a layer of chloroform: however, this process is slower than the transport of most alkali ions [10]. $Ca^{2+}$ transport in liposomes, chromatophores [11] and chloroplasts [12] has also been described.

Lysocellin is a poly(ether) antibiotic [13,14] which mediates the transport of alkali and alkaline earth ions and biological amines through a layer of chloroform [15]. It affects the distribution and transport of $Ca^{2+}$ and other ions in mitochondria [16]. In blood platelets it induces the efflux of serotonin and of $Ca^{2+}$; at higher concentrations platelet aggregation and influx of $Ca^{2+}$ occur [17].

Virginiamycin S is a streptogramin antibiotic which facilitates the transport of various cations, including $Ca^{2+}$, across artificial phospholipid membranes [18].

Bromolasalocid (Ro 20-0006), dihydrolasalocid (Ro 20-5651) and its epimer (Ro-7-9406) are derivatives of lasalocid synthesized by Westley et al. [19]. Their ionophoric activity in artificial systems has been described [20,21]. Bromolasalocid has been reported to mobilize intracellular $Ca^{2+}$ in the rat pituitary [22] and in human lymphoid cells [23].

McN 4308 is a synthetic diglycolamic acid which selectively facilitates transport of $Ca^{2+}$ through a chloroform layer [24].

As well as the ability of the ionophores to transport $Ca^{2+}$ through the platelet membrane we investigated whether platelets responded to the ionophores by the release reaction, aggregation, clot retraction or rapid shape change. The release reaction was estimated by measuring release of $^{14}C$ by platelets labelled with [$^{14}C$]serotonin and by measuring release of material absorbing at 258 nm which consists mainly of adenine nucleotides [25]. Specificity of release was

estimated by measuring the loss of $^3H$ by platelets labelled with [$^3H$]adenine. Adenine is taken up rapidly by platelets and converted in the cytoplasm to adenine nucleotides which form the metabolic pool [26,27]. This pool does not participate in the release reaction. An appreciable loss of $^3H$, therefore, indicates an increase in the permeability of the plasma membrane possibly due to nonspecific cell damage, or a disturbed metabolism.

## Materials and Methods

*Human blood platelets*

These were isolated within 20 h after collection from citrated blood collected for the Central Laboratory of the Blood Transfusion Service of the Swiss Red Cross in Bern [28]. The buffy coats were transferred into a buffered glucose solution; the resulting platelet concentrate contained approx. 40 mM glucose, 12 mM Tes buffer, pH 7.4, and $3 \cdot 10^9$ platelets per ml.

The platelets were washed by repeated centrifugation (9 min, $1500 \times g$, room temperature, pH 6.8) and resuspension. The first two washing solutions contained 120 mM NaCl, 13 mM trisodium citrate and 30 mM glucose, the third was a balanced, buffered salt solution (see below) from which the divalent cations were omitted. The platelets were finally suspended in the complete buffered salt solution, adjusted to the desired platelet concentration by nephelometry and supplemented with 5 mg/ml bovine serum albumin. Before use the suspension was shaken gently at 20°C and its pH kept at 6.8.

*Ionophores*

Ionomycin (Ro 22-1703, sodium salt), $C_{41}H_{71}O_9Na$, $M_r = 731$, was a gift from F. Hoffmann-La Roche and Co., AG, Basle, Switzerland. Beauvericin, $C_{45}H_{57}N_3O_9$, $M_r = 783$, was obtained from Bachem, Liestal, Switzerland. Lysocellin (Ro 21-7270), sodium salt, $C_{34}H_{59}O_{10}Na \cdot 1/2\ CH_3OH$, $M_r = 667$, was a gift from Hoffmann-La Roche. Virginiamycin S, $M_r = 824$, was a gift from Dr. E. Grell, Max Planck Institute, Göttingen (F.R.G.). Lasalocid (Ro 2-2985, X-537A), $C_{34}H_{54}O_8$, $M_r = 591$, dihydrolasalocid (Ro 20-5651) and its epimer (Ro 7-9406), $C_{34}H_{56}O_8$, $M_r = 593$, and bromolasalocid (Ro 20-0006), $C_{34}H_{53}BrO_8$, $M_r = 670$, were gifts from Hoffmann-La Roche. McN 4308, $C_{22}H_{25}NO_4$, $M_r = 367$, was a gift from McNeil Laboratories, Fort Washington, PA. A 23187 was a gift from Eli Lilly, Indianapolis, IN.

