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Intracellular function in rehydrated lyophilized platelets

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Summary. This study aimed to evaluate the effect of cross-linking and lyophilization on intracellular signalling processes in rehydrated, lyophilized (RL) platelets, which are under development as a platelet substitute for transfusion. Exposure of RL platelets to thrombin resulted in enhanced phosphorylation of several proteins, including 18 kDa and 42 kDa kinase substrates that were shown to be the substrates of myosin light chain and protein kinase C respectively. Cross-linking and lyophilization depleted the platelets of free cytoplasmic ADP and ATP, but had less effect on protein-bound nucleotides. The surface membrane of RL platelets was found to be permeable to poly dT probes less than approximately 3 kDa in size; larger nucleotide probes and proteins did not penetrate the surface membrane. Taken together, our results indicate that RL platelets retain some of the haemostatic stimulus-response functions of fresh platelets and are capable of feedback amplification in coagulation.

Keywords: platelets, lyophilization, signalling, kinase, transfusion.

Platelets play a central role in mediating haemostasis; thrombocytopenia and thrombasthenia lead to prolonged bleeding times and bleeding events that require transfusions with donor platelets. Currently, platelets can only be stored for up to 5 d because of microbial contamination and partial loss of viability (see, for example, Read *et al*, 1995 for a discussion of these issues). We have recently discovered and refined a method for lyophilizing platelets for long-term storage, thus circumventing many of the problems of stocking fresh platelets (Read *et al*, 1993,1995). This method is based on the covalent cross-linking of surface membrane proteins and lipids to stabilize membrane structures for freezing, lyophilization and rehydration.

Rehydrated, lyophilized (RL) platelets retain a near normal ultrastructure by electron microscopy and have many of the surface membrane functions of fresh platelets (Read *et al*, 1995). RL platelets adhere to denuded subendothelium, spread on foreign surfaces (Read *et al*, 1995) and the glycoprotein (GP) IIa–IIIb complex binds fibrinogen (Sanders *et al*, 1996). Also, RL platelets support *in vitro* clot lysis by tissue plasminogen activator (Sanders *et al*, 1996). *In vivo*, RL platelets decrease bleeding times in thrombocytopenic rats and participate in thrombus formation in a canine model system (Read *et al*, 1995). Based on studies in which platelets were inoculated with several viruses (including HIV and parvovirus) and strains of bacteria, the cross-linking, freezing and lyophilization process for the preparation of RL platelets yields a transfusion product that is measurably free of bacterial and viral contamination (Bode *et al*, 1997).

The experiments presented here answer the question of whether RL platelets function only as circulating thrombogenic membranes or if the cells are capable of full, or some degree of, stimulation in response to activating agonists. Stimulus-response coupling in platelets involves the activation of surface membrane receptors, including the integrin IIb–IIIa complex, the factor (F)IX-GPIb complex, trimeric G-protein coupling receptors such as the thrombin receptor and, to a lesser extent, receptor tyrosine kinases (see Siess, 1989 for a review). Activated surface membrane receptors directly or indirectly stimulate intracellular protein kinases that, in turn, transfer the terminal phosphate from ATP to specific protein substrates. Phosphorylated protein kinase substrates then play a major role in orchestrating cellular responses such as shape change, secretion and clot retraction. Two key players in platelet stimulus-response coupling are myosin light-chain kinase (MLCK) and protein kinase C (PKC). The thrombin receptor indirectly stimulates both these kinases, then myosin light chain is phosphorlated by MLCK (Walsh *et al*, 1979) and phosphate is attached to pleckstrin by PKC (Brass *et al*, 1997). Phosphorylation of myosin light-chain kinase mediates the actomyosin-based

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force generation that drives granule centralization and clot retraction, while phosphate attachment to pleckstrin leads to an association of this protein with membrane projections, where it potentially regulates PIP_2 generation (Abrams *et al*, 1996). We examined stimulus-response coupling in RL platelets by determining the extent to which MLCK and PKC are activated in response to thrombin, the degree to which cross-linking and lyophilization depleted the cytoplasmic pool of ATP, and the permeability of the surface membrane to ATP and protein components of the kinase signalling system. Experiments were performed to determine if RL platelets have MLCK and PKC that are capable of activation, to determine the levels of the cytoplasmic energy source, ATP, and to determine if the surface membrane was permeable or impermeable to ATP and cytoplasmic protein components.

