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Protection of cells against membrane damage by haemolytic agents: divalent cations and protons act at the extracellular side of the plasma membrane

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The protective effect of Ca^{2+}, Zn^{2+} and H^+ against membrane damage induced by different haemolytic agents has been studied by measuring monovalent cation leakage and haemolysis of erythrocytes, and phosphoryl[^{3}H]choline and adenine nucleotide leakage from Lettre cells prelabelled with [^{3}H]choline. The protective effect of Ca^{2+} and Zn^{2+} on erythrocytes damaged by *Staphylococcus aureus* α-toxin, Sendai virus or melittin is unaffected by the addition of A23187, even though this ionophore greatly increases the uptake of $^{45}Ca^{2+}$ or $^{65}Zn^{2+}$. The same result has been found for the protective effect of Zn^{2+} on Lettre cells damaged by *S. aureus* α-toxin, Sendai virus, melittin or Triton X-100. Leakage of phosphoryl[^{3}H]choline from prelabelled Lettre cells is inhibited if extracellular pH is lowered; lowering the intracellular pH without affecting the extracellular pH, affords little protection. It is concluded that Ca^{2+}, Zn^{2+} and H^+ protect cells against membrane damage induced by haemolytic agents by an action at the extracellular side of the plasma membrane.

Introduction

Haemolytic agents such as certain paramyxoviruses [1,2], bacterial [3] and animal [4] toxins or the membrane attack complex of complement [5] damage cells by the creation of pores or channels, through which ions [1] and metabolites [2,6,7] leak. Such membrane damage does not necessarily result in extensive protein leakage or lysis of non-erythroid cells because these possess mechanisms for recovery [8] and repair [9,10] absent in erythrocytes. The damaging effect of haemolytic agents can be prevented by divalent cations such as Ca^{2+} [11,12] or Zn^{2+} [13,14], as well as by protons [15].

The mechanism of inhibition of leakage by divalent cations and protons is of interest not merely for an understanding of the way in which such ions close trans-membrane channels in general [16,17], but because of the potential therapeutic use of divalent cations like Zn^{2+} against microbial infections [18,19] and other diseases [20,21]. Circumstantial evidence has been presented to suggest that divalent cations and protons protect by binding to the outside of cells [22]. The action of Ca^{2+} in promoting *repair* of complement-induced lesions on the other hand, is from within cells [23]. It is therefore important to establish unequivocally the site at which the *protective* role of divalent cations and protons is exerted. Our strategy has been (a) to assess the intracellular concentration of Ca^{2+}, Zn^{2+} and H^+ in cells protected by these cations against membrane damage induced by various haemolytic agents and (b) to test whether raising intracellular Ca^{2+} or Zn^{2+} by the ionophore A23187 in the presence of haemolytic agent has a protective effect. Because the cells under study (erythrocytes and Lettre cells) do not lend themselves to the use of indicators such as quin2 (Bashford, C.L., unpublished observations) for determining intracellular cations, we have used radioisotopic labelling instead. The evidence to be presented shows that Ca^{2+}, Zn^{2+} and H^+ protect erythrocytes and Lettre cells against membrane attack from the extracellular side of the plasma membrane. Some of these results have been briefly presented at meetings [24–26].

Abbreviations: Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid: DMO, 5,5-dimethyl-2,4-oxazolidinedione; HBS, Hepes-buffered saline.

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Materials and Methods

Measurement of permeability changes. Lettre cells, grown intraperitoneally as an ascitic suspension in Swiss white mice, were removed from the animals, washed in 150 mM NaCl, 5 mM KCl, 5 mM Hepes, 1 mM $MgSO_4$, pH adjusted to 7.4 at 22°C with NaOH (Hepes-buffered saline, HBS) and incubated with [^{3}H]choline for 30–45 min at 37°C in order to label the intracellular phosphorylcholine pool [7]. Erythrocytes were isolated from blood collected from volunteers by venipuncture and stored in heparinised tubes; they were washed three times with Hepes-buffered saline. Washed cells (erythrocytes and Lettre cells) were incubated ($2 \cdot 10^6$–$5 \cdot 10^7$ cells/ml) in Hepes-buffered saline at 37°C with pore-forming agents in the absence or presence of $CaCl_2$ or $ZnSO_4$ with or without the ionophore A23187 [27] and at the pH values specified. Leakage of monovalent cations [28] or phosphoryl[^{3}H]choline [27] and haemolysis, indicated by the haemoglobin content of the supernatant, were assessed after pelleting cells through oil. Uptake of ^{65}Zn or ^{45}Ca was measured in parallel incubations with cells not labelled with [^{3}H]choline.