*Labelled compounds*

$^{45}Ca^{2+}$ was diluted to contain 10 $\mu$Ci/ml and 0.1 mM $CaCl_2$ in saline. [$^3H$]-Inulin: 0.1 mCi/ml, 0.6 mg/ml in saline. [$^3H$]Adenine (hydrochloride salt): 0.5 mCi/ml, 0.5 mM in 0.5 mM HCl. [$^{14}C$]Serotonin: 8 $\mu$Ci/ml, 0.14 mM in 70% ethanol.

*Other substances*

Thrombin (bovine): Hoffmann-La Roche, Basle, Switzerland; 50 NIH units per mg, dissolved in saline. Reptilase reagent: Pentapharm Ltd., Basle, Switzerland. Human fibrinogen: see method of Massini et al. [29].

Buffered salt solution contained 130 mM NaCl, 3 mM KCl, 0.06 mM $CaCl_2$,

0.02 mM $MgCl_2$, 26 mM Tes-NaOH buffer, pH 6.8, and 25 mM glucose.

Scintillator: 2 vols. of xylene plus 1 vol. of Triton X-100 plus 3 mg/ml Permablend III (Packard).

*Measurement of the release reaction and of uptake of $^{45}Ca^{2+}$*

For labelling, 10 ml of a suspension of washed platelets ($4.5 \cdot 10^9$ platelets per ml) or of platelet concentrate were incubated with [$^{14}$C]serotonin (final concentration 0.7 $\mu$M) and [$^3$H]adenine (0.5 $\mu$M) at 20°C and pH 6.8 for 1 h with gentle shaking. On average, 92% of the serotonin and 81% of the adenine were taken up.

For the release experiments, 0.2 ml of this suspension was added to 0.7 ml of prewarmed buffer (37°C) in 1-ml poly(propylene) centrifuge tubes; after 1 min the ionophore (dissolved in dimethylsulphoxide) was added. After 1 min of incubation without stirring the sample was centrifuged in a microcentrifuge (15 s, 11 000 × *g*); with beauvericin, the samples were stirred for 4.5 min at 37°C and 2 $\mu$M imipramin was added to the incubation mixture to prevent re-uptake of released serotonin. Of the supernatant, 0.2 ml was diluted with 0.3 ml of water and mixed with 5 ml of scintillator. The sample was counted in a dual-channel liquid scintillation spectrometer. The release was expressed as percentage of the radioactivity taken up by the platelets:

$$\% \text{ release} = [(A - B)/(C - B)] \times 100$$

where A = radioactivity of the supernatant of the sample, B = radioactivity of the supernatant of a control sample and C = radioactivity of the corresponding aliquot of the platelet suspension. $^{14}$C released represents the release of serotonin and $^3$H released the loss of cytoplasmic nucleotides (metabolic pool) by nonspecific cell damage [26,27,30].

For $Ca^{2+}$ uptake, a non-labelled suspension of washed platelets was used. 0.2 ml was added to 0.7 ml of prewarmed buffer containing [$^3$H]inulin (1.6 $\mu$Ci/ml, 0.1 mg/ml) and $^{45}Ca^{2+}$ (0.08 $\mu$Ci/ml, 0.3 mM). Ionophore was added and the sample incubated and centrifuged as for release. In an aliquot of the supernatant, $^3$H and $^{45}Ca^{2+}$ were measured. The rest of the supernatant was used for assay of release of nucleotides. It was deproteinized in the cold with 0.5 M $HClO_4$ and neutralized with KOH. The absorption spectrum of the extract was recorded. A straight line was drawn through the minima at 300 and 225 nm and the peak at 258 nm above this base-line measured. Release of nucleotides was calculated as indicated above.

