Biochimica et Biophysica Acta, 981 (1989) 277–287
Elsevier

BBA 74423

Reversible electropermeabilisation of human and rat blood platelets: evaluation of morphological and functional integrity 'in vitro' and 'in vivo'

K. Hughes and N. Crawford

Department of Biochemistry and Cell Biology, Hunterian Institute, Royal College of Surgeons of England, London (U.K.)

(Received 2 December 1988)

Key words: Electropermeabilization; Platelet; Drug delivery; (Rat); (Human)

A high-voltage discharge procedure has been developed for permeabilising the plasma membranes of both human and rat blood platelets. The cells can be resealed by incubation at 37°C, show < 4% loss of lactate dehydrogenase (LDH) implying minimal cell lysis and also have well maintained morphological and functional integrity. The prototype apparatus used at field strengths between 6 and 8 kV/cm produces membrane pores which allow free diffusion of low molecular weight substances such as adenine nucleotides, inositol phosphate and fluorescent dyes. Two properties, namely Ca^{2+}-induced secretion of granule stored 5-hydroxytryptamine (5HT) and inositol 1,4,5-trisphosphate (IP_3)-induced release of intracellularly sequestered ^{45}Ca, which are both well expressed immediately after permeabilisation, are essentially abolished after resealing. The efficiency of permeabilisation and resealing can be simply monitored by shifts in 'apparent platelet volume' using a resistive particle counter (Coulter). Permeabilised platelets show a shift in modal volumes from a control range 4–7 fl to 10–15 fl. Resealing restores these modal volumes to the original control range. Encapsulation of the fluorochrome, Lucifer yellow (M_r 550), during permeabilisation revealed that after resealing > 85% of rat platelets, and close to 100% human platelets, contained the encapsulated dye. The initial rates and % aggregation responses of both human and rat platelets to collagen, thrombin and the thromboxane A_2-mimetic U46619 remained essentially normal after permeabilisation and resealing further illustrating the maintenance of functional competence following treatment. Resealed rat platelets reinfused into the circulation after labelling with [^{111}In]indium oxine gave survival curves similar to those of control platelets. Therefore, this reversible permeabilisation procedure may allow the use of autologous or heterologous platelets as carrier vehicles for the delivery of drugs and other agents 'in vivo'.

Introduction

Permeabilisation or poration of the plasma membrane of cells allows experimental manipulation of the composition of their cytosolic compartments and can facilitate the introduction into the cell of non-membrane permeant drugs and other agents. Such permeabilisation can now be achieved with a variety of procedures which include hypotonic lysis [1,2], the application of the detergents saponin or digitonin [3–5], incubation with Sendai virus [6], treatment with EDTA and ATP^{4-} [7,8] or with L-α-lyso-phosphatidylcholine [9,10]. Careful use of these procedures can produce pores in the plasma membrane, without seriously affecting its integrity or that of the boundary membranes of intracellular organelles. The use of the hypotonically permeabilised erythrocyte, or ghosts prepared from them, as carrier vehicles for enzymes and drugs has been extensively explored (for review, see Refs. 11 and 12). More recently much new information has emerged from the application of detergent-based techniques for gaining access to cell cytosolic compartments and particularly in the areas of signal transduction processes, second messenger regulation of intracellular events and the various factors which influence the sequestration and release of Ca^{2+} from intracellular stores in a number of different mammalian cell types.

Abbreviations: LDH, lactate dehydrogenase; 5HT, 5-hydroxytryptamine; PRP, platelet-rich plasma; IP_3, inositol 1,4,5-trisphosphate; TXA_2, thromboxane A_2; PGE_1, prostaglandin E_1; BSA, bovine serum albumin; Pipes, 1,4-piperazinediethanesulphonic acid

Correspondence. K. Hughes, Department of Biochemistry and Cell Biology, Hunterian Institute, Royal College of Surgeons of England, Lincoln's Inn Fields, London, WC2A 3PN, U.K.

As an alternative to these chemical and viral treatments, a number of electrical procedures for perturbing cell membranes have also been developed which result in poration. These have generally involved the application of short duration high-voltage fields to cell monolayers or suspensions (electropermeabilisation or electroporation) resulting in a localised dielectric breakdown of the surface membrane proteo-lipid bilayer and the formation of plasma membrane pores of varying dimension. Variations of this electrical poration procedure are being widely used in transfection studies with animal and plant cells [13] and also for the encapsulation of drugs and other agents into red blood cells for site directed delivery and/or controlled release of the encapsulated agent [14,15]. The principles of electropermeabilisation and the molecular processes involved in the membrane perturbation have been presented and well discussed in the review papers of Zimmerman et al. [16], Knight [17], Knight and Scrutton [18] and Neumann and Rosenheck [19]. The first studies involving the electropermeabilisation of human blood platelets [20,21] used a technique that had been applied earlier to the study of stimulated secretory responses in adrenal medullary cells [22,23]. This platelet permeabilisation procedure revealed new information about the mechanisms involved in the regulation of Ca^{2+} homeostasis and the relationship between $[Ca^{2+}]_i$ and certain platelet secretory functions [24,25].

Our own interest in platelet electropermeabilisation arose from studies of the use of these cells as both biocompatible and biodegradable delivery vehicles for encapsulated small molecular weight hydrophilic drugs and other agents. We reasoned that if such carrier vehicles could be resealed after encapsulation of the agent and at the same time some degree of cellular integrity be maintained, then their survival in the circulation after infusion may substantially follow normal life span characteristics. Delivery of a drug to a target site might thus be prolonged compared to liposome entrapment techniques where intravascular disruption of the liposome structure and leakage can occur and where substantial amounts of the carrier vehicle can also be lost from the circulation during early post-infusion passage through the liver [26]. Although alloimmunisation can develop with multiple platelet transfusions there are records of patients receiving over 1000 platelet concentrates in therapeutic programmes [27].

This present paper documents our development of an electropermeabilisation procedure found to be suitable for reversibly permeabilising human and rat blood platelets. A prototype high-voltage discharge device has been constructed and the conditions for optimal poration and resealing of the cells have been evaluated with respect to the optimal conditions for maintenance of morphological and functional integrity 'in vitro' and 'in vivo'. This functional evaluation has been initially focussed upon the cell's metabolic competence to respond to surface haemostatic stimuli with induction of aggregation and secretory events but the 'in vivo' life span characteristics of permeabilised and resealed (sham-encapsulated) rat platelets have also been studied as an index of their circulating integrity.

Materials and Methods

Reagents

Unless otherwise stated all chemicals were obtained from either BDH Ltd. (Poole, U.K.) or the Sigma Chemical Company (Poole, U.K.). Radiochemical compounds and inositol 1,4,5-trisphosphate were obtained from Amersham International (Amersham, U.K.). Thrombin was purchased from Diagnostic Reagents Ltd. (Thame, U.K.), collagen from Hormon-Chemie (Munich, F.R.G.) and the thromboxane A_2-mimetic U46619 was a gift from Glaxo plc. (Ware, U.K.).

Apparatus

The instrument, used throughout these investigations, was specifically designed to permeabilise blood platelets by repeated high-voltage discharge from precharged capacitors and the prototype was constructed by Rank Bros. Ltd. (Bottisham, U.K.). Its design was substantially based upon the original apparatus of Baker and Knight [22] as used by Scrutton and colleagues [18,20] and Haslam and Davidson [21,25] and has now been in almost daily use for over 18 months. The apparatus is illustrated in Figs. 1a and b but fuller details of the cell design, circuit diagrams, capacitor discharge characteristics etc. are to be reported elsewhere [28]. Briefly, a 4.5 μF capacitor unit is charged appropriately from a high-voltage supply with a maximum charging voltage of 5 kV. Discharge of the stored voltage takes place across a spark gap of 3.25 mm which is bordered by two platinum electrodes. The platelet suspension (800 μl) is placed between these electrodes for repeated cycles of high-voltage discharge. The electric field induced within the cell suspension during each discharge decays exponentially with a half-life of about 13 μs. Alternative cell compartments can be plugged into the top of the device for different cell suspension volumes and for variation of the spark gap distances. The suspension must fully cover the electrode during discharge and the compartment is sealed at the top with a perspex cover lightly smeared with silicone grease.