Adenine nucleotides in cells were measured as follows. Cell pellets were extracted in ice-cold perchloric acid, spun and the supernatants neutralized with $KHCO_3$. After removing potassium perchlorate by spinning, the supernatants were analysed by HPLC, using a 100 × 5 mm Hypersil-APS (5 μm) weak anion exchange column and a Waters Instrument. A linear phosphate gradient (22 mM to 0.7 M) prepared from Aristar potassium phosphate was used. The area under each respective adenine nucleotide peak was integrated using the Jones JCL 6000 chromatography Data System, and quantitated using an external standard of the relevant nucleotide.

Other measurements. The water content of cells pelleted through oil was determined (a) directly as the difference between the wet and dry weight of the pellet [28] or (b) indirectly from the Na^+ and K^+ content of the cell pellet [29]. In these experiments the total Na^+ plus K^+ content of the cell pellet was 160 mM. Intracellular pH of Lettre cells was assessed by the distribution of [^{14}C]DMO [30] and verified both by determining the chemical shift of intracellular inorganic phosphate by ^{31}P nuclear magnetic resonance [29] and by using intracellular Neutral red [31] as a pH indicator.

Pore-forming agents used. Sendai virus was grown in embryonated eggs for three days [7]. *Staphylococcus aureus* α-toxin was isolated [32] from strain Wood 46 (NCTC 7121) and kindly donated by Dr. Joyce de Azavedo, Moyne Institute, Trinity College, Dublin. Melittin was purified [33] and kindly donated by Dr. R.C. Hider, King's College, London. Triton X-100 was a commercial sample (BDH).

Other reagents. [^{3}H]Choline, ^{65}Zn and ^{45}Ca were from Amersham International. A23187 was from Sigma.

Results

Erythrocytes: Ca^{2+} and Zn^{2+}

Haemolysis of erythrocytes is induced by *S. aureus* α-toxin, Sendai virus or melittin in the presence of 10^{-4} M Ca^{2+}; $3 \cdot 10^{-2}$ M Ca^{2+} strongly inhibits haemolysis (Fig. 1). The presence of A23187 does not increase the protective effect of Ca^{2+}: if anything, haemolysis is somewhat enhanced (Fig. 1). Simultaneous measurement of $^{45}Ca^{2+}$ in those cells that had not haemolysed (i.e., that had sedimented through oil) showed little effect of haemolytic agent, but a 50–100-fold increase by A23187 (Fig. 2). Note that A23187 does not itself cause haemolysis (except in the case of rabbit erythrocytes; upper panel of Fig. 1). What these results therefore show is that a very large increase in intracellular Ca^{2+} causes little change on the protective effect of extracellular Ca^{2+} against haemolysis by α-toxin, Sendai virus or melittin.

Confirmation that even at high extracellular Ca^{2+} little Ca^{2+} enters erythrocytes can be obtained by measuring K^+ efflux. Fig. 3 (upper panel) shows that 10^{-2} M Ca^{2+} causes no K^+ efflux, whereas in the presence of

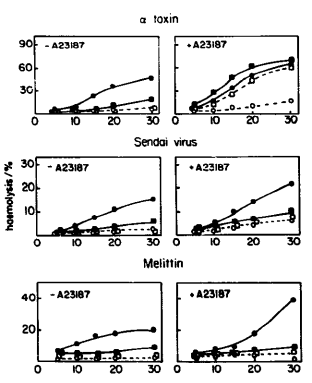

Fig. 1. Protection of erythrocytes by Ca^{2+}. $2 \cdot 10^8$ cells/ml in Hepes-buffered saline at 37°C without (○, □) or with (●, ■) α-toxin (1.1 μg/ml) or Sendai virus (80 HAU/ml) or melittin ($3 \cdot 10^7$ M) and 10^{-4} M (○, ●) or $3 \cdot 10^{-2}$ M (□, ■) $CaCl_2$ in the absence or presence of 10^{-6} M A23187 as indicated. Rabbit erythrocytes were used with α-toxin and human erythrocytes with Sendai virus and melittin.

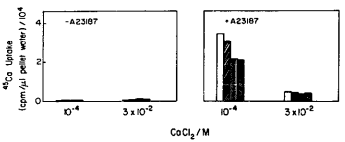

Fig. 2. ^{45}Ca uptake by erythrocytes. $2 \cdot 10^8$ cells/ml were incubated at 37 °C for 20 min in Hepes-buffered saline without (□) or with α-toxin (▨, 1.1 µg/ml) or Sendai virus (▩, 80 HAU/ml) or melittin (▧, $3 \cdot 10^{-7}$ M) and 10^{-4} M or $3 \cdot 10^{-2}$ M $CaCl_2$ containing $^{45}Ca^{2+}$ (900 cpm/µl) in the absence or presence of 10^{-6} M A23187 as indicated. Rabbit erythrocytes were used with α-toxin and human erythrocytes with Sendai virus and melittin.