MATERIALS AND METHODS

Preparation of radiolabelled fresh and RL platelets. Human platelet concentrates (3 d old) were obtained from the American Red Cross. Platelets were centrifuged (15 min at 3000 ***g***) once with citrated saline (5·4 mmol/l of trisodium citrate, 146 mmol/l of NaCl, pH 6·8), then incubated for 30 min at 37°C in citrated saline with 29·6 MBq of ^{32}Pi (Amersham) at $3{\cdot}2 \times 10^8$ cells/ml. The cells were then washed with centrifugation (15 min at 3000 ***g***) with citrated saline. Platelets were used fresh or were cross-linked, lyophilized, stored at −80°C for 2 d and then rehydrated in the original volume of H_20 (Read *et al*, 1995).

Activation of RL platelets. Activation studies were performed with RL platelets after 2 d of storage. Fresh platelets were analysed immediately after radiolabelling. RL and fresh platelets were suspended at a concentration of 8×10^6 cells/ml in citrated saline with 1 mmol/l of $CaCl_2$ and incubated with stirring at 37°C. Thrombin (1000 units/ml) or an equal volume of citrated saline was added to RL and fresh platelets for a final thrombin concentration of 10 or 0 units/ml. After 15 min, cells were pelleted by centrifugation at 10 000 ***g*** for 15 s, suspended in ice-cold citrated saline with 1 mmol/l of para-nitrophenol, 1 mmol/l of sodium orthovanidate and 1% Triton X-100 at 4×10^7 cells/ml, and immediately frozen at −80°C until electrophoresis.

Electrophoresis, Western analysis and autoradiography. Isoelectric focusing utilized a 10% pH 3–10, 90% 5–7 ampholine mixture according to previously described methods (O'Farrell, 1975). Sodium dodecyl polyacrylamide gel electrophoresis (SDS-PAGE) electrophoresis was performed in 10% polyacrylamide gel (Laemmli, 1970). Western analysis was performed (Tobin *et al*, 1979) with the anti-phosphotyrosine antibody 4G10 (Upstate Biotechnologies, Lake Placid, NY, USA), anti-myosin light-chain antibody MY-21 (Sigma Chemical Co, St. Louis, MO, USA) or anti-pleckstin antisera 352 (kindly provided by Dr Charles Abrams, University of Pennsylvania, USA). Chemiluminescence was used to detect secondary antibodies with an ECL kit from Amersham Life Sciences (UK).

Analysis of cytoplasmic nucleotide pool in RL platelets. Fresh platelets were radiolabelled with ^{32}Pi, cross-linked and lyophilized as described above. Cytoplasmic levels of ATP and ADP were measured with polyethyleneimine thin layer chromatography (TLC). Ethanol solubility was utilized to determine the ratio of free vs. protein-bound nucleotides. The intracellular nucleotide pool was examined before cross-linking, after cross-linking, and after lyophilization and rehydration. With each sample, 8×10^7 cells were centrifuged and the pellet was suspended in 20 μl of H_2O. TLC analysis and sample preparation utilized the procedure of Crabtree & Henderson (1971). An aliquot (10 μl) of each pellet resuspension was mixed with 10 μl of 0·42 mol/l perchloric acid. After incubating for 30 min at 25°C, the acid was neutralized by adding KOH to 0·21 mol/l and then the samples were spotted on a polyethyleneimine cellulose TLC plate. TLC plates were developed with 2 mol/l sodium formate, pH 3·4, and then autoradiography was performed. ATP, ADP and monophosphate spots were scraped and subjected to liquid scintillation counting to measure cytoplasmic levels of the nucleotide. Quantification of free and protein bound cytoplasmic nucleotides utilized the method of Holmsen (1972); 10 μl of each platelet pellet (4×10^7 cells) was diluted to 100 μl with ethanol and then centrifuged at 10,000 ***g*** for 5 min. The pellet and supernatant were subjected to liquid scintillation counting to measure protein-bound and unbound radiolabelled compounds respectively.