The sediment of each sample was solubilized with 0.5 ml of 0.5% Triton X-100, transferred to a counting vial, mixed with 5 ml of scintillator and counted in the $^3$H/$^{14}$C channels of the spectrometer. The amount of $^{45}Ca^{2+}$ contained in the sediment and corrected for the enclosed medium was calculated in the following way:

$$\text{Uptake of } ^{45}Ca^{2+} = [Ca^{2+}] \left( \frac{\text{cpm } ^{45}Ca^{2+} \text{ in sediment}}{\text{cpm } ^{45}Ca^{2+} \text{ per } \mu\text{l supernatant}} - \frac{\text{cpm } ^3H \text{ in sediment}}{\text{cpm } ^3H \text{ per } \mu\text{l supernatant}} \right)$$

and is given in μmol $^{45}Ca^{2+}$ per $10^{11}$ platelets [2]. The solvent of the ionophores (dimethylsulphoxide, up to 1/600th of sample volume) has no measurable effect on platelets.

*Aggregation*

Aggregation was measured turbidimetrically using a Bryston aggregometer. It was performed in poly(propylene) centrifuge tubes so that release could be assayed after aggregation, using labelled platelets and measuring the radioactivity of the supernatants.

*Clot retraction*

Clot retraction was allowed to proceed at 37°C in 10 × 100 mm test-tubes coated inside with 12% gelatine (to facilitate detachment of the clot). To 0.5 ml of platelet concentrate, diluted with buffered saline to 0.9 ml, was added the ionophore. The mixture was clotted with reptilase at room temperature, then incubated at 37°C. The clot length was measured during up to 2 h. Thrombin control: clotted with 10 units/ml thrombin instead of reptilase. Final concentrations: $1.5 \cdot 10^9$ platelets per ml, 50 vol% platelet concentrate; 34 mM Tris-HCl, pH 7.4, at 37°C; 10 vol% reconstituted reptilase; NaCl to isotonicity.

*Shape change*

Platelet concentrate, buffered to pH 7.4, was incubated at 37°C for 2 h to allow the platelets to regain the discoid form. It was then treated with ionophore for 30 s, fixed with 4 vols. of 0.1% glutardialdehyde at 37°C for at least 10 min, applied onto poly(lysine)-coated slides and viewed and photographed with an oil-immersion, phase-contrast microscope at approx. 1200 × magnification.

*Uptake of $^{45}Ca^{2+}$ by resting platelets*

When a substance did not activate platelets its activity to induce $Ca^{2+}$ uptake in resting platelets was investigated. A suspension of washed platelets in buffered salt solution containing 5 mg/ml bovine serum albumin was covered and shaken at 37°C and pH 6.8. Ionophore or its solvent and 0.3 mM $^{45}CaCl_2$ were added and incubation was continued for 9 h. Every hour, a sample of 0.6 ml was mixed with 0.02 ml [$^3$H]inulin and centrifuged in the microcentrifuge through a layer of 0.2 ml of separating oil (10 vols. of dinonylphthalate plus 25 vols. of dibutylphthalate). Aliquots of the supernatant were counted: the sediments were solubilized in 0.5% Triton X-100 and counted too. The $^{45}Ca^{2+}$ taken up, corrected for $^{45}Ca^{2+}$ entrapped, was calculated as described above. The results were transformed by linear regression analysis and expressed as μmol/h $Ca^{2+}$ taken up by $10^{11}$ platelets.