Preparation of platelet suspensions

Blood samples were taken into plastic tubes from human volunteers with minimal venous stasis and all donors had declared themselves free from aspirin-containing medicaments for at least 9 days. Anticoagulation was effected by inversion mixing with one-ninth volume of 3.8% trisodium citrate. Platelet-rich plasma

(PRP) was prepared by centrifugation (200 × *g* for 15 min) at room temperature. The PRP was removed and acidified by dropwise addition of 0.3 M citric acid to pH 6.4 [29]. Platelets were pelleted by centrifugation (750 × *g* for 10 min) and washed once by resuspension in a buffer consisting of 36 mM citric acid, 5 mM glucose, 90 mM NaCl, 10 mM EDTA adjusted to pH 6.5 with NaOH. Prostaglandin E_1 (PGE_1) was included in this buffer (100 nM) to facilitate resuspension. After again pelleting at 750 × *g* for 10 min the platelets were finally resuspended in a buffer containing (except where stated) 150 mM potassium glutamate, 20 mM K-Pipes, 5 mM K^+-ATP, 7 mM $MgCl_2$, 5 mM glucose, 2 mM EGTA, adjusted to pH 7.4 with NaOH. Routinely, cell densities of approx. $3 \cdot 10^8$ platelets/ml buffer were used for electropermeabilisation. All platelet preparation procedures were performed within 2 h of donation.

In the case of rat platelets the same preparative protocol was followed except that blood samples were taken by aortic puncture from Hypnorm/Hypnovel-anaesthetised male Wistar animals and a one-fifth volume of 3.8% trisodium citrate was used for the anticoagulation. The rat PRP was harvested by 10 min centrifugation at 500 × *g*.

Measurement of adenine nucleotide losses and liberated lactate dehydrogenase (LDH) activity during high-voltage discharge

The nucleotide pool of the platelet cytosol was prelabelled by incubating PRP at 37°C for 1 h in the presence of 0.33 μM [^{3}H]adenine (10 μCi/ml) and 1 μM unlabelled adenine. Platelets were isolated and washed under pH 6.5 conditions as described earlier. Five minutes after cycles of high-voltage discharge the platelets were isolated from a 500 μl aliquot of the suspension by centrifugation (12000 × *g* for 2 min). Duplicate aliquots of supernatant were taken for scintillation counting to measure the released nucleotides. The amount of radioactivity retained in the cells was also determined by Millipore filtration (pore size 0.45 μm) of aliquots of cell suspension. Supernatants prepared exactly as for the nucleotide release studies were also assayed for LDH activity in the presence of 0.2% (v/v) Triton X-100 using a modification of the procedure of Wroblewski and La Due [30]. Briefly, 2.5 ml of 50 mM phosphate buffer (pH 7.4) was added to a 10 mm light path quartz cuvette, followed by 200 μl of 45 mM sodium pyruvate and 200 μl 3 mM NADH, both dissolved in the same phosphate buffer. A 200 μl sample of cell supernatant was then added to the cuvette and, after mixing, change in absorbance was monitored in a spectrophotometer at a wavelength of 340 nm. Total LDH activity in the cell suspension was determined as that released after exposing the whole cells to 0.2% (v/v) Triton X-100 for 20 min before the centrifugation.

Measurement of 5-hydroxytryptamine [5HT] secretion

These experimental procedures were essentially based upon those described by Knight and Scrutton [31]. Platelets in PRP were incubated for 5 min at 37°C with 0.5 μCi/20 ml of 5-hydroxy[^{14}C]tryptamine. This was immediately followed by a further 5 min incubation after increasing the label concentration to 1.0 μCi/20 ml in order to promote the loading of the 5HT storing dense granule pool. The cell suspension was then acidified to pH 6.4 as described earlier and after pelleting by centrifugation the platelets were resuspended ($3 \cdot 10^8$ cells/ml) in the high-K^+ permeabilisation medium containing 10 mM EGTA. After the high-voltage discharge cycles had been applied to this suspension, 10 mM $CaCl_2$ was added to give a free [Ca^{2+}] of approx. 10 μM in order to intracellularly stimulate platelet 5HT secretion. Imipramine (5 μM) was added to the platelet suspension to block any reuptake of secreted amine. Five minutes after the addition of $CaCl_2$, a 500 μl aliquot of cell suspension was centrifuged (12000 × *g* for 2 min) and released [^{14}C]5HT determined in duplicate 200 μl aliquots of the supernatant by scintillation counting. In other studies freshly permeabilised cells were resealed by incubation at 37°C for periods up to one hour before adding the $CaCl_2$.

Cell counting and measurement of 'apparent volume' distribution (Coulter Volume) of platelet populations

Cell counts were made on platelet suspensions after dilution in Isoton II using a Coulter Model ZM Counter with a 50 μm orifice. For volume distribution studies 10-μl aliquots of untreated, freshly permeabilised or permeabilised and resealed platelets were added to 20 ml of Isoton II and the volume profiles determined with the Coulter Counter and Channelyser 256. The Coulter Counter was precalibrated using standard latex particles of uniform diameters giving upper and lower thresholds of 36 and 2 fl.

Encapsulation of Lucifer yellow into permeabilised platelets

The techniques for these studies were based upon those described by Mir et al. [32]. Platelet suspensions after exposure to cycles of high-voltage discharge were incubated at 37°C in the presence of known concentrations (usually approx. 500 μg/ml) of Lucifer yellow (M_r 550). After one hour for resealing, the preparation was acidified to pH 6.5 by dropwise addition of 0.3 M citric acid. Platelets were then pelleted by centrifugation (700 × *g*, 10 min) and washed by resuspension in permeabilisation buffer which had been previously adjusted to pH 6.5. After centrifugation the cells were then resuspended. This washing/resuspension cycle was repeated three times. Encapsulation of Lucifer yellow was semi-quantified by fluorescence spectrometry. Control experiments for any Lucifer yellow associated with

the surface of non-permeabilised cells and those which were permeabilised and resealed before exposure to Lucifer yellow were included with all preparations. The internalised fluorochrome was also monitored using fluorescence microscopy and photomicrographs were prepared.

Platelet aggregometry

Platelet aggregation responses after addition of agonists was followed using a Payton 300B dual channel aggregometer. For these studies untreated or permeabilised and resealed platelets in EGTA-free permeabilisation buffer ($3 \cdot 10^8$/ml) were added to aggregometry cuvettes held at 37°C with stirring. After 4 min an appropriate quantity of agonist was added and the aggregation response optically recorded as a change in light transmission. The agonists routinely used were thrombin in the range 0.04–2.0 U/ml, collagen 1–30 μg/ml and the thromboxane A_2-mimetic U46619 at 1.5 μM.