Surface membrane permeability to fluorescent probes in RL platelets. A series of 3′ fluoroscein isothiocyanate (FITC)-labelled poly-dT oligonucleotides were synthesized (UNC Department of Pathology synthesis facility) with the following molecular masses: dT-FITC, 0·640 kDa; dT_2-FITC, 0·949 kDa; dT_5-FITC, 1·84 kDa; dT_7-FITC, 2·474 kDa; dT_{11}-FITC, 3·70 kDa; dT_{17}-FITC, 5·53 kDa; dT_{29}-FITC, 9·21 kDa; dT_{44}-FITC, 13·80 kDa. Fresh and RL platelets at 8×10^8 cells/ul in citrated saline were incubated for 5 min with 1 μmol/l of each poly dT-FITC probe. Penetration of the probes into the cytoplasm was ascertained by performing fluorescence microscopy at 60× magnification with oil immersion. Probes were judged as impermeable if the platelet interior was not fluorescent.

Surface membrane permeability of lactate dehydrogenase. RL and fresh platelets were prepared as detailed previously in this section, then incubated at 8×10^7 cells/ml in citrated saline for 10 min at 37°C. Part of the sample was centrifuged at 100 000 ***g*** for 1 h. The supernatants were retained and the pellets were resuspended in the original volume of citrated saline or citrated saline with 100 mmol/l octyglucoside. Samples were subjected to lactate dehydrogenase analysis as described by Fischer *et al* (1987).

RESULTS

We performed two types of experiments to determine the effect of cross-linking and lyophilization on intracellular functions in platelets. First, activation-dependent protein kinase activities were compared in RL and fresh platelets. Fresh platelets were incubated with ^{32}Pi to radiolabel the cytoplasmic ATP pool. Half of the ^{32}Pi-labelled cells were used immediately for the activation experiments as a 'fresh platelet' control. The other half of the radiolabelled platelets

