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Stimulation of human platelets by carrageenans

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The rank order of four carrageenans tested as inducers of human platelet aggregation was the same (iota > lambda > gelcarin > kappa) as their relative inflammatory potencies in vivo. All four carrageenans caused some precipitation of plasma proteins, and induced aggregation in platelet-rich plasma or washed platelet suspensions. The second phase of aggregation was citrate-dependent and associated with secretion of 5-hydroxytryptamine and lysosomal enzymes. Platelets could provide a useful model for investigating the actions of carrageenans on cell membranes.

Carrageenans are sulphated polysaccharides that exhibit a wide range of biological actions (Di Rosa 1972). They are known particularly for their inflammatory activity which has led to their use in models of acute and chronic inflammation (Robertson & Schwartz 1953; Winter et al 1962). The mechanisms responsible for carrageenan-induced inflammation are not fully understood, although it has been suggested that they induce secretion of mediators such as histamine, 5-hydroxytryptamine and prostaglandins (Vinegar et al 1976) from inflammatory cells, and activate production of bradykinin (Noordhoek et al 1977) and complement (Di Rosa et al 1971).

Carrageenans induce platelet secretion and aggregation in rabbits and Vargaftig & Lefort (1977) suggested that this might be responsible for the hypotension which follows intravenous injection of carrageenans. The effects of carrageenans on platelets may contribute in some measure to their inflammatory activity: platelets contain and release several inflammatory mediators, although their role in inflammation is usually minor and restricted to the intravascular events (Gordon 1979). However, more important than the contribution of platelets in inflammation is their value as models for investigating the mechanisms by which stimuli induce the secretion of cellular constituents (MacIntyre 1976). We have used suspensions of platelets in vitro to study aggregation and secretion induced by carrageenans.

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Different carrageenans vary in their inflammatory and hypotensive potency (Di Rosa 1972) but the reasons for this are not clear and as yet no comparative investigation on isolated cells has been carried out. In the present study we have compared the activity of four carrageenan preparations as inducers of platelet aggregation and secretion, and have used potential pharmacological inhibitors to investigate some of the mechanisms responsible for the effects of carrageenans.

MATERIALS AND METHODS

Materials

Iota, lambda, gelcarin and kappa carrageenans were obtained from Marine Colloids Ltd., U.S.A. and were dissolved in phosphate buffered saline at 60 °C. The following compounds were dissolved in saline at room temperature: aspirin (acetylsalicylic acid powder), from Addenbrookes' Hospital Pharmacy, Cambridge, U.K.; aminophylline and quinacrine, from Sigma Chemical Co., Kingston-upon-Thames, U.K.; EDTA, from BDH Chemicals Ltd., Poole, U.K. Prostaglandin E_1, a gift from Dr J. E. Pike, Upjohn Co. Kalamazoo, MI, U.S.A. was prepared as previously described (MacIntyre & Gordon 1975). 5-Hydroxy [side chain-2-^{14}C]tryptamine creatinine sulphate([^{14}C]-5-HT), 58 mCi mmol^{-1}, and [8-^{3}H]-adenine ([^{3}H]adenine), 24 Ci mmol^{-1}, were obtained from The Radiochemical Centre, Amersham, U.K.

Methods

Platelet aggregation was measured photometrically (Born 1962) in 0·1 ml samples of human platelet-rich plasma (PRP) as previously described (Gordon & Drummond 1974). Plasma-free platelet suspensions in Tyrode buffer were prepared from citrated PRP by gel filtration on Sepharose 2B (Salzman et al 1976).

Cell free plasma was prepared by centrifugation (1000 g; 10 min; 20° C) of PRP.