A23187, 10^{-5} M causes 50% K^+ loss (though without haemolysis); this is a manifestation of the well-known 'Gardos' effect (reviewed in Ref. 34). Calculation of intracellular Ca^{2+} (lower panel of Fig. 3) on the basis of $^{45}Ca^{2+}$ content (see Fig. 2) shows that between 10^{-6} and 10^{-3} M intracellular Ca^{2+} *induces* K^+ leakage, and cannot therefore simultaneously *protect* against K^+ leakage (e.g., Table IV of Ref. 22) or haemolysis (Fig. 1). This is shown in a direct way for α-toxin by the plots of Fig. 4, in which intracellular Ca^{2+} was again calculated from $^{45}Ca^{2+}$ uptake data. Although such calculations require several assumptions in regard to the specific activity of free intracellular Ca^{2+}, it is clear that there is no correlation between calculated intracellular Ca^{2+} and α-toxin-induced haemolysis, since the values lie on two well-separated curves depending on the presence or

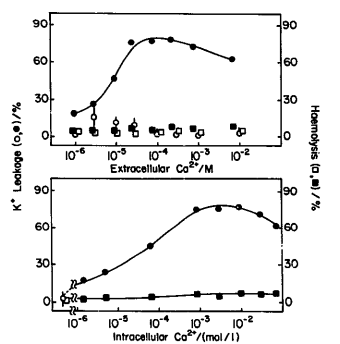

Fig. 3. Leakage of erythrocytes induced by Ca^{2+}. $2.5 \cdot 10^8$ cells/ml were incubated in Hepes-buffered saline at 37 °C for 30 min with $CaCl_2$ as indicated containing ^{45}Ca (900 cpm/µl) without (○,□) or with (●,■) 10^{-6} M A23187.

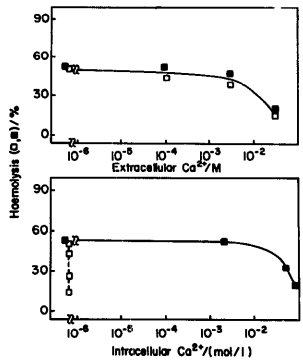

Fig. 4. Inhibition of *S. aureus* α-toxin induced haemolysis by Ca^{2+}. $2.2 \cdot 10^8$ rabbit erythrocytes/ml were incubated at 37 °C for 30 min in Hepes-buffered saline with 1.1 µg α-toxin/ml and $CaCl_2$ as indicated containing ^{45}Ca (900 cpm/µl) without (□) or with (■) 10^{-6} M A23187. Haemolysis values have been scaled so that haemolysis of cells not treated with toxin (see Fig. 1) has a value of 0.

absence of A23187 (lower panel of Fig. 4). In the case of extracellular Ca^{2+}, the values fall on approximately the same curve irrespective of the presence or absence of A23187 (upper panel of Fig. 4). Such a result is found for Sendai virus and melittin also (Table I).

Experiments similar to those carried out with $^{45}Ca^{2+}$ were conducted with $^{65}Zn^{2+}$. Unlike Ca^{2+}, Zn^{2+} plus A23187 does not induce K^+ efflux (upper panels of Fig. 5), even though there is extensive stimulation of $^{65}Zn^{2+}$ uptake by A23187 (15–50-fold at 10^{-6} M Zn^{2+}; 3–5-fold

TABLE I

INHIBITION BY Ca^{2+} OF HAEMOLYSIS INDUCED BY PORE-FORMING AGENTS

Cells were incubated with pore-forming agents at 37 °C as described in the legends to figures. The extracellular and concomitant intracellular Ca^{2+} concentrations were calculated from the radioactivity of the supernatant and cell pellet and the specific activity of $^{45}Ca^{2+}$ used in the incubations.