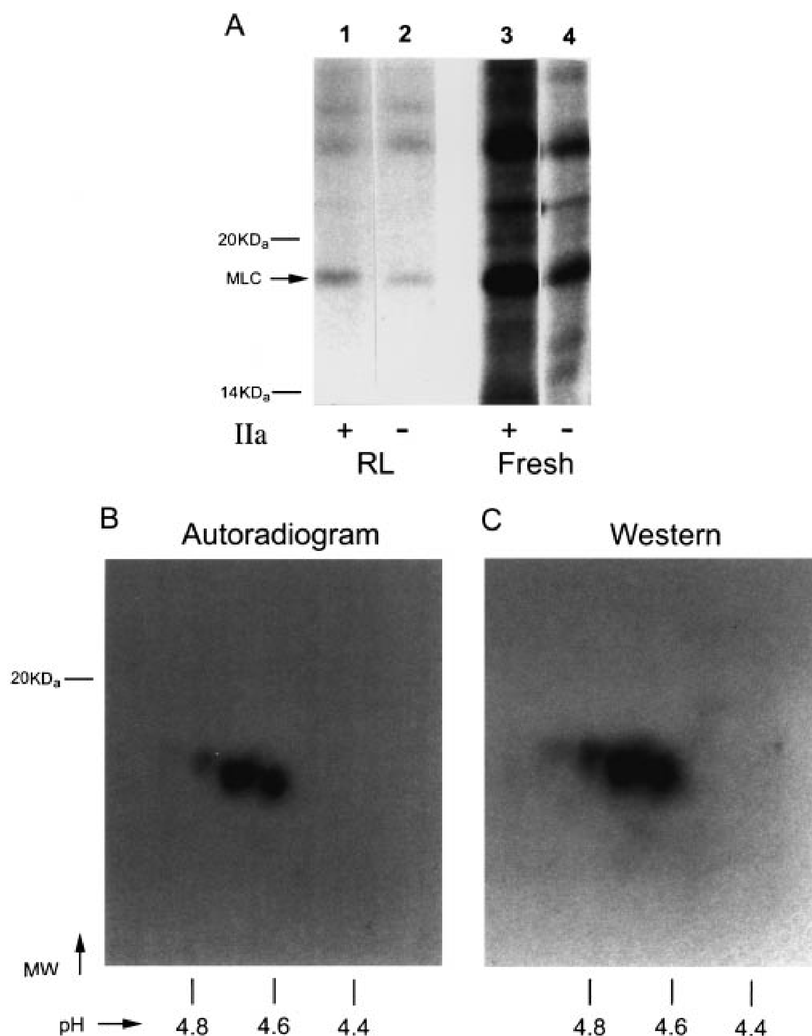

Fig 1. Phosphorylation of myosin light chain in RL platelets. A. Autoradiogram after SDS–PAGE electrophoresis of fresh and RL platelets. B. Autoradiogram after 2D-IEF SDS–PAGE. C. Western analysis with anti-myosin light-chain monoclonal antibody of the same membrane as in B.

were cross-linked and lyophilized. After two days of storage at −80°C, platelets were rehydrated and activation experiments were performed with the same procedure as with the fresh control platelets. RL and fresh platelets were treated with thrombin or control buffer, stirring at 37°C, and then quenched with ice-cold citrated saline with detergent and phosphatase inhibitors for electrophoresis. Activation-dependent changes in the activities of protein kinase C and myosin light-chain kinase were measured by two-dimensional isoelectrofocusing (2D-IEF) SDS–PAGE, autoradiography and Western analysis to detect phosphate transfer to protein substrates. Figure 1A demonstrates that, as with fresh platelets, thrombin activation of RL platelets resulted in enhanced phosphorylation of a 18 kDa protein. Two-dimensional isoelectric focusing SDS–PAGE showed better resolution of the 18 kDa phosphoprotein (Fig 1B), which was identified with Western analysis as myosin light chain (Fig 1C). A comparison of the intensity of the autoradiographic myosin light chain spots indicated that, in response to thrombin, myosin light chain phosphorylation was reduced in RL platelets when compared with fresh platelets. The autoradiographic density at the top of the gel lanes for RL platelets (Fig 1A) showed that a considerable portion of the phosphoproteins was cross-linked into random high molecular weight complexes that did not enter the gel. Figure 2A shows that an 42 kDa protein was also phosphorylated in an activation-dependent manner in RL platelets, although to a reduced extent when compared with fresh cells. Three isoforms of the 42 kDa phosphoprotein were resolved with 2D-IEF SDS-PAGE (Fig 2B). The identity of the 42 kDa phosphoproteins as pleckstrin isoforms was confirmed by Western blot analysis (Fig 2C).

The reduced energy-dependent phosphorylation observed with RL when compared with fresh platelets raises the hypothesis that the nucleotide pool is reduced by cross-linking and lyophilization procedures. To test this hypothesis, fresh platelets were incubated with ^{32}Pi to radiolabel the cytoplasmic nucleotide pool, cross-linked, lyophilized and rehydrated. Portions of the platelets were subjected to TLC analysis before cross-linking (fresh platelets), after cross-linking (cross-linked platelets), and after lyophilization and rehydration (RL platelets). Figure 3 shows that the cytoplasmic pool of ADP, ATP and monophosphates was dramatically depleted when compared with fresh platelets.