For measurement of secretion, PRP was pre-incubated at 37° C for 30 min with 1 μM [^{14}C]-5-HT (which is incorporated into the platelet dense granules) and 0·1 μM [^{3}H]adenine (which is incorporated into the cytoplasmic pool of adenine nucleotides). Aggregation induced by carrageenans was terminated by the addition of 4 volumes ice-cold 0·4% (w/v) EDTA in iso-osmotic saline and rapid centrifugation (14 700 g; 30 s). Subsamples of cell-free supernatant were transferred into scintillation vials for radioactive determination of adenine and 5-HT release. Replicate subsamples were also taken for fluorimetric measurement (Gordon 1975) of release of β-N-acetyl-glucosaminidase, a lysosomal enzyme which is a constituent of the platelet alpha granules. Platelet pellets were digested for 30 min at 37° C with 19M formic acid before transferring to scintillation vials, or with 1% Triton x-100 for lysosomal enzyme assays. Radioactivity was measured in a Nuclear Chicago Mk 2 Liquid Scintillation Counter under conditions giving optimal discrimination between ^{3}H and ^{14}C. Fluorimetric determinations were carried out in a Farrand Mk 2 Spectrophotofluorimeter. Increases in supernatant radioactivity or lysosomal enzyme activity were expressed as percentages of the activity in a pellet of control (unstimulated) platelets.

RESULTS AND DISCUSSION

Platelet aggregation in stirred PRP or platelet suspensions is measured as a progressive decrease in optical density. All the carrageenans induced aggregation in PRP which was preceded by a large increase in optical density. This initial increase in optical density was also observed in cell free plasma, but not in gel-filtered platelets (Fig. 1) and presumably represents a change in plasma proteins. Precipitation of plasma proteins is observed after intravenous injection of carrageenan, accompanied by the appearance of intravascular aggregates containing carrageenan, protein and platelets (Dr P. R. M. Steele, unpublished observations). The proteins with which carrageenans interact have not yet been determined; it was suggested that fibrinogen might be the main protein involved (Schimpf et al 1969) but Vargaftig & Lefort (1977) concluded that this was unlikely, because a defibrinating agent had no effect, and precipitation occurred in both plasma and serum. These authors suggested that lipoproteins were more probable candidates, as mucopolysaccharides can precipitate lipoproteins in both plasma and serum (Amenta & Waters 1960).

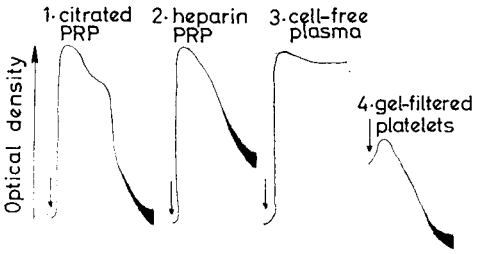

FIG. 1. Effects of carrageenan on human platelets. Iota carrageenan (50 μg ml^{-1}) was added to stirred 0·1 ml samples of (1) citrate PRP; (2) heparin PRP; (3) cell free plasma; (4) gel-filtered platelets and optical density changes were monitored in a platelet aggregometer. Similar results were obtained with gelcarin, kappa and lambda carrageenans.

All carrageenans tested produced a biphasic aggregation response in citrated PRP. No secretion occurred during the first phase of aggregation but the second phase was associated with selective release of platelet granule contents—less than 10% release of the cytoplasmic constituent, adenine, accompanied 75% release of [^{14}C]-5-HT and 35% release of β-N-acetyl glucosaminidase (Fig. 2). Other platelet stimuli such as ADP, adrenaline and synthetic prostaglandin analogues produce biphasic aggregation responses in citrated PRP. Primary aggregation induced by low concentrations of these stimuli is

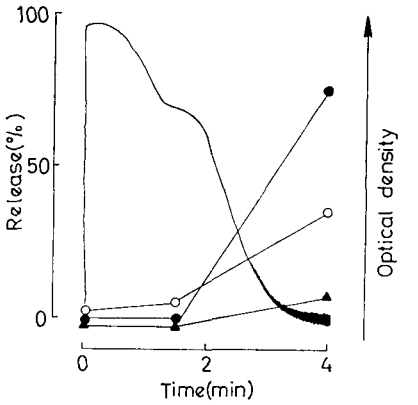

FIG. 2. Platelet aggregation and secretion of granule constituents induced by carrageenan. Iota carrageenan (50 μg ml^{-1}) was added to stirred 0·1 ml samples of PRP. Platelet aggregation was recorded photometrically and at 0, 1·5 and 4 min after the addition of agonist, replicate subsamples of PRP were taken for measurement of release of [^{14}C]-5-HT (●—●), β-N-acetyl glucosaminidase (○—○) and [^{3}H]adenine (▲—▲). (See methods). Results are mean values of duplicate determinations. Similar results were obtained with gelcarin, kappa and lambda carrageenans.

reversible, and higher concentrations are required to initiate the second phase (Fig. 3a) which is irreversible and is associated with release of granule constituents. In contrast, the first phase of carrageenan aggregation in citrated PRP was irreversible and always followed by a second phase. The delay before onset of this secondary aggregation response was inversely related to the carrageenan concentration (Fig. 3b).