Pore-forming agent	Ca^{2+} concn. required for 50% inhibition of haemolysis			
	extracellular Ca^{2+} (mol/l)		resultant intracellular Ca^{2+} (mol/l)	
	−A23187	+A23187	−A23187	+A23187
α-Toxin	$1.8 \cdot 10^{-2}$		$< 10^{-6}$	$6.6 \cdot 10^{-2}$
Sendai virus	$3.0 \cdot 10^{-2}$		$< 10^{-3}$	$2.3 \cdot 10^{-1}$
Melittin	$3.5 \cdot 10^{-2}$		$< 10^{-4}$	$5.6 \cdot 10^{-2}$

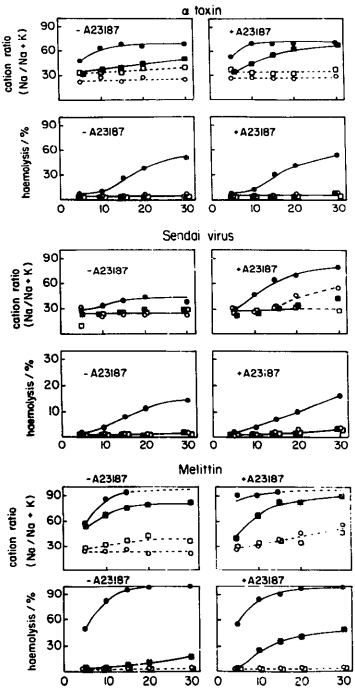

Fig. 5. Protection of erythrocytes by Zn^{2+}. $2 \cdot 10^8$ cells/ml in Hepes-buffered saline at 37 °C without (○, □) or with (●, ■) α-toxin (1.1 μg/ml) or Sendai virus (80 HAU/ml) or melittin ($8 \cdot 10^{-7}$ M) and 10^{-6} M (○, ●) or 10^{-4} M (□, ■) $ZnSO_4$ in the absence or presence of 10^{-6} M A23187 as indicated. Rabbit erythrocytes were used with α-toxin and human erythrocytes with Sendai virus and melittin.

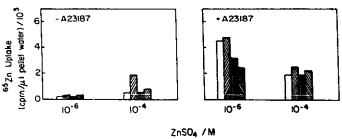

Fig. 6. ^{65}Zn uptake by erythrocytes. $2 \cdot 10^8$ cells/ml were incubated at 37 °C for 20 min in Hepes-buffered saline without (□) or with α-toxin (▨, $8 \cdot 10^{-7}$ M) or Sendai virus (▩, 80 HAU/ml) or melittin (▩, $8 \cdot 10^{-7}$ M) and 10^{-6} M or 10^{-4} M $ZnSO_4$ containing ^{65}Zn (30 cpm/μl) in the absence or presence of 10^{-6} M A23187 as indicated. Rabbit erythrocytes were used with α-toxin and human erythrocytes with Sendai virus and melittin.

at 10^{-4} M Zn^{2+}; Fig. 6). Since high concentrations of Zn^{2+} themselves induce ion leakage [22], including that of Zn^{2+}, the effect of A23187 on uptake of $^{65}Zn^{2+}$ at 10^{-4} M is not so marked as at 10^{-6} M (Fig. 6). Measurement of ion leakage and haemolysis induced by pore-forming agents shows the following. As with Ca^{2+}, the protective effect of Zn^{2+} against ion leakage or haemolysis induced by α-toxin, Sendai virus or melittin is little affected by the presence of A23187; if anything, ion leakage and haemolysis are increased, not decreased, by A23187 (Fig. 5). Fig. 7 illustrates the lack of correlation between intracellular Zn^{2+} and K^+ leakage or haemolysis induced by α-toxin. As for inhibition of haemolysis by Ca^{2+} (Fig. 4), the values for inhibition by Zn^{2+} with/without A23187 lie on quite separate curves for intracellular Zn^{2+} (lower panel of Fig. 7), in contrast to extracellular Zn^{2+} (upper panel of Fig. 7). Table II documents such data for haemolysis induced by Sendai virus and melittin.

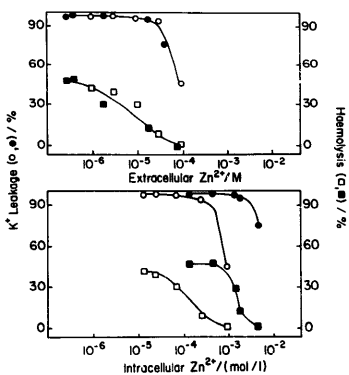

Fig. 7. Inhibition of *S. aureus* α-toxin-induced leakage of erythrocytes by Zn^{2+}. $1 \cdot 10^8$ rabbit erythrocytes/ml were incubated at 37 °C for 10 min in Hepes-buffered saline with 2 μg α-toxin/ml and $ZnSO_4$ as indicated containing ^{65}Zn (30 cpm/μl) without (○, □) or with (●, ■) 10^{-6} M A23187. K^+ leakage and haemolysis have been scaled so that leakage of cells not treated with toxin have a value of 0.