Induced ^{45}Ca release from platelet internal stores with inositol 1,4,5-trisphosphate (IP_3)

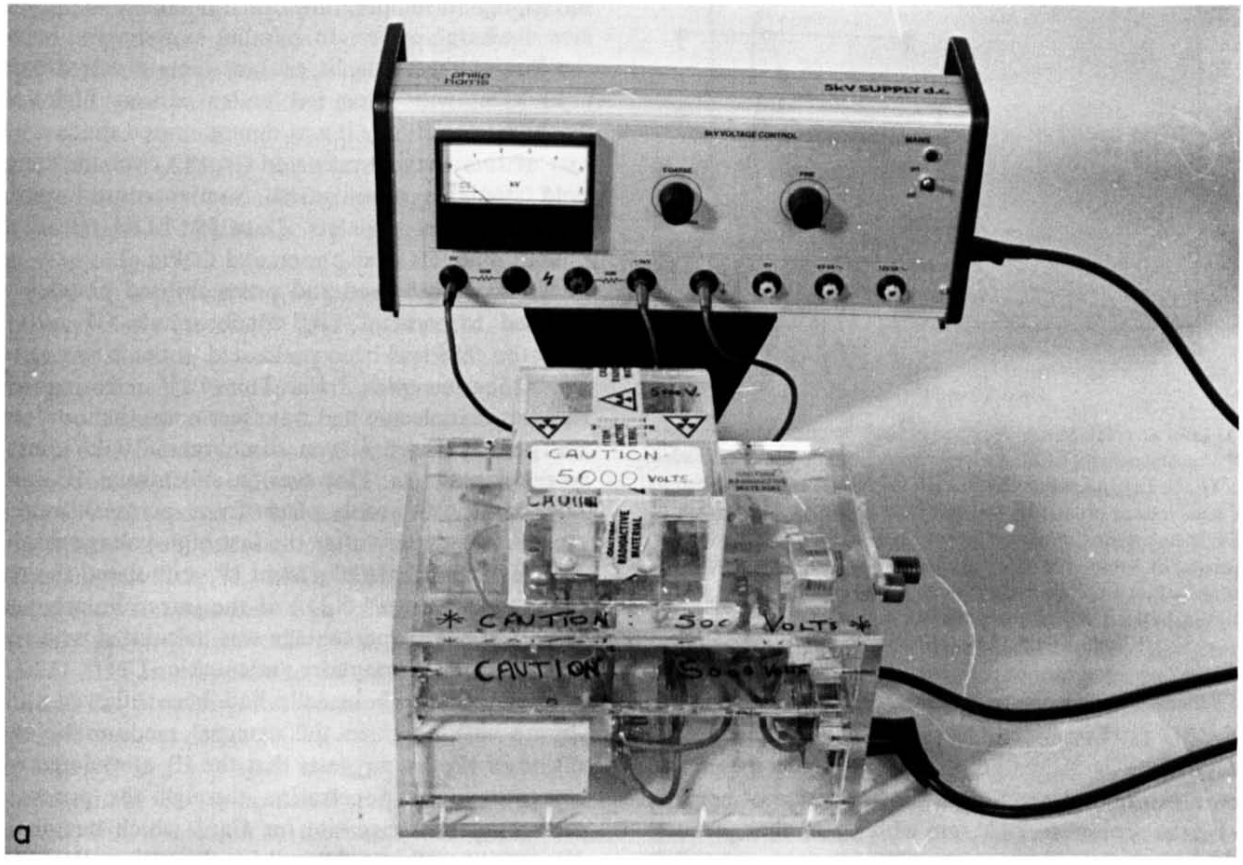
Platelet intracellular stores were preloaded with ^{45}Ca using the buffer medium recommended by Brass [33]. After pelleting the platelets from 20 ml PRP, they were resuspended in 20 ml of a buffer consisting of 140 mM KCl, 1 mM glucose, 1 mM $MgCl_2$, 0.42 mM NaH_2PO_4, 11.9 mM $NaHCO_3$, 10 mM Pipes containing 1 mg/ml BSA and 100 nM PGE_1 adjusted to pH 6.5. To this suspension 20 μM $CaCl_2$ was added containing 2 μCi/ml ^{45}Ca (5–50 Ci/g Ca^{2+}) and the mixture incubated at room temperature for 4 h. After this incubation period the platelets were harvested by centrifugation (500 × *g*, 20 min) and washed once in a buffer consisting of 120 mM KCl, 10 mM Pipes, 5 mM EGTA, 5 mM $MgCl_2$, 100 nM PGE_1 (pH 6.5). After further pelleting at 750 × *g* for 10 min the platelets were resuspended in the high K^+ permeabilisation buffer (without EGTA) to a cell density of $3 \cdot 10^8$/ml in readiness for the cycles of high-voltage discharge. All IP_3 induced Ca^{2+} release studies were made within about 30–90 min of this resuspension. After IP_3 induced release from the permeabilised cells, the retained ^{45}Ca was measured by taking 400-μl aliquots of the suspension for transfer to Millipore membrane filters (pore size 0.45 μm). The cells washed twice 'in situ' with 10-ml aliquots of an ice-cold buffer containing 120 mM KCl, 10 mM Pipes, 5 mM $MgCl_2$ and 5 mM EGTA (pH 7.4). The activity associated with the cells retained on the filter was determined by scintillation counting. All ^{45}Ca release data were expressed as percentages released with respect to the total ionophore-releasable Ca^{2+} pool determined in parallel experiments using 12.4 μM A23187.

Measurement of platelet life spans 'in vivo'

For these experiments all buffer solutions were presterilized by ultrafiltration and sterile apparatus was used during all the 'ex vivo' handling procedures. Male Wistar rats (weight range 250–350 g) were used with the platelets collected from one exsanguinated donor rat infused into the recipient rat after [^{111}In]indium oxine labelling. The platelet labelling procedure was a modification of that described by Hawker et al. [34]. Briefly, the platelets from 3 ml PRP were resuspended in 5 ml sterile high-K^+ permeabilisation buffer. After the sequence of high-voltage discharge cycles (seven times 6.8 kV/cm) and resealing by incubation for 1 h at 37°C, the platelet suspension was acidified to pH 6.4 (citric acid). The cells were then pelleted at 700 × *g* for 10 min and resuspended in 1 ml of a sterile buffer consisting of 137 mM NaCl, 2.6 mM KCl, 11.9 mM $NaHCO_3$, 0.32 mM Na_2HPO_2, 2 mM $MgCl_2$, 5.5 mM glucose (pH 6.4), containing 25 U/ml preservative-free heparin and 100 nM PGE_1. For the radiolabelling procedure, 100 μCi of [^{111}In]indium oxine was added to the suspension and the mixture incubated for 2 min at room temperature before addition of 5 ml of a 1 : 1 mixture of cell free rat plasma and permeabilisation buffer both preacidified to pH 6.4. The platelets were then pelleted (700 × *g*, 10 min) and resuspended in 1 ml of platelet free plasma prepared without pH 6.4 adjustment. The radiolabelled platelets in this plasma were infused into the circulation of the recipient male rats by tail-vein injection using a 21-gauge needle. Blood samples (approx. 200 μl) were taken from these rats by cardiac puncture (23-gauge needles) at timed intervals after infusion of the labelled cells. Platelet counts were performed on these samples and the radioactivity associated with the platelets determined by radioactive counting.

Results

The exposure of washed platelet suspensions to cycles of high-voltage discharge in the range 5–12 kV/cm results in a loss of adenine nucleotides and their metabolites from the cytosolic pool. These losses were investigated by prelabelling the platelets with [^{3}H]adenine before exposure to the high-voltage discharge treatment. Fig. 2 shows the nucleotide loss data from experiments with human and rat platelets in which the field strength was varied from 5 to 12 kV/cm for a fixed number of discharge cycles (seven) with short intervals for capacitor recharging between each discharge mode. For rat platelets only 10% loss of nucleotides occurs at field strength 5 kV/cm with maximum release of the label (approx. 89%) occurring at a field strength of around 6–7 kV/cm. Further increases in the discharge voltage above this value appear to result in no further losses of the label. With human platelets some loss of