## Results and Discussion

Ionomycin induces the release of serotonin and of adenine nucleotides (Table I); the greater part of cytoplasmic nucleotides (i.e., the metabolic pool which is labelled in these experiments with $^3$H) is retained. The ratio of released nucleotides to released serotonin is approx. 3 : 4. This ratio is near to the value

TABLE I

UPTAKE OF $^{45}Ca^{2+}$ AND RELEASE REACTION INDUCED BY IONOMYCIN

0.9 ml samples of platelet suspension were incubated at 37°C for 1 min with ionomycin, then centrifuged. Labelling and assays: see Methods. Final concentrations: $10^9$ platelets per ml; 34 mM Tris-HCl, pH 7.4, at 37°C; 0.3 mM $CaCl_2$; 1 mg/ml bovine serum albumin; 22 vol% buffered salt solution; NaCl to isotonicity. For $Ca^{2+}$ uptake: in addition 0.08 μCi/ml $^{45}Ca^{2+}$; 0.8 μCi/ml, 0.1 mg/ml [$^3$H]inulin; platelets not labelled. The ionophore was added as 3 mM solution in dimethylsulphoxide.

| | Control | Ionomycin (μM) | | | |
|---|---|---|---|---|---|
| | | 1 | 3.3 | 10 | 20 |
| Release of $^{14}C$ (serotonin) (%) | 0 | 12 | 46 | 75 | 84 |
| Release of nucleotides (%) | 0 | 13 | 36 | 55 | 66 |
| Loss of $^3H$ (cytoplasmic nucleotides) (%) | 1 | 1 | 3 | 9 | 16 |
| Uptake of $^{45}Ca^{2+}$ (μmol per $10^{11}$ platelets) | 0.22 | 0.41 | 0.61 | 0.92 | 1.63 |

of 2 : 3 which would be expected for a true secretion since almost all the serotonin, but only about two-thirds of nucleotides are contained in the secretory granules [27].

Table II shows that metabolic energy is required for release to occur: release is prevented when both oxidative phosphorylation and glycolysis are blocked by preincubation of the platelets with antimycin A and 2-deoxyglucose in the absence of glucose, respectively.

Replacement of $Ca^{2+}$ by the $Ca^{2+}$-chelator, EGTA, inhibits release of both [$^{14}$C]serotonin and nucleotides moderately, showing that external $Ca^{2+}$ is not absolutely required for induction of the release reaction (Table III).

This ionophore also induces a quick uptake of external $Ca^{2+}$ at concentrations at which release is induced (Table I). Uptake of $Ca^{2+}$ does not require metabolic energy (Table II). These findings suggest that ionomycin induces the secretion of the compounds stored in the dense bodies [30] by rendering the membranes permeable to $Ca^{2+}$ and thereby abolishing the $Ca^{2+}$ gradients between external medium, and the cytoplasmic and granular compartments.

TABLE II

EFFECT OF STARVATION ON RELEASE REACTION AND $Ca^{2+}$ UPTAKE INDUCED BY IONOMYCIN AND THROMBIN

G, preincubated for 1 h at 20°C with 25 mM glucose; A + D, preincubated without glucose but with 1 μM antimycin A and 50 mM 2-deoxyglucose. Sample treatment and concentrations: see Table I and Methods.

| | | Control | Ionomycin (3.3 μM) | Trombin (1 unit/ml) |
|---|---|---|---|---|
| Release of $^{14}C$ (serotonin) (%) | G | 0 | 54 | 80 |
| | A + D | 3 | 8 | 5 |
| Release of nucleotides (%) | G | 0 | 43 | 54 |
| | A + D | 6 | 4 | 7 |
| Uptake of $^{45}Ca^{2+}$ (μmol per $10^{11}$ platelets) | G | 0.11 | 0.42 | 0.45 |
| | A + D | 0.14 | 0.49 | 0.16 |

TABLE III

DEPENDENCE ON $Ca^{2+}$ OF THE RELEASE REACTION INDUCED BY IONOMYCIN

As in Table I, except that the incubation mixture contained either 0.3 mM $Ca^{2+}$ or 2 mM EGTA. For assay of nucleotides, the sediments were homogenized and deproteinized and the absorption spectra compared to the spectra of control sediments as described in Methods.

| | Release (%) | | | | | |
|---|---|---|---|---|---|---|
| | [$^{14}$C]serotonin | | | Nucleotides | | |
| Ionomycin ($\mu$M): | 3 | 10 | 20 | 3 | 10 | 20 |
| $Ca^{2+}$ | 30 | 60 | 71 | 43 | 59 | 69 |
| EGTA | 34 | 42 | 49 | 40 | 38 | 39 |

Ionomycin also induces aggregation of platelets, as shown in Fig. 1, and clot retraction: 9 $\mu$M ionomycin induces clot retraction at a rate similar to 10 units/ml thrombin or 3.6 $\mu$M A23187. EDTA (5 mM) inhibits clot retraction with both ionophores.