TABLE II

INHIBITION BY Zn^{2+} OF HAEMOLYSIS INDUCED BY PORE-FORMING AGENTS

Cells were incubated with pore-forming agents at 37 °C as described in the legends to figures. The extracellular and intracellular Zn^{2+} concentrations were calculated from the radioactivity of the supernatant and cell pellet and the specific activity of the $^{65}Zn^{2+}$ used in the incubations.

Pore-forming agent	Zn^{2+} concn. required for 50% inhibition of haemolysis			
	extracellular Zn^{2+} (mol/l)		resultant intracellular Zn^{2+} (mol/l)	
	−A23187	+A23187	−A23187	+A23187
α-Toxin	$6.9 \cdot 10^{-6}$		$1.1 \cdot 10^{-4}$	$1.5 \cdot 10^{-3}$
Sendai virus	$2.8 \cdot 10^{-6}$		$3.3 \cdot 10^{-5}$	$3.6 \cdot 10^{-3}$
Melittin	$8.0 \cdot 10^{-6}$		$1.2 \cdot 10^{-4}$	$5.2 \cdot 10^{-3}$

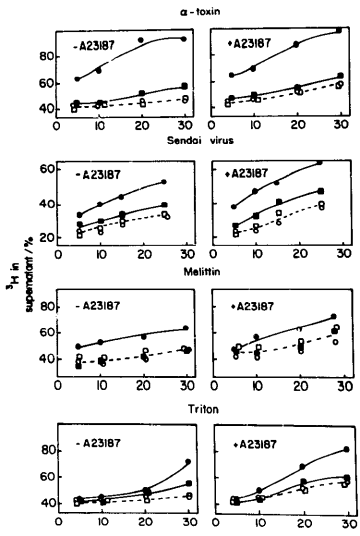

Fig. 8. Protection of Lettre cells by Zn^{2+}. (4–7)·10^6 cells/ml, prelabelled with [^{3}H]choline in Hepes-buffered saline at 37 °C without (○,□) or with (●,□) α-toxin (1 μg/ml) or Sendai virus (after a preincubation of 5 min at 23 °C with 3000 HAU/ml) or melittin ($3 \cdot 10^{-7}$ M) or Triton X-100 (0.003%) and 10^{-6} M (○, ●) or 10^{-4} M (□, ■) $ZnSO_4$ in the absence or presence of 10^{-6} M A23187 as indicated.

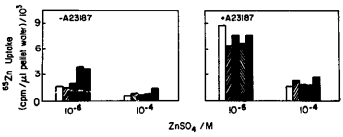

Fig. 9. ^{65}Zn uptake by Lettre cells. $4 \cdot 10^6$ cells/ml were incubated at 37 °C for 20 min in Hepes-buffered saline without (□) or with α-toxin (▨, 1 μg/ml) or Sendai virus (▩, after a preincubation of 5 min at 23 °C with 3000 HAU/ml) or melittin (▩, $3 \cdot 10^{-7}$ M) or Triton X-100 (□, 0.003%) and 10^{-6} M or 10^{-4} M $ZnSO_4$ containing ^{65}Zn (30 cpm/μl) in the absence or presence of 10^{-6} M A12187 as indicated.

Lettre cells: Zn^{2+}

Uptake measurements of $^{45}Ca^{2+}$ by Lettre cells are much more difficult to interpret than those of erythrocytes, due to the complexity of $^{45}Ca^{2+}$ distribution among different intracellular compartments. Nevertheless preliminary data [24] suggest that A23187 has little effect on the protection by Ca^{2+} against leakage of intracellular cations or metabolites such as phosphoryl [^{3}H]choline induced by *S. aureus* α-toxin, Sendai virus, influenza virus (pH 5.5), melittin or polylysine. As with erythrocytes, any effect of A23187 is to promote, not to inhibit, leakage.

Kinetics of $^{65}Zn^{2+}$ uptake by Lettre cells are not as complex as those of $^{45}Ca^{2+}$ uptake, and the effect of A23187 on $^{65}Zn^{2+}$ uptake and leakage of phosphoryl [^{3}H]choline at different concentrations of extracellular Zn^{2+} has been measured. In this instance the protective effect of Zn^{2+} against Triton X-100-induced leakage at sublytic concentrations [15] has been explored as well. A23187 has little effect on the protective action of Zn^{2+} against leakage induced by *S. aureus* α-toxin, Sendai virus, melittin or Triton X-100 (Fig. 8), even though it

TABLE III

INHIBITION BY Zn^{2+} OF LETTRE CELL ^{3}H LEAKAGE INDUCED BY PORE-FORMING AGENTS

Cells were incubated with pore-forming agents at 37 °C as described in the legends to figures. The extracellular and concomitant intracellular Zn^{2+} concentrations were calculated from the radioactivity of the supernatant and cell pellet and the specific activity of the $^{65}Zn^{2+}$ used in the incubations.