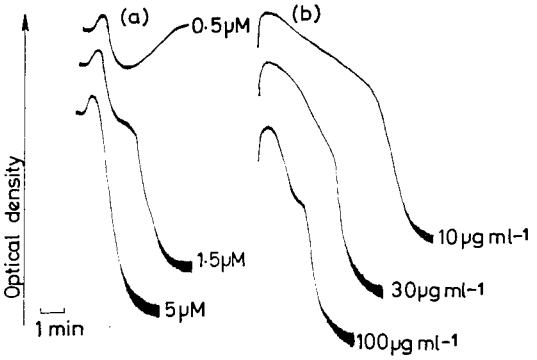

FIG. 3. Comparison of human platelet aggregation responses to ADP and carrageenans. Platelet aggregation induced by (a) ADP or (b) iota carrageenan. Similar results were obtained with gelcarin, kappa and lambda carrageenans.

The aggregation response to carrageenan in heparinized PRP or gel-filtered platelets was irreversible and monophasic, and was not accompanied by secretion (Fig. 4a), but the addition of 10 μl citrated cell-free plasma or of citrate (final concentration 13mM) produced two phases of aggregation, and secretion of granule constituents (Fig. 4b, c). Experiments with EDTA (q.v.) showed that this effect of citrate was not due to chelation of divalent cations. A similar requirement for citrate has been found with platelet aggregation and secretion induced by ADP (Mustard et al 1975).

The four carrageenan preparations showed considerable variation in their potency as stimuli for platelet aggregation and secretion (Table 1). These potency differences might provide clues as to the mechanism of action of carrageenans in platelets. Carrageenans can activate complement and some other complement activators (e.g. endotoxin) induce platelet aggregation and secretion with a pattern broadly similar to that obtained with carrageenans (MacIntyre et al 1977). This suggested that platelet responses induced by carrageenan might be mediated via complement activation as has been previously proposed for collagen-induced platelet aggregation (Chater 1976). However, the rank order of potency

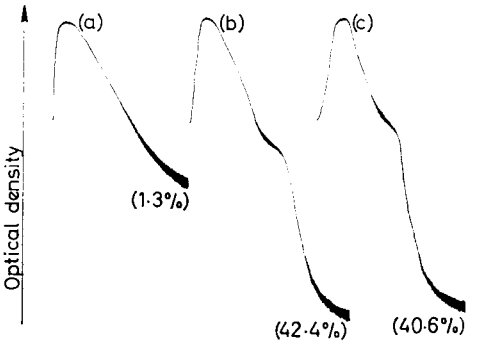

FIG. 4. Requirement for citrate in carrageenan-induced platelet secretion. Samples (0·1 ml) of heparin PRP were pre-incubated for 2 min at 37 °C before the addition of (a) 10 μl of iso-osmotic saline (b) 10 μl of citrated cell-free plasma (c) 10 μl of 0·13 M trisodium citrate; 15 s later iota carrageenan (50 μg ml^{-1}) was added. Figures in parentheses indicate the percentage release of [^{14}C]-5-HT. Results are mean values of duplicate determinations. Similar results were obtained using gel-filtered platelets in place of heparin PRP.

of carrageenans as complement activators (Schorlemmer et al 1977) differs from their relative activity as platelet stimuli—lambda carrageenan is more potent than iota in producing complement activation.

Two further observations suggest that the platelet responses to carrageenans and to collagen are not complement dependent. Firstly, collagen and carrageenan, but not endotoxin, can induce aggregation in plasma-free platelet suspensions; secondly, collagen and carrageenan (again, unlike endotoxin) are very effective against human platelets, which are immune-adherence negative, as well as against immune-

Table 1. Potency of carrageenans as inducers of platelet aggregation.