A conspicuous early manifestation of the activation of platelets is the so-called rapid shape change [31] which is characterized by transformation from discs to spheres bearing spikes and pseudopods. This process has also been ascribed to an increase in the intracellular $Ca^{2+}$ concentration [1,32]. Indeed, Le Breton and Feinberg [33] have reported direct evidence for such an

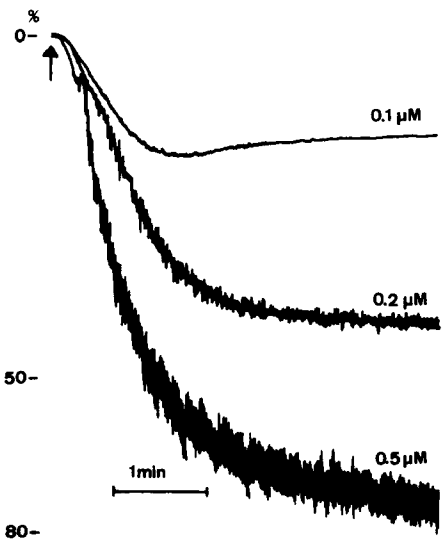


Fig. 1. Aggregation induced by ionomycin. Washed platelets were incubated in 0.3 ml containing (final concentrations): $10^9$ platelets per ml; 34 mM Tris-HCl, pH 7.4, at 37°C; 4 mM KCl; 1 mM $MgCl_2$; 2 mM $CaCl_2$; 8 mM glucose; 1.7 mg/ml bovine serum albumin; 1.3 mg/ml human fibrinogen; NaCl to isotonicity. After 1 min stirring in the aggregometer, 0.1—0.5 $\mu$l ionomycin (concentration as indicated) was added (arrow). Transmission was recorded for 4 min. Transmission settings were 0% for platelet suspension and 100% for incubation medium.