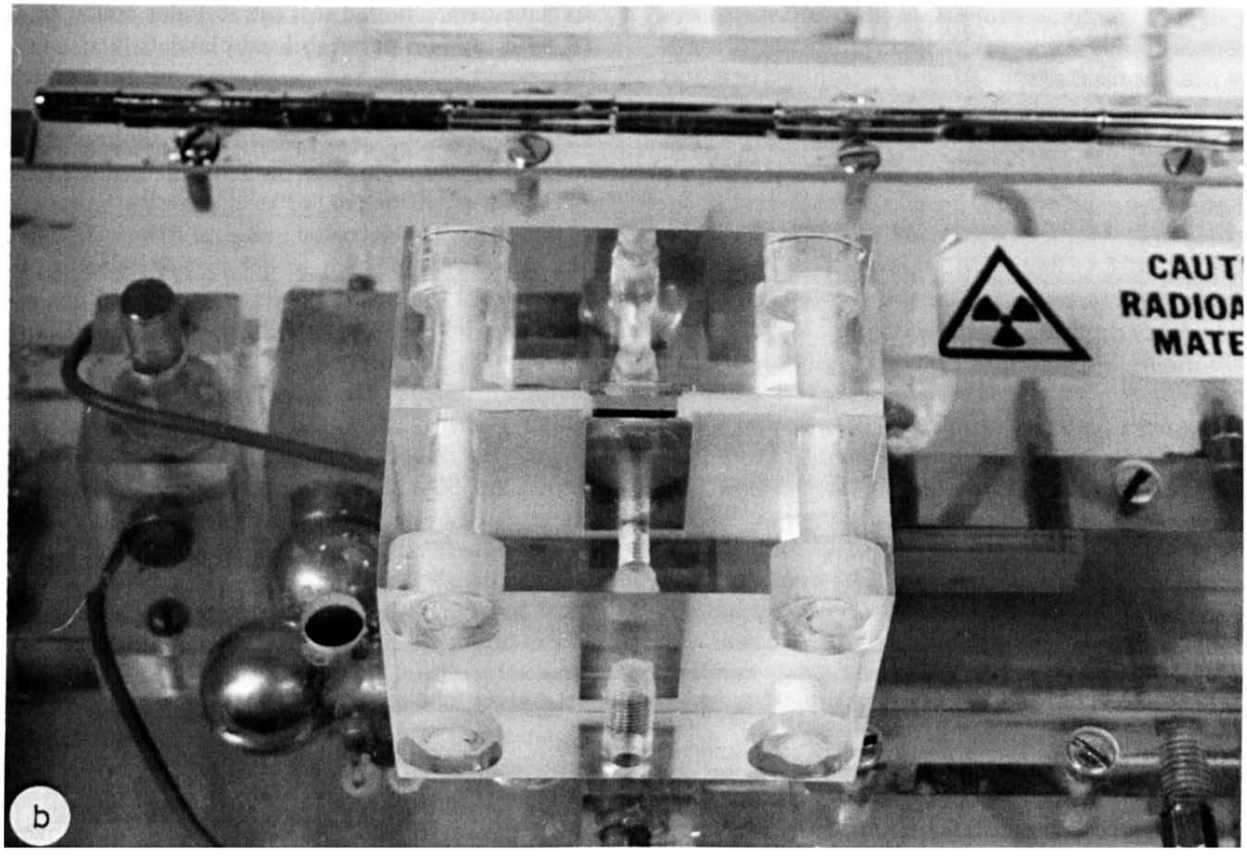


Fig. 1. The high-voltage discharge apparatus. (a) The prototype electropermeabilisation device with high-voltage supply unit connected. (b) Enlarged view of the discharge chamber (capacity 800 μl). The chamber units are interchangeable with others of different spark gap and capacity. Their design allows them to be readily disassembled for inspection and cleaning.

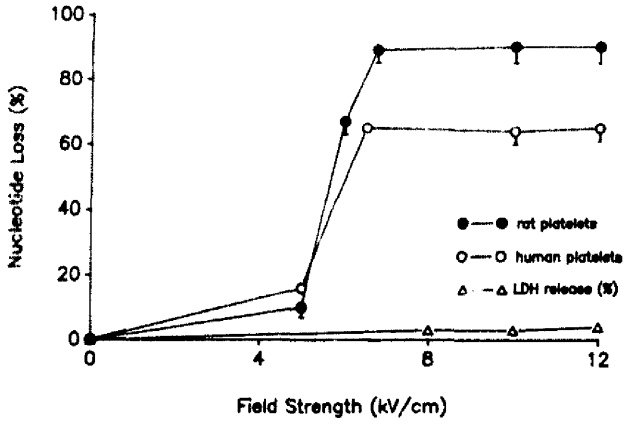


Fig. 2. Loss of [^{3}H]adenine metabolites and lactate dehydrogenase (LDH) from platelets during electropermeabilisation. Platelets loaded with [^{3}H]adenine and suspended in high K^+ buffer were subjected to seven high-voltage discharges at fixed field strengths. The release of adenine metabolites from either human (○) or rat (●) cells into the supernatant at 5 min after electropermeabilisation was expressed as a percentage of total radioactivity present in control untreated platelets. Percentage LDH release (△) was related to that released after treatment with 0.2% (v/v) Triton X-100.

nucleotide (16%) takes place at 5 kV/cm and as the field strength is increased the loss of label begins to become substantial reaching a plateau value of approx. 65% loss at around 6–7 kV/cm which also changes little with increasing field strengths. The insertion of a fast response temperature probe into the cell suspension immediately after each discharge revealed no measurable change in temperature over the course of 12 successive discharge cycles. In parallel experiments in which the loss of the cytosolic enzyme lactate dehydrogenase (140 kDa) was measured under various high-voltage discharge conditions it was demonstrated that very little loss of this enzyme occurred (< 4%) over the range of field strengths investigated. Similar results were obtained with rat platelets. Data for LDH release from human platelets is also included in Fig. 2.

Non-permeabilised and permeabilised platelets were exposed to inositol 1,4,5-trisphosphate (IP_3, 20 μM) after the cells had been preloaded at the intact cell level by incubation with ^{45}Ca. This Ca^{2+}-releasing second messenger molecule had no effect upon the untreated or the permeabilised (seven discharges; 10 kV/cm) and resealed platelets. However, a substantial IP_3-induced release of ^{45}Ca took place from permeabilised cells tested immediately after the last high-voltage discharge. In five experiments 20 μM of IP_3 stimulated the release of 29 ± 5% (mean ± S.D.) of the intracellularly sequestered Ca^{2+}. This percentage was calculated with respect to the total ionophore releasable Ca^{2+} (12.4 μM A23187). Since these cells had been allowed to equilibrate with Ca^{2+} in the external medium before addition of IP_3 this suggests that the IP_3-stimulated release was due to its penetration through the pores to an intracellular storage site for Ca^{2+} which has an intact IP_3-sensitive release channel for the cation. Earlier studies have demonstrated this intracellular action of added IP_3 with saponin-permeabilised platelets [4].

TABLE I

Ca^{2+}-induced secretion of prelabelled [^{14}C]5HT from control (untreated), permeabilised and resealed human and rat platelets

Species	Membrane status [a]	Field strength (kV/cm) [b]	External free $[Ca^{2+}]$ [c]	% Total 5HT, mean ± S.D. (n = 4)	
				found in external medium [d]	released by Ca^{2+} [e]
Human	control	0	none	14 ± 3	none
Human	control	0	10 μM	14 ± 3	none
Human	P	10	none	15 ± 4	1
Human	P	10	10μM	58 ± 4	51
Human	P/R	10	10 μM	27 ± 3	15
Human	P	15	none	14 ± 3	none
Human	P	15	10 μM	52 ± 5	44
Human	P/R	15	10 μM	48 ± 4	39
Rat	control	0	none	27 ± 2	none
Rat	control	0	10 μM	27 ± 1	none
Rat	P	6.8	none	30 ± 3	4
Rat	P	6.8	10 μM	93 ± 2	90
Rat	P/R	6.8	10 μM	37 ± 2	14

[a] Control = untreated platelets, P = permeabilised, P/R = permeabilised and resealed (incubated 37°C 1 h).
[b] Permeabilisation conditions = seven cycles of high-voltage discharge at stated field strengths.
[c] None = EGTA only present. Free $[Ca^{2+}]$ adjusted by Ca^{2+}-EGTA buffers.
[d] Total [^{14}C]5HT determined after solubilisation of controls with Triton X-100.
[e] % Ca^{2+}-induced secretion calculated by reference to controls of column (d)

$$\text{viz. } \%(e) = \frac{\text{experimental counts} - \text{control counts (d)}}{\text{total counts (d)} - \text{control counts (d)}} \times 100.$$