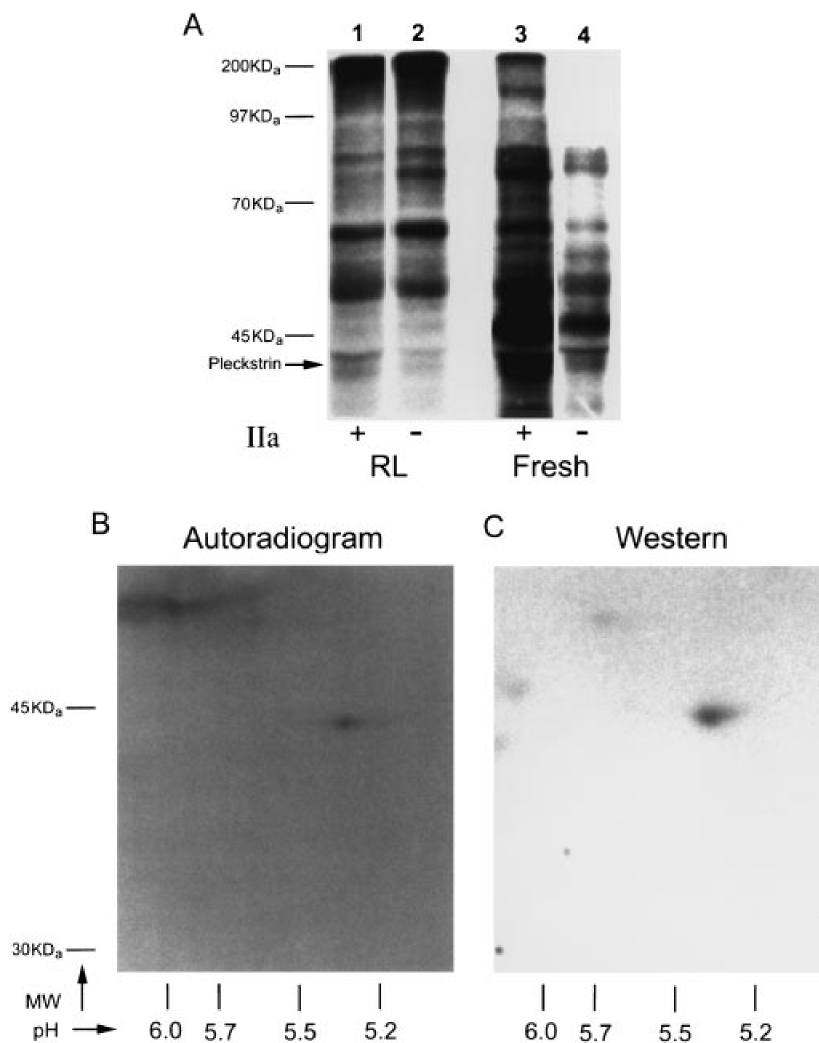

Fig 2. Phosphorylation of pleckstrin in RL platelets. A. Autoradiogram after SDS–PAGE electrophoresis of fresh and RL platelets. B. Autoradiogram after 2D-IEF, SDS–PAGE. C. Western analysis with anti-pleckstrin monoclonal antibody of the same membrane as in B.

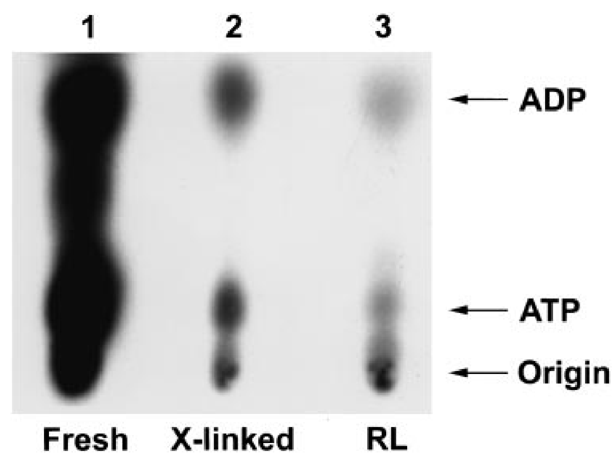

Fig 3. Nucleotides in RL platelets. Polyethylenimine cellulose TLC and autoradiography before cross-linking (fresh platelets), after cross-linking (X-linked platelets) and after lyophilization and rehydration (RL platelets).

Quantification (see Table I) of the radioactivity of the resolved phosphate compounds indicated that, after cross-linking, the cytoplasm was largely depleted of nucleotides. The ethanol insolubility of the ATP and ADP that remained in cross-linked and RL platelets indicated that this pool of nucleotide was bound to proteins.