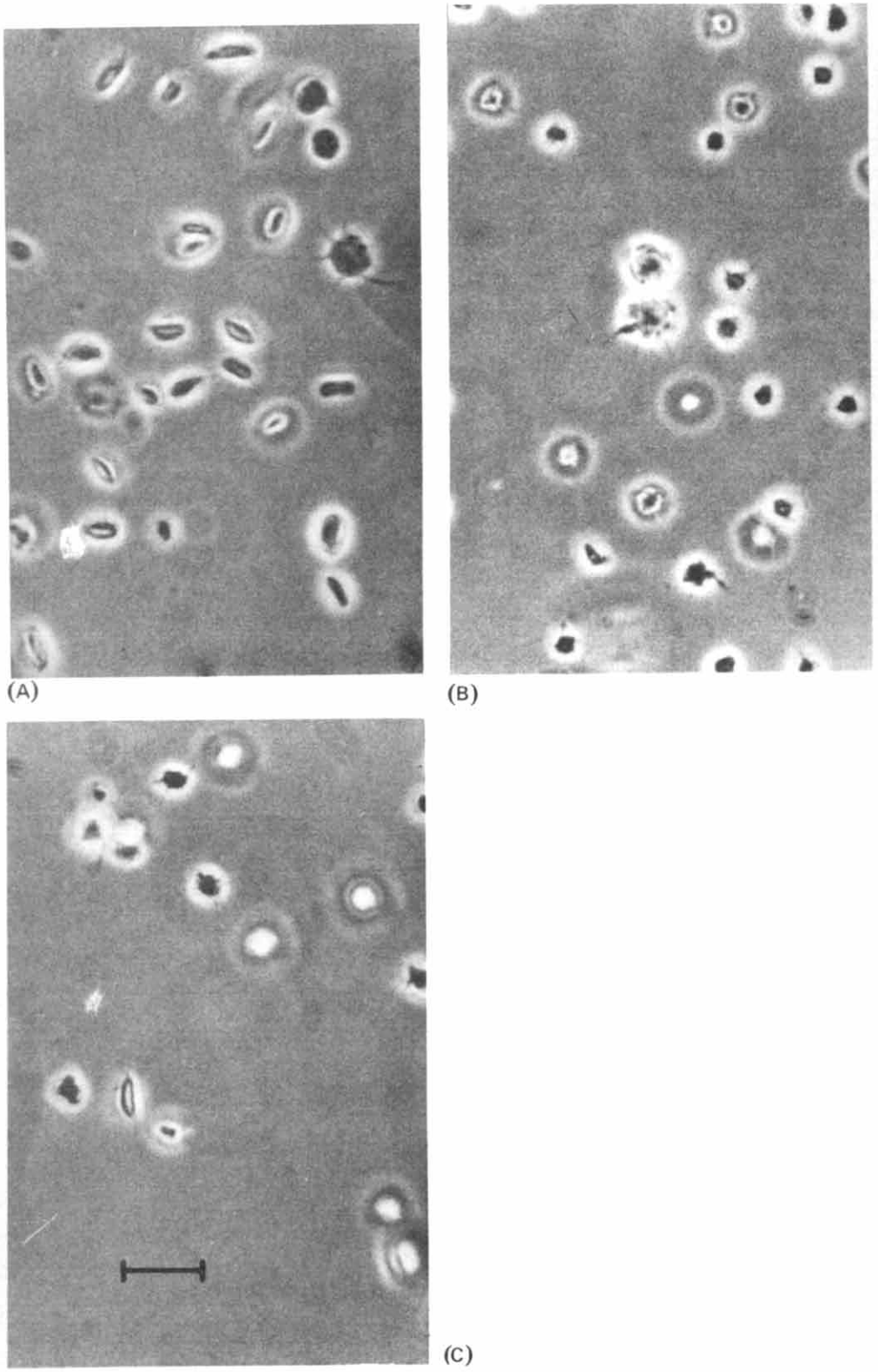


Fig. 2. Induction of shape change by ionomycin and ADP. (A) Platelet concentrate incubated for 2 h at 37° C and pH 7.4. (B) Treated for 30 s with 6 μM ionomycin and 4 mM EDTA. (C) Treated for 30 s with 2 μM ADP and 4 mM EDTA. Fixation and microphotography, see Methods. Bar in C, 10 μm.

increase when shape change is induced by ADP. Fig. 2 shows that platelet shape is changed by ionomycin in a manner similar to ADP.

Taken together, these findings suggest strongly that ionomycin acts on platelets as a true $Ca^{2+}$ ionophore, i.e., by facilitating the passage of $Ca^{2+}$ through the membranes from regions of high to regions of low concentration, and thereby increasing the cytoplasmic $Ca^{2+}$ concentration which is very low in resting platelets [34]. All the processes which occur in activated platelets are elicited by ionomycin. Moreover, the way the release reaction is influenced by various inhibitors is characteristic for true ionophores: metabolic energy is required for release but not for uptake of $Ca^{2+}$ since ionophores abolish ion gradients across membranes passively; the $Ca^{2+}$-chelator, EGTA, inhibits release only slightly because the ionophore can penetrate internal membranes and thereby mobilize $Ca^{2+}$ stored in organelles.

In contrast, clot retraction is inhibited completely by chelating $Ca^{2+}$, presumably because of the long time required for this process during which $Ca^{2+}$ mobilized from organelles by the ionophore diffuses through the plasma membrane and is trapped by the chelator, so that the final cytoplasmic $Ca^{2+}$ concentration is too low to support clot retraction [2].

The conclusion that ionomycin is a true $Ca^{2+}$ ionophore is in agreement with the results of Martonosi and collaborators [9] who reported that it increases the $Ca^{2+}$-permeability of the membranes of rabbit sarcoplasmic reticulum vesicles.