In studies involving Ca^{2+}-induced release of granule stored components, platelets were prelabelled with [^{14}C]5HT by the procedure outlined in Methods. When human cells were permeabilised in the absence of Ca^{2+} in the external medium no secretion of [^{14}C]5HT took place (Table I). Also, if the platelets were first permeabilised and then resealed by incubation for 1 h at 37°C, addition of 10 μM Ca^{2+} to the external medium resulted in only a 15% release of [^{14}C]5HT. However, if Ca^{2+} (10 μM) is present during permeabilisation 51% of the intracellularly stored [^{14}C]5HT is secreted. In these experiments human platelets were exposed to seven discharge cycles at 10 kV/cm. After exposure of human platelets to higher voltages (i.e., 15 kV/cm) the cells do not reseal with incubation, even after 2 h at 37°C. That the reduction of Ca^{2+}-induced 5HT secretion after resealing cells exposed to 10 kV/cm is not an artefact of this incubation is illustrated by the values for the 15 kV/cm samples. Here 10 μM Ca^{2+} produced essentially the same secretion response after 1 h at 37°C (39%) as the freshly permeabilised cells (43%, see Table I). The release of 5HT in response to 10 μM Ca^{2+} from permeabilised rat platelets after seven discharge cycles at 6.8 kV/cm was very high (90%), but again lower (14%) when exposed to Ca^{2+} after resealing (again Table I). The reduction in Ca^{2+}-stimulated secretion of [^{14}C]5HT correlated with the length of the incubation period for resealing but, with human cells, after only 20 min incubation the secretion was substantially reduced. With rat platelets this effect was even more rapid.

A series of investigations was made of apparent Coulter volume distribution profiles before and after permeabilisation and after resealing. These studies showed a phenomenon that was subsequently adopted for a routine assessment of the extent of permeabilisation and efficacy of resealing. After electropermeabilisation of platelets suspended in the high-K^+ buffer and subsequent transfer of an aliquot to Isoton II for Coulter counting and volume distribution profiling the platelets showed a marked shift to apparent higher modal volumes (10–15 fl) when compared to control suspensions of unpermeabilised platelets which had modal volumes of approx. 4–7 fl (see Fig. 3). At no time in these experiments was there a loss in total cell count. Incubation of the permeabilised cells at 37°C to reseal and then transfer of an aliquot into Isoton II resulted in a volume distribution profile which was essentially the same as the original distribution profile prepared from the untreated cells and with identical apparent modal volume values (also shown in Fig. 3). These volume shifts correlate with the biochemical evidence for pore formation. If volume distribution curves are prepared with platelets remaining in the high-K^+ permeabilisation buffer instead of their transfer to Isoton II no shift in apparent modal volume is observed. Similarly these modal volume shifts are not seen with profiles per-

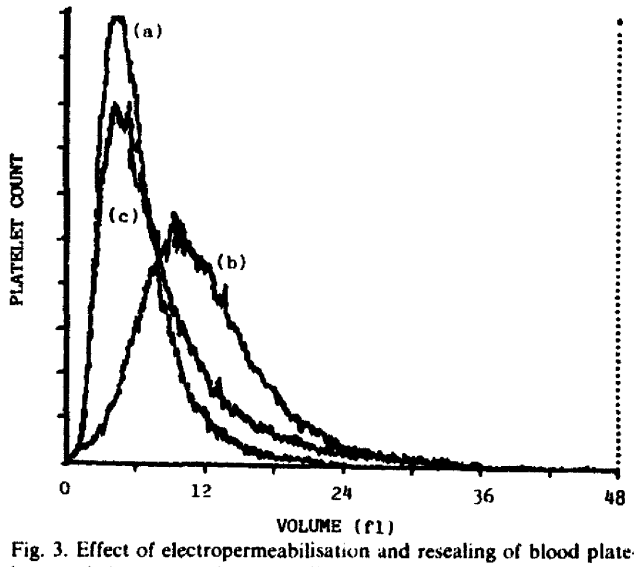


Fig. 3. Effect of electropermeabilisation and resealing of blood platelets on their 'apparent' volume distribution. (a) Volume distribution of control (unpermeabilised) platelets after transfer of 10 μl of suspension into 20 ml of Isoton II counting medium. (b) Volume distribution profile of freshly permeabilised platelets suspended in Isoton II showing large increases in apparent cell volume. (c) Volume profile for resealed platelets suspended in Isoton II (resealing conditions 1 h at 37°C). Note: Volume profiles for control and freshly permeabilised platelets produced after dilution in high-K^+ buffer instead of Isoton II exactly overlapped with curve (a).

formed in Isoton III. The magnitude of these apparent modal volume shifts shown by the permeabilised platelets can be varied by altering the relative proportions of Isoton II and high-K^+ buffer in the mixture into which the aliquots are transferred for the volume distribution profiling. Substitutions and deletions of the various constituents of either the permeabilising buffer or Isoton II (the composition of which is 136 mM NaCl, 5.4 mM KCl, 13.4 mM Na_2HPO_4, 1.2 mM NaH_2PO_4, 0.8 mM EDTA, 11.9 mM NaF and 3.3 g/l 2-phenoxyethanol) failed to reveal a satisfactory explanation for these observed apparent volume shifts seen after permeabilisation and their restoration to normal following resealing. The phenomenon, however, was found to be highly reproducible and consistent in the magnitude of the volume shifts with both human and rat platelets. We refer to these volume shifts seen with the permeabilised cells as 'apparent' because planimetry measurements of the areas of a large number of platelets seen in scanning electron micrographs revealed no significant differences between control (untreated) and permeabilised/resealed cells suspended in Isoton II. The area ratio for permeabilised versus control platelets P : C was 1.15. If one assumed sphericity this translates to a volume increase of less than 30% whereas the apparent modal volume shifts recorded with the Coulter counter suggested a greater than 2-fold increase. The area ratios permeabilised/resealed versus control platelets P/R : C gave values generally less than 1.0 compatible with the restora-

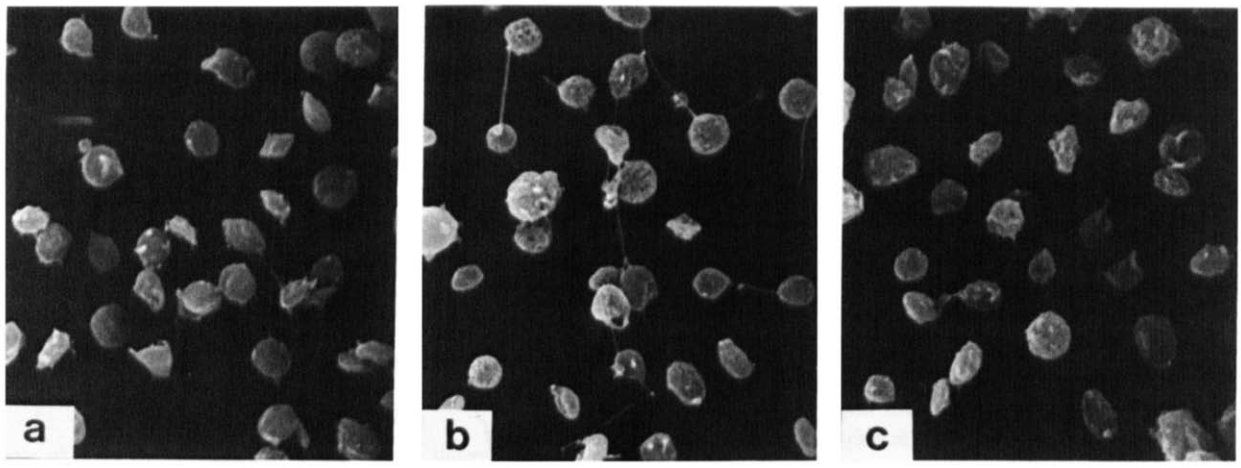


Fig. 4. Scanning electron microscopy of control, electropermeabilised and resealed human platelets. (a) Control platelets showing normal morphology with good discoidicity (×5000). (b) Freshly electropermeabilised platelets (seven times 10 kV/cm) showing filopodia extending between cells (×5000). Planimetry of such micrographs showed an area increase of approx. 15%, insufficient to account for the large increase in Coulter volume. (c) Resealed platelets (×5000). Planimetry revealed area measurements approximating those of control (a) platelets. Note: For all preparations the cells were fixed for electron microscopy after transfer to Isoton II.

tion of the volume profile after resealing (scanning electron micrographs, Fig. 4).