Depletion of cytoplasmic ATP and ADP in RL platelets

Table I. Effect of cross-linking and lyophilization on cytoplasmic nucleotides.

	Fresh	X-link	RL
ATP	100%	9%	3%
ADP	100%	14%	4%
Monophosphates	100%	1%	< 0·5%
Pi	100%	< 0·5%	< 0·5%

Nucleotide levels were normalized to the values for fresh cells. Detection limit was 0·5%.

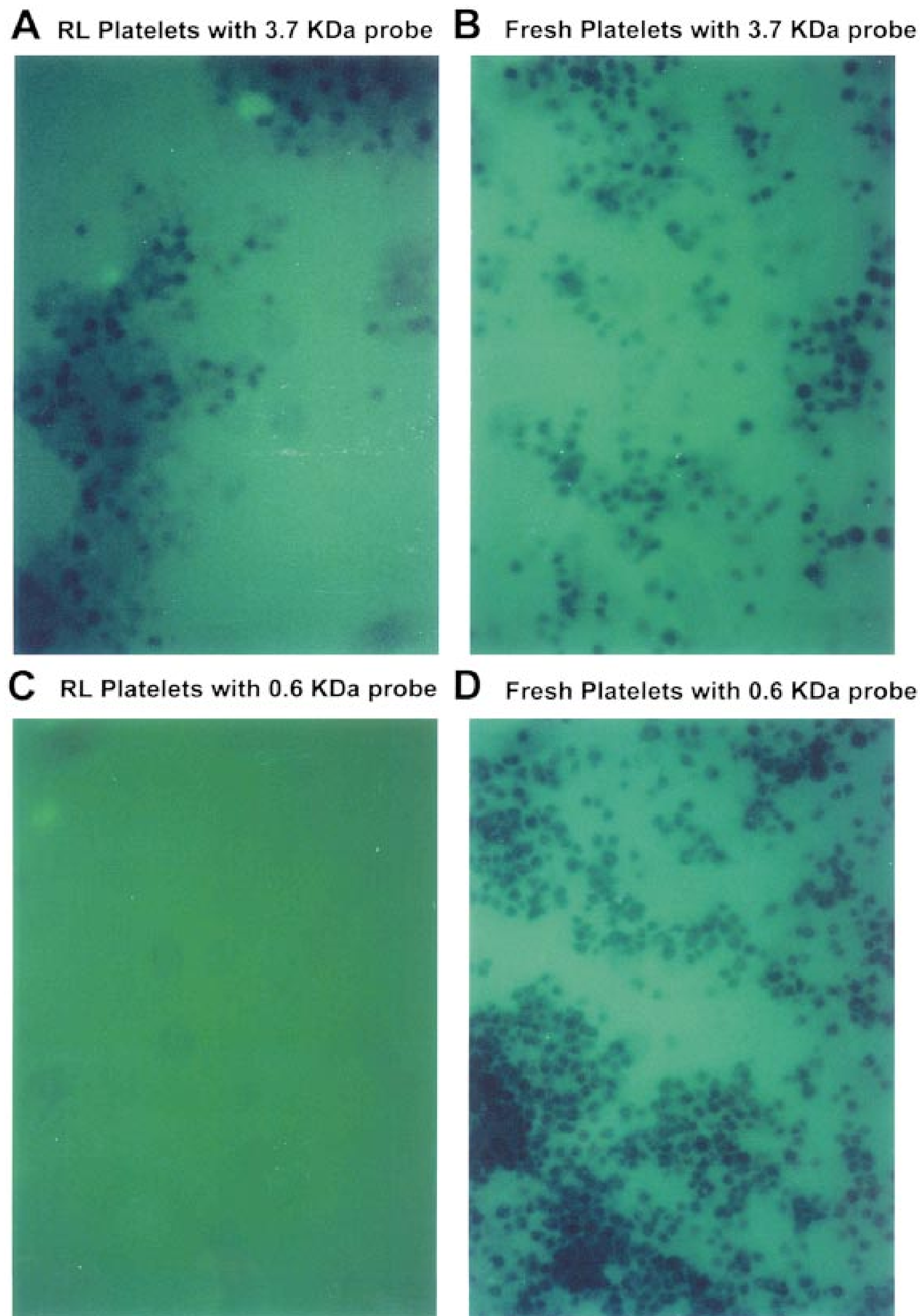

Fig 4. Integrity of the surface membrane. Permeability of surface membrane of fresh and RL platelets to 3·7 kDa and 0·6 kDa FITC-oligo-dT size probes.