Beauvericin induces strong aggregation at 20 $\mu$M (Fig. 3). At higher concentrations, transmission decreases after a quick rise: this phenomenon has not been explored further.

At 100 $\mu$M, beauvericin induces release of serotonin (77%) and of nucleo-

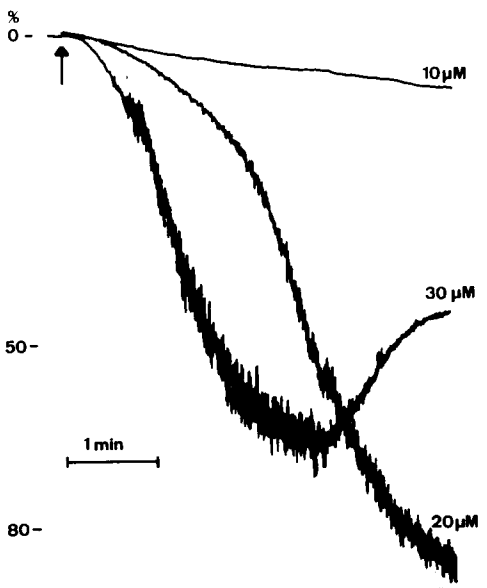


Fig. 3. Aggregation induced by beauvericin. Method and concentrations as in Fig. 1. Beauvericin was added as 30 mM solution in dimethylsulphoxide.

tides (51%) and also a considerable loss of cytoplasmic nycleotides (33%). Uptake of $^{45}Ca^{2+}$ is stimulated too. At 50 $\mu$M, beauvericin has less effect, and at 20 $\mu$M is ineffective. Stirring is required for these processes. Starvation (i.e., preincubation of the platelets with antimycin and deoxyglucose) inhibits release. Unexpectedly, the uptake of $^{45}Ca^{2+}$ is also inhibited by starvation. This effect is not typical nor to be expected for $Ca^{2+}$ ionophores (see Tables II and IV and discussion of ionomycin effects). In contrast to ionophores, thrombin and other non-ionophoric stimulants do not stimulate $Ca^{2+}$ uptake if the release reaction is inhibited [35]. It is possible that beauvericin, in spite of its ionophoric activity in other systems (see Introduction), does not act as a $Ca^{2+}$ ionophore on platelets but primarily rather induces aggregation, upon which 'aggregation-induced release' follows [36—38]. Release is accompanied by a secondary stimulation of $Ca^{2+}$ uptake [35]. The requirement for stirring substantiates this hypothesis since release cannot occur in these instances when aggregation is inhibited by omission of stirring [36,39].

Lysocellin (100 $\mu$M) induces 92% release of serotonin but no significant release of stored nucleotides (8%) nor uptake of $^{45}Ca^{2+}$. 33 $\mu$M lysocellin induces 32% release of serotonin; lower concentrations are ineffective. Serotonin release is not impaired in starved platelets. When platelets are stirred in the aggregometer in the presence of 50—500 $\mu$M lysocellin, transmission increases to some extent but microscopic inspection shows no aggregates but loose platelets, some of which are swollen. Also, platelets labelled with [$^3$H]-adenine lose most of the $^3$H during incubation with lysocellin under these conditions.

These findings do not support the hypothesis [17] that lysocellin induces the release reaction by increasing the intracellular $Ca^{2+}$ concentration. It seems to induce the loss of stored serotonin in a passive way, possibly by rendering the membranes permeable for serotonin. The changes seen in the aggregometer indicate that it also disturbs the osmotic balance of platelets. This damage might prevent induction of aggregation by the serotonin released.

Virginiamycin S does not induce release of serotonin nor uptake of $^{45}Ca^{2+}$ at concentrations up to 100 $\mu$M. Also, resting platelets incubated for 6 h with 100 $\mu$M virginiamycin S have not taken up more $^{45}Ca^{2+}$ than control platelets.