Pore-forming agent	Zn^{2+} concn. required for 50% inhibition of ^{3}H-leakage			
	extracellular Zn^{2+} (mol/l)		resultant intracellular Zn^{2+} (mol/l)	
	−A23187	+A23187	−A23187	+A23187
α-Toxin	$1.0 \cdot 10^{-5}$		$4.0 \cdot 10^{-4}$	$1.9 \cdot 10^{-3}$
Sendai virus	$7.0 \cdot 10^{-7}$		$4.9 \cdot 10^{-5}$	$1.5 \cdot 10^{-4}$
Melittin	$3.4 \cdot 10^{-5}$		$3.8 \cdot 10^{-4}$	$2.0 \cdot 10^{-3}$
Triton X-100	$1.7 \cdot 10^{-6}$		$9.1 \cdot 10^{-4}$	$9.1 \cdot 10^{-3}$

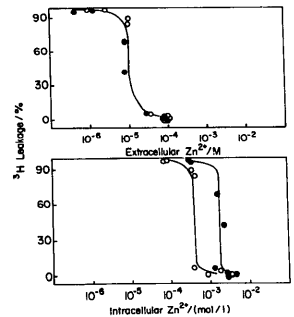

Fig. 10. Inhibition of *S. aureus* α-toxin-induced leakage of Lettre cells by Zn^{2+}. $8 \cdot 10^6$ cells/ml, prelabelled with [^{3}H]choline, were incubated at 37°C for 20 min in Hepes-buffered saline with 0.5 μg α-toxin/ml and $ZnSO_4$ as indicated containing $^{65}Zn^{2+}$ (30 cpm/μl) without (○) or with (●) 10^{-6} M A23187. ^{3}H leakage has been scaled so that leakage of cells not treated with toxin has a value of 0.

causes a significant increase in intracellular $^{65}Zn^{2+}$ (5–8-fold at 10^{-6} M Zn^{2+}; 2–3-fold at 10^{-4} M Zn^{2+}; Fig. 9); if anything, leakage is stimulated not inhibited by A23187.

A plot of α-toxin-induced phosphoryl[^{3}H]choline leakage versus intracellular Zn^{2+} shows two well-separated curves (lower panel of Fig. 10), in contrast to extracellular Zn^{2+} (upper panel of Fig. 10). The data for Sendai virus, melittin and Triton X-100 show the same effect (Table III).

Lettre cells: H^+

Two types of experiment were conducted in order to assess the effect of intracellular H^+ (measured with the weak acid [^{14}C]dimethyl-2,4-oxazolidinedione) on phosphoryl[^{3}H]choline leakage from Lettre cells. In the first, extracellular pH was varied by the use of different citrate-phosphate buffers. In the second, intracellular pH was varied by omitting Na^+ from the extracellular medium to minimise Na^+–H^+ exchange, and by adding nigericin to stimulate K^+–H^+ exchange. Since it is the Na^+–H^+ exchange system that is largely responsible for the control of intracellular pH in non-erythroid cells [35], it was thought that by preventing this system from working, intracellular pH might be lowered while maintaining extracellular pH constant. This proved to be the case (Table IV). One can then directly see whether it is intracellular or extracellular pH that affects phosphoryl [^{3}H]choline leakage induced by *S. aureus* α-toxin, melittin, Sendai virus or Triton X-100. Table IV shows that

TABLE IV

PROTECTION OF LETTRE CELLS FROM PORE-FORMING AGENTS BY H^+

Cells were incubated at 37°C with pore-forming agents in phosphate-citrate buffered isotonic saline (a) or 150 mM choline chloride, 5 mM KCl, 1 mM $MgSO_4$ buffered with 5 mM Hepes (b) or 5 mM 4-morpholineethanesulphonic acid (Mes) (c) with or without 10^{-6} M nigericin as indicated.