indicates that the cross-linking and/or lyophilization procedures permeabilize the surface membrane. We tested this hypothesis by performing two types of experiments that characterize the permeability of the surface membrane. First, we determined the permeability of the plasma membrane of RL platelets to a series of FITC poly-dT oligonucleotides with molecular masses from 0·64 kDa to 13·8 kDa. Each probe was added to suspensions of unactivated RL and fresh platelets, and cells were examined with fluorescent microscopy. Cells that are impermeable to the fluorescent probes appear dark against a fluorescent background, while permeable cells have a fluorescent cytoplasm that does not contrast with the fluorescent extracellular medium. Figure 4 demonstrates that the plasma membranes of RL, but not fresh, platelets were permeable to the 0·64 kDa probe. The surface membranes of RL and fresh platelets were impermeable to the larger 3·7 kDa probe. The FITC poly-dT nucleotides with molecular masses up to

Table II. Permeability of the plasma membrane of RL and fresh platelets to size probes.

Probe	Molecular mass (kDa)	RL platelets	Fresh platelets
dT-FITC	0·64	+	–
dT_2-FITC	0·95	+	–
dT_5-FITC	1·84	+	–
dT_7-FITC	2·47	+	–
dT_{11}-FITC	3·70	–	–
dT_{17}-FITC	5·53	–	–
dT_{29}-FITC	9·21	–	–
dT_{44}-FITC	13·80	–	–

2·47 kDa penetrated the surface membrane into the cytoplasm, while the surface membrane of fresh platelets was impermeable to all probes (Table II).

The permeability of the surface membrane was also examined by determining if intracellular lactate dehydrogenase leaked into the extracellular space. RL and fresh platelets were centrifuged. Starting samples, pellets and supernatants were subjected to lactate dehydrogenase analysis. No lactate dehydrogenase activity was measured in the supernatants of intact RL and fresh platelets without permeabilizing with octylglucoside, while the octylglucoside-treated RL and fresh cells reduced NAD^+ (Table III). This result showed that the surface membrane of RL platelets was impermeable to lactate dehydrogenase.

DISCUSSION

Our results show that intracellular processes were partially operational in RL platelets. The finding that the protein kinase C substrate pleckstrin is phosphorylated upon treatment of RL platelets with thrombin indicates that the thrombin receptor is able to activate the phospholipases and/or calcium functions that lead to protein kinase C stimulation (see, for example, Brass *et al*, 1997). Phosphorylated pleckstrin associates with and promotes the formation of membrane ruffles and projections (Me *et al*, 1997). Also, phosphorylated pleckstrin appears to play a negative-feedback role in the platelet activation process by inhibiting phosphoinositide 3-kinase (Abrams *et al*, 1996). Our finding that pleckstrin is phosphorylated in response to thrombin indicates that these pathways were at least partially functional in RL platelets. Myosin light-chain kinase is activated when it forms a complex with calcium/calmodulin (Walsh *et al*, 1979). Also, the formation of the calmodulin-myosin light-chain kinase complex is stabilized when myosin light-chain kinase is phosphorylated by ERK 1/2 (Klemke *et al*, 1997). Thus, the phosphorylation of myosin light chain in thrombin-activated RL platelets indicates that calcium/calmodulin and MAP kinase signalling pathways were preserved in part in these cells. The result of myosin light-chain kinase activation is the phosphorylation of myosin light chain and the induction of actomyosin force generation (Lebowitz & Cooke, 1978).

Table III. Lactate dehydrogenase permeability of RL platelets.