When platelets were permeabilised with seven discharges of 6.8 kV/cm and subsequently incubated in the presence of the non-membrane penetrating fluorescent dye Lucifer yellow (M_r 550) the dye became encapsulated within the cytosolic compartment. After repeated washing of these platelets to remove much of the surface associated dye they showed a 4-fold (mean $\pm$ S.D. 3.9 ± 0.6, $n = 6$) higher concentration of the

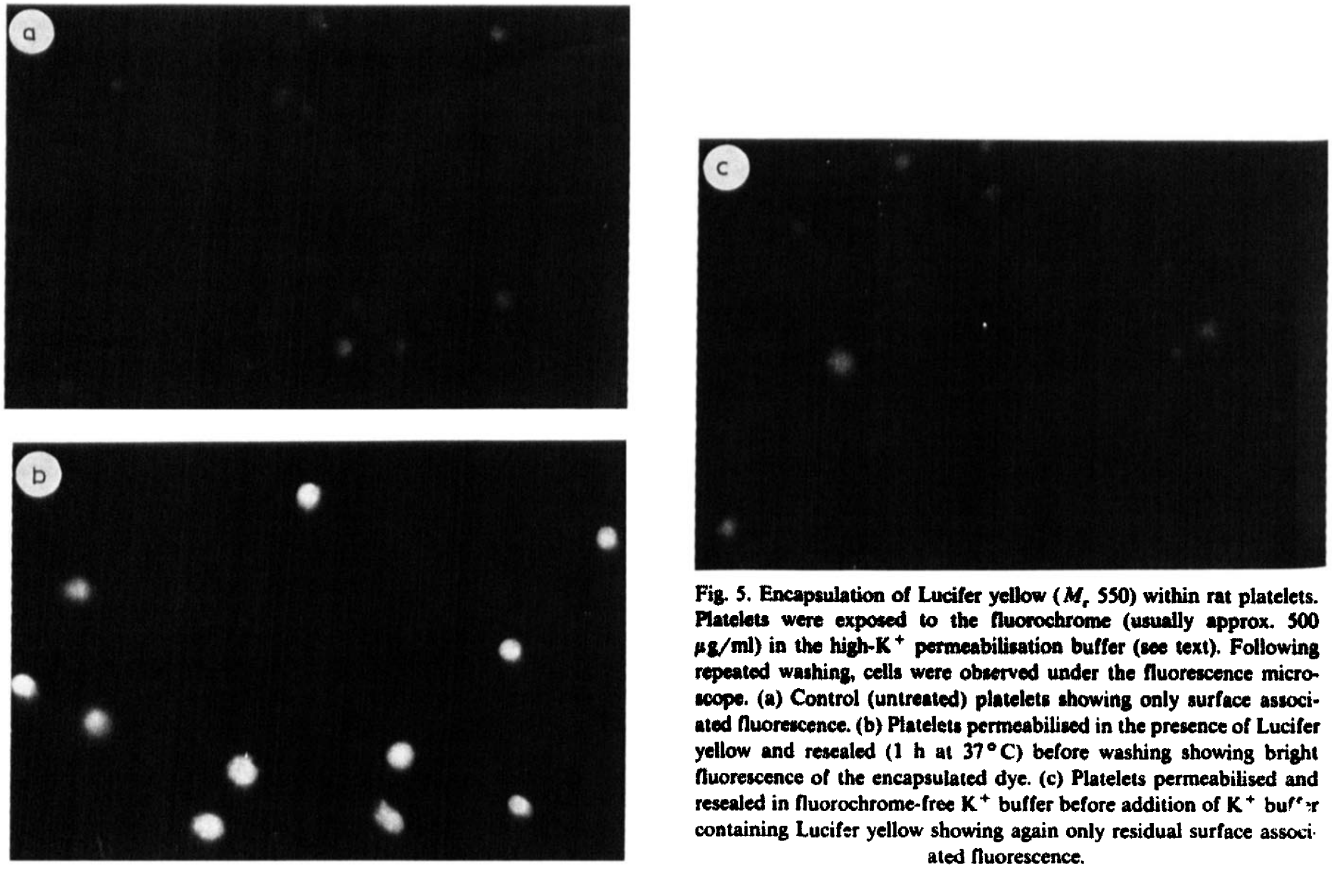


Fig. 5. Encapsulation of Lucifer yellow (M_r 550) within rat platelets. Platelets were exposed to the fluorochrome (usually approx. 500 μg/ml) in the high-K^+ permeabilisation buffer (see text). Following repeated washing, cells were observed under the fluorescence microscope. (a) Control (untreated) platelets showing only surface associated fluorescence. (b) Platelets permeabilised in the presence of Lucifer yellow and resealed (1 h at 37°C) before washing showing bright fluorescence of the encapsulated dye. (c) Platelets permeabilised and resealed in fluorochrome-free K^+ buffer before addition of K^+ buffer containing Lucifer yellow showing again only residual surface associated fluorescence.

fluorochrome/unit cell than was found for similarly washed control (non-permeabilised) platelets or platelets permeabilised and resealed before exposure to the fluorescent dye. Fluorescence microscopy and counting of these permeabilised and resealed platelets also revealed that on a mean basis > 85% of rat platelets and approaching 100% human platelets contained encapsulated Lucifer yellow (Fig. 5). Similar experiments were performed with [6,6′(n)-^{3}H]sucrose present in the high K^+ buffer during permeabilisation. After resealing and washing, these platelets had also internalised a significant amount of radiolabel when compared with untreated or permeabilised/resealed platelets similarly exposed (data not shown).

Both the permeabilised and resealed rat and human platelets (7 kV/cm) gave essentially normal aggregation responses to the agonists thrombin and collagen (Fig. 6), and also to the thromboxane A_2 mimetic U46619 (1.5 μM). However, with human platelets exposed to 10 kV/cm the dose-response curve shows marginally lower aggregation responses for collagen but full responses for thrombin. Platelet shape change was not observed in these studies, even with control unpermeabilised platelets, in contrast to cells permeabilised irreversibly with saponin [4].

It was also found that the capacity to transport adenine into the platelet from the external environment, was fully restored to the levels recorded for control (untreated cell) platelets after they have been resealed by incubation at 37 °C.

In an 'in vivo' study in which permeabilised and resealed platelets were reinfused into rats after labelling 'ex vivo' with [^{111}In]indium oxine it can be seen (Fig. 7) that although a proportion of the cells may be lost either at the injection site, by entering the splenic pool or be removed from the circulation immediately post-infusion by cells of the reticuloendothelial system, those that circulate show similar life span characteristics to the control untreated rat platelets similar labelled 'ex vivo' and infused. The half life ($t_{1/2}$) of the control untreated platelets labelled with ^{111}Indium was approx. 2.0 days and of the reversibly permeabilised platelets approx. 1.5 days when the [^{111}In]indium oxine counts are referenced to the samples taken 2 h post-infusion.