	pH_{out}	pH_{in}	Nigericin	^{3}H leakage (% of maximum)			
				α-toxin	Sendai virus	melittin	Triton X-100
(a)	7.5	7.4		86	100	100	100
	6.5	6.8		100	55	83	47
	5.5	6.5		45	–	22	–
(b)	7.4	7.2	–	83	100	83	100
	7.4	6.9	+	83	91	100	89
(c)	6.4	6.9	–	100	–	67	–
	6.4	6.3	+	91	–	56	–

at pH_o 6.5 (citrate-phosphate buffered saline), at which pH_i is 6.8, there is approximately 50% inhibition of leakage induced by Sendai virus or Triton X-100, whereas at pH_o 7.4 (Hepes-buffered choline chloride plus nigericin), at which pH_i is 6.9, there is at best 10% inhibition of leakage. Similarly at pH_o 5.5 (citrate-phosphate buffered saline), at which pH_i is 6.5, there is 65% and 78% inhibition of leakage induced by α-toxin and melittin, respectively, whereas at pH_o 6.4 (Hepes-buffered choline chloride plus nigericin), at which pH_i

TABLE V

ADENINE NUCLEOTIDE CONTENT OF LETTRE CELLS PERMEABILIZED WITH SENDAI VIRUS OR TRITON X-100

$6.7 \cdot 10^6$ cells/ml prelabelled with [^{3}H]choline were incubated in HBS containing 10^{-4} M $ZnSO_4$ at 22°C for 5 min in the absence (no agent) or presence of 100 HU Sendai virus/ml or 0.005% Triton X-100. Pelleted cells were resuspended to the same concentration in HBS containing either 5 mM EGTA or 0.5 mM $ZnSO_4$ or 5 mM $CaCl_2$ and incubated at 37°C for 20 min at which point cells were pelleted, extracted with 4% perchloric acid and content of adenine nucleotide and ^{3}H assessed. The leakage of phosphoryl[^{3}H]choline caused by Sendai virus in the presence of EGTA was 80% (1 and 3% in the presence of 0.5 mM Zn^{2+} and 5 mM Ca^{2+}, respectively) and that caused by Triton X-100 was 36% (12 and 16% in the presence of 0.5 mM Zn^{2+} and 5 mM Ca^{2+}, respectively).

	Adenine nucleotides (nmol/10^8 cells)	
	ATP	AMP + ADP + ATP
No agent + EGTA	70	336
Sendai virus + EGTA	7	120
+ Zn^{2+}	59	329
+ Ca^{2+}	58	371
Triton X-100 + EGTA	< 3	83
+ Zn^{2+}	25	189
+ Ca^{2+}	23	212

is 6.3, there is only 9% and 44% inhibition, respectively. It can be concluded that it is extracellular pH not intracellular pH, that inhibits leakage from cells damaged by pore-forming agents.

Lettre cells: leakage of intracellular nucleotides

It might be argued that leakage of phosphoryl-[^{3}H]choline is not representative of cell damage, as might be assessed by leakage of total adenine nucleotides or loss of ATP, for example. An experiment was therefore performed to test this point. Table V shows the result. Cells were pretreated with agent in the presence of an inhibitory concentration of Zn^{2+}, and then spun to remove excess agent and Zn^{2+}. In this mode, any subsequent leakage of adenine nucleotide or phosphoryl[^{3}H]choline is a measure of the extent to which Zn^{2+} blocks in a *reversible* manner (i.e., not by the displacement of agent, for example). Cells treated in this way lose adenine nucleotides including ATP, as well as phosphoryl[^{3}H]choline, when resuspended in EGTA. When resuspended in 0.5 mM Zn^{2+} or 5 mM Ca^{2+}, loss of nucleotides and phosphoryl[^{3}H]choline is inhibited. Note that loss of uridine, guanine, cytosine and other nucleotides is similarly inhibited by Zn^{2+} or Ca^{2+} (data not shown). This result therefore confirms an earlier observation [7] that agents such as Sendai virus, that cause leakage of phosphoryl[^{3}H]choline, cause similar leakage of sugar phosphates and nucleotides.

Discussion

Two general conclusions can be drawn from the results presented here. First, the protective action of Ca^{2+} on erythrocytes against membrane damage inflicted by haemolytic agents as varied as *S. aureus* α-toxin, Sendai virus or melittin is in every case unaffected by the presence of A23187 (Fig. 1). Yet A23187 induces a dramatic increase in the intracellular concentration of Ca^{2+} (Fig. 2). Indeed, in the absence of A23187 most of the $^{45}Ca^{2+}$ associated with erythrocytes is probably not intracellular at all; this conclusion is based on the results of EPR and NMR experiments with Mn^{2+} [36,37], an analogue of Ca^{2+} [38]. The protective effect of Zn^{2+} on erythrocytes (Fig. 5) and Lettre cells (Fig. 8) is likewise unaffected by the presence of A23187, though this increases intracellular ^{65}Zn dramatically (Figs. 6 and 9). The fact that most of the intracellular Zn^{2+} is probably protein-bound does not affect the issue, since it is extremely unlikely from data such as those of Figs. 6 and 9 and Tables II and III, that the effective concentration of free, intracellular Zn^{2+} is the same with or without A23187. Taken together with the results of a preliminary study showing that A23187 does not potentiate the protective effect of Ca^{2+} against pore-formation in Lettre cells either [24], one may conclude that Ca^{2+} and Zn^{2+} exert their protective effect at the extracellular side of the plasma membrane. Experiments with BHK cells in monolayer culture (Salacinski, H. and Pasternak, C.A., unpublished experiments) are in complete accord with this conclusion.