Bromolasalocid (Ro 20-0006) induces the release of serotonin at 20 $\mu$M; at 50 $\mu$M it induces the release of nucleotides and the uptake of $^{45}Ca^{2+}$. One of the two epimers of dihydrolasalocid, Ro 20-5651, has similar effects at 50 $\mu$M whereas the other epimer, Ro 7-9406, has no effect on platelets at this concentration (the epimers differ in the conformation of the hydroxyl group which results from the reduction of the keto group of lasalocid). The release of nucleotides is inhibited if the platelets are starved whereas release of serotonin is not inhibited. These effects are similar to lasalocid itself. The effects of lasalocid and its derivatives, of A 23187 and thrombin are summarized in Table IV.

Evidently, lasalocid and its two active derivatives induce the passive transport of serotonin along its concentration gradient at low concentrations and, at higher concentrations, induce transport of $Ca^{2+}$ into the cytoplasm which results in the activation of platelets. The 'aminophoric' activity of lasalocid has already been described by Pressman [6] and has been suggested as operating in platelets in our earlier paper [2].

TABLE IV

EFFECTS OF A 23178, LASALOCIDES AND THROMBIN ON PLATELETS: INFLUENCE OF STARVATION

Two aliquots of platelet suspension were incubated with either 25 mM glucose (G) or 1 μM antimycin A and 50 mM 2-deoxyglucose (A + D) for 1 h at 20°C. 0.9 ml samples were incubated with thrombin or ionophore for 1 min at 37°C. Labelling and assays: see Methods. Final concentrations: see Table I. The ionophores were added in ethanol or dimethylsulphoxide; the solvents (1/1000th vol.) had no effect.

| | | Control | A 23187 (4 μM) | Lasalocid (50 μM) | Ro 20-0006 (50 μM) | Ro 20-5651 (50 μM) | Thrombin (1 unit/ml) |
|---|---|---|---|---|---|---|---|
| Release of [$^{14}$C]serotonin (%) | G | 4 | 71 | 95 | 98 | 82 | 68 |
| | A + D | 4 | 14 | 91 | 93 | 65 | 5 |
| Release of nucleotides (%) | G | 0 | 63 | 54 | 62 | 54 | 56 |
| | A + D | 2 | 14 | 10 | 10 | 12 | 12 |
| Uptake of $^{45}Ca^{2+}$ (μmol per $10^{11}$ platelets) | G | 0.16 | 1.09 | 1.57 | 1.13 | 1.64 | 0.32 |
| | A + D | 0.21 | 1.47 | 1.48 | 0.47 | 2.00 | 0.20 |

McN 4308 does not induce platelet aggregation nor the release reaction at concentrations of up to 330 $\mu$M. Neither does it induce retraction of clots formed by reptilase. However, at 200 $\mu$M it increases the uptake of $^{45}Ca^{2+}$ by resting platelets approx. 2-fold. This uptake, which is very slow compared to the uptake rate induced by activating $Ca^{2+}$ ionophores [2], presumably does not allow the intracellular $Ca^{2+}$ concentration to reach the threshold needed for activation of the platelets, since platelets dispose of a system which can remove $Ca^{2+}$ from the cytoplasm [40].

## Conclusions

In the last few years many compounds have been shown to exert $Ca^{2+}$-ionophoric activity. Of the eight substances which were included in the present study only one, ionomycin, seems to act on platelets exclusively as a $Ca^{2+}$ ionophore in the sense that all the observed effects can be explained by its ability to transport $Ca^{2+}$ through membranes.

Lasalocid and two of its derivatives act like $Ca^{2+}$ ionophores but, in addition, deplete the storage organelles of serotonin. Beauvericin seems to activate platelets not by transporting $Ca^{2+}$ into platelets but primarily by inducing aggregation. Lysocellin is so toxic that activation is precluded. McN 4308 transports $^{45}Ca^{2+}$ into platelets but this process seems to be too slow for activation. Finally, virginiamycin S does not transport $^{45}Ca^{2+}$ into platelets at all. Evidently these two compounds act differently on the platelet membrane than on the artificial membranes through which they transport $Ca^{2+}$ [24,18].

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