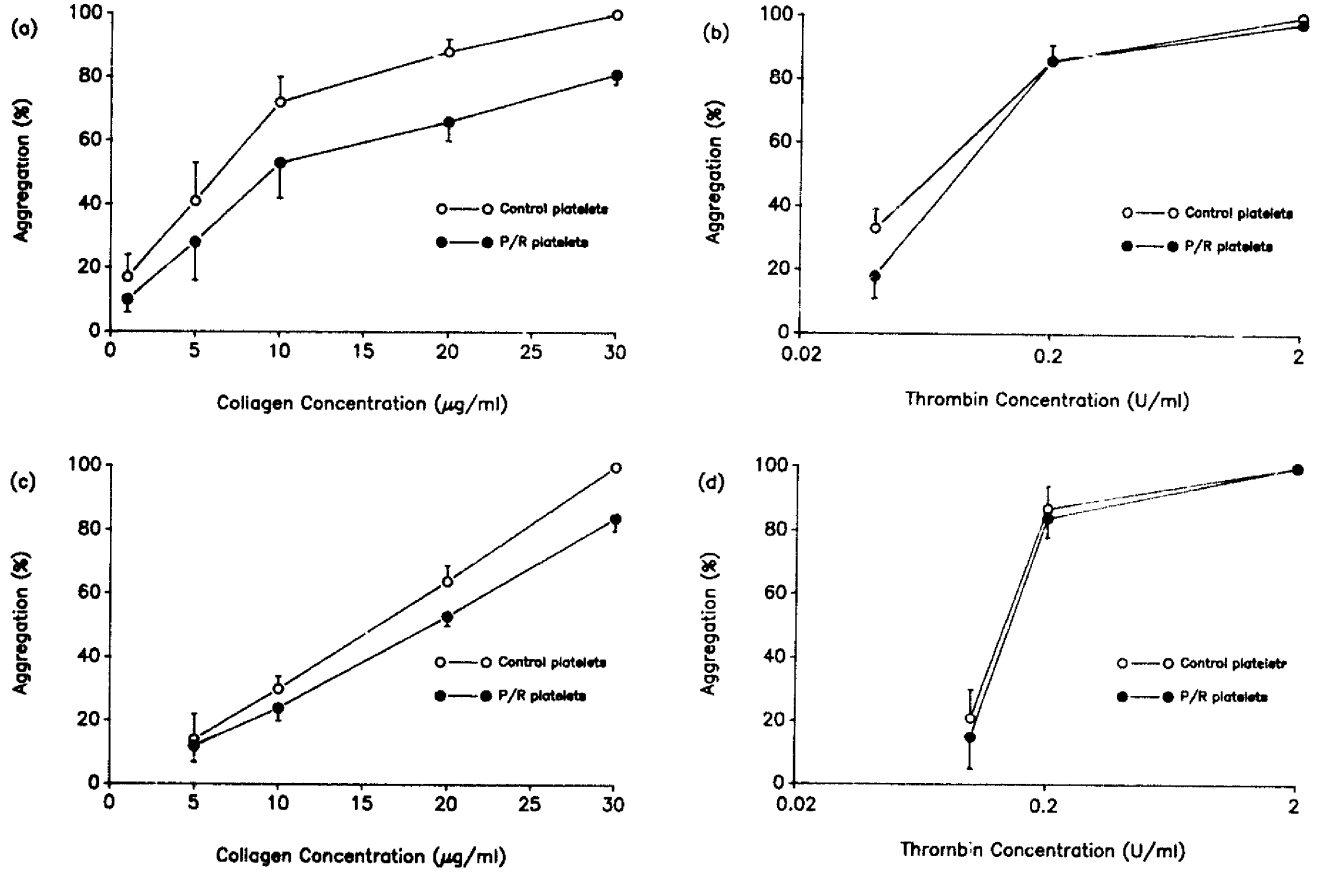


Fig. 6. Dose-response curves for collagen- and thrombin-induced aggregation responses in control (untreated) and permeabilised/resealed (P/R) platelets. (a,b) human platelets – resealed cells, permeabilised by seven discharge cycles at 10 kV/cm, showed aggregation responses for collagen that were slightly reduced below control values. With seven cycles at 7 kV/cm the responses for control and treated cells to both agonists gave essentially overlapping dose response curves. (c,d) rat platelets – resealed rat cells, permeabilised by seven cycles at 7 kV/cm, showed approaching normal aggregation with both collagen and thrombin. (means ± S.D., $n \geq 4$).

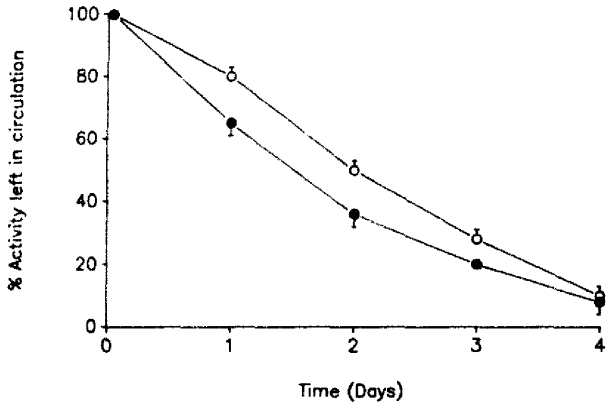


Fig. 7. Life span studies with control and permeabilised/resealed rat platelets labelled with [^{111}In]indium oxine 'ex vivo' and reinfused. The dpm for circulating ^{111}In measured in daily blood samples (approx. 200 μl) was expressed as a percentage of that recorded in samples taken 2 h post infusion. (●——●), P/R = permeabilised and resealed platelets. (○——○), control (untreated) platelets. Mean ± S.D., $n = 4$.

Discussion

With the apparatus we have developed, human and rat platelets can be reversibly permeabilised by a sequence of high-voltage discharges through a suspension of the cells held between two parallel platinum plates. The optimum poration voltage for human platelets is between 7 and 10 kV/cm and for rat platelets between 6 and 7 kV/cm. In both species 5–7 discharge cycles are adequate for maximum diffusability of low molecular weight species between the cytosolic compartment of the platelet and the external medium, as determined by the adenine nucleotide release studies. Though the pore size has not been determined precisely those formed under these conditions facilitate transfer of substances in the molecular mass range of at least 500–600 Da. The cytosol marker enzyme lactate dehydrogenase (M_r 140000) is substantially retained (3–4% loss) under these permeabilisation conditions indicating that minimal cell lysis occurs during discharge cycles.

Permeabilisation can be demonstrated by the accessing of the platelet cytosol to the Ca^{2+} releasing second messenger molecule IP_3. Preloading the cells with ^{45}Ca revealed that after permeabilisation substantial amounts of internally sequestered calcium can be mobilised by adding IP_3 to the external medium. Inositol 1,4,5-trisphosphate is an established second messenger molecule for a range of platelet functions which follow agonist/receptor interactions at the surface membrane. It is normally generated intracellularly and is responsible for mobilising Ca^{2+} from the platelet's dense tubular system, which must therefore remain intact and functional during the electropermeabilisation procedure. These membrane elements sequester Ca^{2+} by a ($Ca^{2+} + Mg^{2+}$)-ATPase-associated uptake pump analogous to similar processes in the endoplasmic reticulum of other cells. No such Ca^{2+} release occurs with control untreated platelets illustrating that membrane permeability to IP_3 is electrically induced in this system.