Second, simultaneous measurement of pH_i and pH_o under different conditions shows that it is a fall in pH_o, not in pH_i, that correlates with inhibition of leakage induced in Lettre cells by *S. aureus* α-toxin, Sendai virus, melittin or Triton X-100 (Table IV). Hence H^+, like Ca^{2+} and Zn^{2+}, protects Lettre cells from the extracellular side of the plasma membrane.

What is the nature of the protective action of Ca^{2+}, Zn^{2+} and H^+ against membrane damage by pore-forming agents? In a previous report [15] we suggested that pores might involve negatively-charged groups at the external side of the plasma membrane which, when chelated (by M^{2+}) or protonated (by H^+), would cause the pores to 'close'. Evidence in favour of such a hypothesis has come from two types of experiment. First, it has been shown that the surface (zeta) potential of erythrocytes and Lettre cells is indeed reduced by Ca^{2+}, Zn^{2+} and H^+ at concentrations at which these ions inhibit haemolytic agent-induced leakage [22]. However, the surface potential is affected equally by Ca^{2+} and Mg^{2+}, whereas inhibition of leakage is not [22]. Since much of the surface potential as measured by electrophoresis is due to charged groups in the glycocalyx region–up to 10 nm from the edge of the lipid/aqueous interface [39]—this suggests that the inhibitory action of Ca^{2+} (and perhaps of Zn^{2+} and H^+ also), is not exerted there, but in the region of the phospholipid bilayer itself, rather like binding by UO_2^+ [40]. The relative insensitivity of agent-affected erythrocytes to the protective action of Ca^{2+}, compared with that of Lettre cells [22,41,42], might suggest that non-erythroid cells such as Lettre cells have more anionic phospholipids in their outer leaflet than erythrocytes. Currently there is little information on this point (e.g., see Ref. 43); what data exist, do not support the idea that appreciable amounts of anionic phospholipids are present in the outer leaflet of any cell type (Ref. 44, and personal communication from Zwaal, R.F.A.).

The second type of experiment, namely conductivity measurements with planar bilayers, also points to an action near the outer surface of the phospholipid bilayer. When haemolytic agents such as *S. aureus* α-toxin [45], cytolysin from sea anemone venom [46,47], or cytolysin from cytotoxic lymphocytes [48,49] are added to phospholipid bilayers, discrete channels are formed. In the presence of Zn^{2+} [42] and H^+ [22], as well as of Ca^{2+} [15], such channels tend to be in the closed, rather than in the open, configuration. An earlier experiment on the effects of H^+ on Triton X-100-induced channels [50] is compatible with this interpretation. Although there are some discrepancies between the results of

conductivity experiments with planar phospholipid bilayers and leakage experiments with whole cells that relate to the voltage-dependency of channel closure in bilayers [15,22,45] and the effective concentration of Ca^{2+} and Zn^{2+} required for channel closure in bilayers [42], in general the results are in accord with the hypothesis that Ca^{2+}, Zn^{2+} and H^+ protect cells by a direct action at the extracellular side of the plasma membrane. Such a conclusion is entirely consistent with the protective role of Ca^{2+} in regard to the electrical behaviour of excitable cells in the absence of any pore-forming agent [16,17,51].

The nature of the ligands to which Ca^{2+}, Zn^{2+} or H^+ bind must await elucidation of the molecular structure of pores: while *S. aureus* α-toxin is capable of forming well-defined 'channels' [52], this does not appear to be true of melittin [53] or Triton X-100. Moreover, neither melittin [54] nor Triton X-100 possess anionic sites capable of binding divalent cations or protons. In these cases, at least, Ca^{2+}, Zn^{2+} and H^+ probably affect some endogenous component of the plasma membrane. Currently, the phospholipids of the outer leaflet appear to be the most likely site. The observation that divalent cations [55] and protons [56] alter the structural disposition of phospholipid bilayers so as to reduce the amount of water associated with their headgroups [57,58] is compatible with such a hypothesis.

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