Carrageenan type and structure	Threshold concn (μg ml^{-1})	EC 50 (μg ml^{-1})*
Iota		
Alternating residues of β-D-galactose-4-sulphate and 3,6 anhydro α-D-galactose-2-sulphate	3	10
Lambda		
β-D-Galactose-4-sulphate	10	40
Kappa		
Alternating residues of β-D-galactose-4-sulphate and 3,6 anhydro-α-D-galactose	100	>300
Gelcarin		
Mixture of kappa and lambda	50	200

* EC50 = Concn inducing a half maximal aggregation response.

adherence positive species of platelets, such as rabbit. The balance of the evidence available therefore suggests that complement activation is not directly responsible for the stimulatory actions of carrageenans on platelets, although in the presence of plasma complement activation may occur, which can enhance the response of platelets (for review see Brown 1976).

We endeavoured to obtain further information about the mechanisms involved in the responses of platelets to carrageenans by using a range of pharmacological agents with defined modes of action (Fig. 5). Both phases of aggregation in citrated

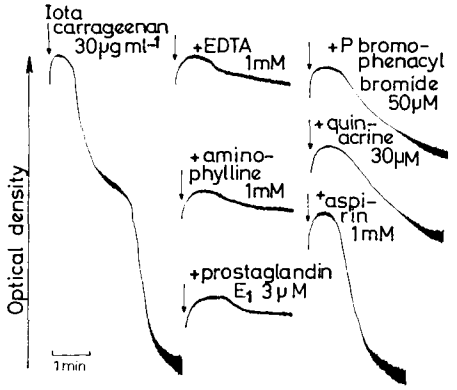

FIG. 5. Inhibition of carrageenan induced platelet aggregation. Samples (0·1 ml) of citrate PRP were pre-incubated for 2 min at 37 °C with EDTA (1mM), aminophylline (1 mM), PGE_1 (3 μM), Quinacrine (30 μM), *p*-bromophenacyl bromide (50 μM) or aspirin (1·1 mM) before the addition of iota carrageenan (30 μg ml^{-1}). Control samples received vehicle in place of drug.

PRP were abolished by EDTA, which chelates divalent cations, and by PGE_1 or aminophylline, which elevate intracellular cAMP concentrations. Aspirin, which blocks platelet PG synthesis by acetylating the cyclo-oxygenase component enzyme (Roth & Majerus 1975) inhibited only the second phase of aggregation; at no concentration of aspirin was any inhibition of the first phase observed. Carrageenan-induced aggregation is therefore apparently subject to the same regulatory mechanisms as aggregation responses induced by other platelet stimuli such as ADP and adrenaline: both phases are modulated by divalent cations and cAMP whereas only the secretory phase is dependent on arachidonate metabolites. Quinacrine and *p*-bromophenacyl bromide inhibit phospholipase A_2 activity (Volwerk et al 1974; Flower & Blackwell 1976) and prevent the liberation, from membrane phospholipids, of arachidonic acid, the major substrate for platelet PG synthase. These drugs, in contrast to aspirin, inhibited the first phase of aggregation as well as abolishing the second phase, indicating that they exert an action additional to their effect on arachidonate metabolism. Higher concentrations of these drugs (>100 μM) abolished both phases of carrageenan-induced aggregation but at these concentrations some inhibition of ADP-induced aggregation was also seen. Our findings are consistent with the suggestion made by Vargaftig (1977) that phospholipase A_2 releases not only arachidonic acid but also other fatty acids which can be metabolised to form mediators of platelet aggregation by an enzyme system distinct from PG synthase. Lapetina et al (1978) have recently suggested that arachidonate liberation from phospholipids without metabolism by cyclo-oxygenase may be a sufficient stimulus for platelet aggregation. Our results are also in accord with this hypothesis.

The rank order of potency of the carrageenans as platelet stimuli (iota > lambda > gelcarin > kappa) is in accord with their comparative activity as acute or chronic inflammatory agents in vivo (Robertson & Schwartz 1953; Winter et al 1962), which supports the concept that blood platelets may serve as useful in vitro models for studying the inflammatory activity of carrageenans. Further work is needed to establish whether the same mechanisms are responsible for the effects of carrageenans on platelets and on other cells such as leukocytes and macrophages, but the evidence available at present suggests that carrageenans exert a direct effect on cell membranes (which may involve phospholipase A_2 activation) and also an indirect effect through a change in plasma proteins. In addition, the marked variation in activity of different carrageenans in this study indicates that great care is required when quantitatively interpreting any results obtained with uncharacterized carrageenans.

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