Supernatants	Intact cells	Permeabilized cells
RL	$< 0{\cdot}2\%$	100%
Fresh	$< 0{\cdot}2\%$	100%

Lactate dehydrogenase activities were normalized to the values for permeablized (octylglucoside-treated) whole cells. Detection limit was 0·2%.

Surface integrins on RL platelets bind and internalize fibrinogen (Sanders *et al*, 1996; Merricks *et al*, 1998). Fibrinogen internalization requires integrin clustering (Hato *et al*, 1998) and can involve endocytotic transport into alpha granules (James *et al*, 1977; Harrison *et al*, 1989). These processes are mediated by the actin-based cytoskeleton and require energy-dependent actomyosin force generation (James *et al*, 1977). The results we report, when considered with previous findings that RL platelets extend pseudopodia and centralize secretory granules, provide evidence that intracellular processes involving F-actin and actomyosin force generation are partially operational in RL platelets.

Intracellular kinase signalling and actomyosin-based force generation are ATP-dependent processes. We found that RL platelets were largely depleted of free cytoplasmic ATP and ADP (as judged as ethanol-soluble nucleotides) and contained only a fraction of the amount of protein-bound (perchlorate-soluble) nucleotides (Holmsen, 1972). This contrasts with fresh platelets, in which approximately half of the ATP is protein bound (Holmsen, 1972). The residual protein-bound ATP in RL platelets is evidently able to support the partial protein kinase activities that we report. Our permeability studies with fluorescent poly dT probes and lactate dehydrogenase showed that the surface membrane of RL platelets was permeable to molecules with molecular masses less than approximately 3 kDa, suggesting that cytoplasmic ATP diffuses out of the cells. However, our studies showed that the surface membrane of RL platelets was impermeable to larger poly dT probes, as well as nucleoside-binding proteins such as kinases, actin and myosin. The finding that surface membrane nucleotide permeability is associated with the cross-linking suggests that chemical modification of lipids and/or surface membrane proteins by the cross-linker disrupts the bilayer structure so as to form permanent or transient pores. While Figure 3 qualitatively indicates that our preparation is approximately homogenous with respect to fluorescent probe permeability, the possibility exists that subpopulations with different size pores exist. The chemical modification by the cross-linker of surface membrane lipids and integral proteins might disrupt the bilayer structure by increasing the curvature of the surface membrane. Alternatively, cross-linking of proteins and/or lipids might interfere with the packing of hydrocarbon lipid moieties and hydrophobic peptide side-chains in the bilayer interior. These results help explain why signalling in, and the activation response of, RL

platelets is diminished, and provides a rational basis for preserving and/or recovering native functions of these cells.

The activation of intracellular kinases in RL platelets demonstrates that these cells are capable of intracellular stimulus-response signalling and, thus, do more than simply serve as a thrombogenic membrane. The potential consequence of intracellular signalling is the capability for providing positive-feedback amplification during thrombus formation. Our finding that RL platelets both degranulate and increase surface membrane thrombogenicity in an activation-dependent manner provides evidence for intracellular signalling and positive feedback (unpublished observations). Degranulation in platelets is the end-point of intracellular signalling events that involve protein kinase C activation (Elzagallaai *et al*, 2000), actin reorganization (Marcu *et al*, 1996) and the assembly of VAMP-SNAP-syntaxin membrane fusion complexes (Reed *et al*, 1999; Chen, 2000). Increases in surface membrane thrombogenicity occur when an activation-dependent intracellular calcium signal stimulates 'scrambalase' activities that flip phosphatidylserine from the inner to the outer surface of the platelet (Bevers *et al*, 1996). The demonstration of partial stimulus-response coupling with RL platelets distinguishes these cells from other platelet substitutes, including thromboerythrocytes (Coller *et al*, 1991), plateletsomes (Rybak & Renzulli, 1993), infusible platelet membranes (Chao *et al*, 1996), synthocytes (Middleton *et al*, 1998) and fibrinogen-coated microcapsules (Levi *et al*, 1999). Because the thrombogenicity of the surface of platelet substitutes (other that RL platelets) are approximately static, these substitutes are not capable of providing positive-feedback amplification in coagulation responses, as do native platelets.

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