Under the electrical conditions we have described, a large proportion of both human and rat blood platelets become permeabilised to hydrated calcium ions in solution (we believe, in the light of other studies here described, approaching 100%), hence 5HT secretion is stimulated if free external Ca^{2+} is present at sufficient concentration (10 μM). After the last discharge cycle platelets of both species reseal during incubation at 37 °C and this can be followed by using the reduction in Ca^{2+}-induced 5HT secretion response with time. The permeabilisation and resealing protocols can also be more simply monitored by volume profiling with a resistive particle counter (Coulter) where freshly permeabilised cells show an 'apparent' upward shift in modal volumes when they are transferred immediately after high-voltage treatment into the Isoton II diluent. Monitoring of the resealing process at 37 °C is also possible as volume distributions become essentially normal with the modal volume being reestablished to exactly that of unpermeabilised controls. After extensive investigations we have as yet no satisfactory explanation for this phenomenon, however, it is sufficiently reliable to be used for routine monitoring of permeability and resealing with platelets treated under the experimental conditions described and it correlates well with the biochemical and functional evidence of both permeabilisation and restoration of membrane integrity.

From studies made after prelabelling the intact platelet nucleotide pool with [^{3}H]adenine it is clear that the pore size allows for free diffusion outward of these low molecular weight adenine metabolites to the external medium. However, the inclusion of ATP at 5 mM in the high-K^+ permeabilisation buffer during the voltage discharge sequences appears to be sufficient to maintain metabolic competence after resealing since the resealed platelets are responsive to conventional platelet agonists (thrombin, collagen and U46619) displaying both aggregation and induced-secretion of 5HT. After permeabilisation by high-voltage discharge (7–10 kV/cm human and 6–7 kV/cm rat) the membranes are able to reorganise and reseal without significantly effecting the receptors for these agonists or the signal transduction mechanisms associated with the receptors. Higher field strengths (15 kV/cm for human cells and > 8 kV/cm for rat cells) result in membrane breakdown beyond the capacity to reseal during incubation at 37 °C.

In these studies the penetration of Ca^{2+} (10 μM) into the cell has been shown to trigger the exocytotic release of a substantial amount of a prelabelled storage pool of 5HT (platelet dense bodies or 5HT granules). This would suggest that the capacity of the permeabilised

platelet plasma membrane and granule boundary membranes to participate in fusion events is not compromised by reversible electropermeabilisation.

The studies with the fluorescent dye Lucifer yellow show that encapsulation within human and rat platelets of low molecular weight species is possible. Quantitatively, encapsulation studies with this dye have revealed in fluorescence microscopy that > 85% rat platelets and approx. 100% human platelets could be permeabilised and resealed in order to encapsulate hydrophilic species of size greater than that of a nucleotide.

Perhaps one of the most sensitive procedures for assessing platelet integrity after 'ex vivo' manipulation is by studying their circulating life span characteristics after re-infusion. In the case of rat platelets, labelled with [^{111}In]indium oxine, those which circulate showed very similar life-span characteristics to those of untreated platelets similarly labelled and infused further indicating the high degree of integrity retained within a large population of platelets following high-voltage treatment.

In conclusion, the results presented in this paper illustrate that human and rat blood platelets can be reversibly electropermeabilised without significant loss in metabolic and functional potential. The size of the membrane pores formed suggests that free diffusion and encapsulation of molecular species to a minimum upper limit of 500–600 Da is possible.

Our view is that the use of the blood platelet as a fully biocompatible and biodegradable carrier vehicle for 'in vivo' delivery of drugs and other agents may have considerable potential. The preservation of cellular integrity throughout the encapsulation procedures might also prolong the action of internalised agents beyond that normally possible with liposome transport systems.

Acknowledgements

We are grateful to the Imperial Cancer Research Fund for their generosity in supporting this programme. One of us (K.H.) is a recipient of an I.C.R.F. Bursarship award. We are grateful to Dr. C. Mattock for many fruitful discussions concerning the design and operation of the apparatus, to Dr. T.E. Bates for his advice and assistance with the platelet lifetime studies and we also thank Miss Heather Watson for her kindness and patience in the preparation of this manuscript.

References

1 Ihler, G.M. (1983) Pharmacol. Therap. 20, 151–157.
2 Pitt, E., Johnson, C.M., Lewis, D.A., Jenner, D.A. and Offord, R.E. (1983) Biochem. Pharmacol. 32, 3359–3368.
3 Hirata, M., Suematsu, E., Hashimoto, T., Hamachi, T. and Koga, T. (1984) Biochem. J. 223, 229–236.
4 Authi, K.S., Evenden, B.J. and Crawford, N. (1986) Biochem. J. 233, 709–718.
5 Purdon, A.D., Daniel, J.L., Stewart, G.J. and Holmsen, H. (1984) Biochim. Biophys. Acta 800, 178–187.
6 Impraim, C.C., Foster, K.A., Micklem, K.J. and Pasternak, C.A. (1980) Biochem. J. 186, 847–860.
7 McClennan, G.B. and Winegrad, S. (1978) J. Gen. Physiol. 72, 737–764.
8 Cockroft, S. and Gomperts, B.D. (1979) Nature (Lond.) 279, 541–542.
9 Miller, M.R., Castellot, J.J. and Pardee, A.B. (1978) Biochemistry 17, 1073–1080.
10 Mire, A.R., Wickremasinghe, G., Michalevicz, R. and Hoffbrand, A.V. (1985) Biochim. Biophys. Acta 847, 159–163.
11 Zimmermann, U. (1983) in Targeted Drugs (Goldberg, E.P., ed.), Wiley, New York.
12 Lewis, D.A. and Alphar, H.O. (1984) Int. J. Pharm. 22, 137–146.
13 Bradshaw, H.D., Parson, W.W., Sheffer, M., Lioubin, P.J., Mulvihill, E.R. and Gordon, M.P. (1987) Anal. Biochem. 166, 342–348.
14 Pitt, E., Johnson, C.M. and Lewis, D.A. (1983) Biochem. Pharmacol. 32, 3359–3368.
15 Pitt, E., Lewis, D.A. and Offord, R.E. (1983) Biochem. Pharmacol. 32, 3355–3358.
16 Zimmermann, U., Pilwatt, G., Holzapfel, C. and Rosenheck, K. (1976) J. Membr. Biol. 30, 135–152.
17 Knight, D.E. (1981) in Techniques in Cellular Physiology (Baker, P.F., ed.), P113, pp. 1–20, Elsevier/North-Holland Biomedical Press, Amsterdam.
18 Knight, D.E. and Scrutton, M.C. (1986) Biochem. J. 234, 497–506.
19 Neumann, E. and Rosenheck, K. (1972) J. Membr. Biol. 10, 279–290.
20 Scrutton, M.C., Knight, D.E. and Niggli, V. (1985) Adv. Biol. Med. 192, 171–183.
21 Haslam, R.J. and Davidson, M.M.L. (1984) Biochem. J. 222, 351–361.
22 Baker, P.F. and Knight, D.E. (1978) J. Physiol. (Lond.) 284, 30p.
23 Baker, P.F. and Knight, D.E. (1981) Philos. Trans. R. Soc. (London Ser. B) 296, 83–103.
24 Haslam, R.J. and Davidson, M.M.L. (1984) J. Recept. Res. 4, 605–619.
25 Haslam, R.J. and Davidson, M.M.L. (1984) FEBS Lett. 174, 90–95.
26 Jonah, M.M., Cerny, E.A. and Rahman, Y.E. (1975) Biochim. Biophys. Acta 401, 336–348.
27 Djerassi, I. (1966) Transfusion 6, 55–61.
28 Hughes, K., Mattock, C. and Crawford, N. (1989) unpublished observations.
29 Lagarde, M., Byron, P.A., Guichardant, M. and Dechavanne, M. (1980) Thromb. Res. 17, 581–588.
30 Wroblewski, F. and La Due, J.J. (1955) Proc. Soc. Exp. Biol. Med. 90, 210–213.
31 Knight, D.E. and Scrutton, M.C. (1980) Thromb. Res. 20, 437–446.
32 Mir, L.M., Banoun, H. and Paoletti, C. (1988) Expt. Cell Res. 175, 15–25.
33 Brass, L.F. (1984) J. Biol. Chem. 259, 12563–12570.
34 Hawker, R.J., Hawker, L.M. and Wilkinson, A.R. (1980) Clin. Sci. 58, 243–248.