(K_d = 1.8 nM) and that from the competition binding curves (K_d = 1.2 nM) was also found.

The identity of the high affinity binding sites as β-adrenergic receptors was further supported by (1) the ability of both agonist and antagonist to cause stereospecific displacement of [^{3}H]DHA binding, (2) the very low affinity of the α-adrenergic agonist, phenylephrine, (3) the order of potency of β-adrenergic agonists to displace bound radioligand [(−)-isoproterenol > (−)-epinephrine > (−)-norepinephrine] typical for β-2 adrenergic receptors [11].

The presence of adenylate cyclase activity has been demonstrated in human erythrocytes, but with extremely low catecholamine sensitivity [15]. However, these cells seem to be relatively enriched in guanine regulatory protein [16–18]. The present study demonstrates the existence of β-adrenergic receptors functionally coupled to adenylate cyclase in human erythrocytes by (1) the concentration-dependent cAMP elevation by (−)-isoproterenol, (2) the concentration-dependent and stereospecific inhibition of (−)-isoproterenol stimulation by propranolol, (3) stereospecificity of isoproterenol stimulation and (4) the order of agonist potencies [(−)-isoproterenol > (−)-epinephrine > (−)-norepinephrine] in cAMP elevation, typical for β-2 adrenergic receptors [11].

Human leucocytes have β-adrenergic receptors [2–4]. The stimulation of these receptors could have contributed to the observed cAMP levels, but only to a minor degree, since the ratio between the number of erythrocytes and the number of leucocytes in the incubation mixture was approximately 7000:1. The ratio between cAMP levels in erythrocytes and leucocytes is approximately 1:25 under comparable incubation conditions [19].

Even if the reported cAMP levels in the human erythrocytes are low, the basal and maximal levels represent an accumulation of about 90 and 300 molecules cAMP per sec per cell, respectively. Such changes in cAMP levels are probably enough to exert biological effects. Cyclic nucleotides have been proposed to play a role in the deformability of the human red cell [20]. The effect of adrenergic amines on human red cell haemolysis exhibited a β-2 adrenergic profile [21]. It was also observed that the effect of catecholamines was blocked by propranolol, but mimicked by exogenous cAMP.

The present study shows that intact human erythrocytes possess a population of functional β-2 adrenergic receptors. However, further studies are necessary to establish the physiological role of these receptors.

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Adriamycin-iron catalysed phospholipid peroxidation: a reaction not involving reduced adriamycin or hydroxyl radicals

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The anthracycline antitumour antibiotic adriamycin is widely used in modern cancer therapy. Its mode of action can be related to its ability to intercalate with cell DNA and it is thought to cause strand scissions in the DNA molecule [1]. Strand scissions occur after the drug has undergone a reductive cycle in which electrons are transferred to dioxygen, resulting in the formation of oxygen radicals [1, 2].

Clinical use of adriamycin is limited by its toxic side effects, the most serious of which is a cumulative dose-dependent cardiomyopathy [3]. The mechanisms leading to cardiac toxicity are not fully understood, but evidence strongly points to oxygen radical damage (for a review see [4]). Lipid peroxidation, an oxygen radical chain reaction of membrane lipids, has been implicated as a cause of drug mediated cell damage [5]. The hydroxyl radical (OH·) is frequently implicated as the species responsible for initiating the adriamycin-stimulated lipid peroxidation seen in microsomal preparations [6–8]. A recent study has, however, shown that OH· radicals do not participate in iron-dependent phospholipid peroxidation [9]. Adriamycin is here shown to greatly stimulate this latter type of peroxidation in a reaction which does not require reduction of adriamycin or the participation of hydroxyl radicals.

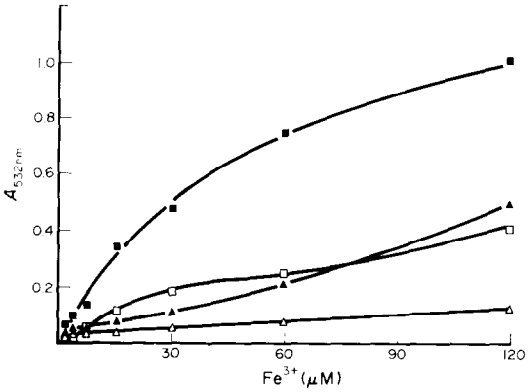

Fig. 1. Peroxidation of phospholipid measured as TBA reactivity. Reactions were catalysed by: ■ ■ adriamycin, 0.03 mg/ml + ferric chloride in a xanthine oxidase/hypoxanthine superoxide-generating system; □ □ adriamycin, 0.03 mg/ml + ferric chloride; ▲ ▲ ferric chloride in a xanthine oxidase/hypoxanthine superoxide-generating system; △ △ ferric chloride. The results are a mean of two separate experiments which were reproducible to ±5%.

Materials and methods

Superoxide dismutase (bovine erythrocyte 3000 units/ml), catalase (bovine liver 27,000 units/ml), xanthine oxidase (grade I, 28.1 units/ml), albumin (human fatty acid free), hypoxanthine and diethylenetriaminopentaacetic acid (DETAPAC) were obtained from Sigma Chemical Co. Ltd. Units of enzyme activity were as defined in the Sigma catalogue. Desferrioxamine was from Ciba-Geigy Ltd. Adriamycin (doxorubicin hydrochloride) was from Farmitalia Carlo Erba (absorbance of a 1% solution at 495 nm showed the sample to be approximately 70% active). The pH of the solution was 5.0. All other chemicals were of AnalaR grade, where available, and obtained from BDH Ltd.

Peroxidation of phospholipid liposome. Bovine brain phospholipids were prepared as previously described [10]. Multilamellar vesicles were formed by vigorously vortex-mixing 5 mg phospholipid in 1 ml 0.15 M NaCl, pH 7.4, for 2 min.

(a) Iron(III)-catalysed peroxidation. To 0.2 ml of liposomal suspension were added 20 μl adriamycin (1 mg/ml) and 20 μl ferric chloride to give a final iron concentration as indicated in Fig. 1 and Tables 1 and 2. Where appropriate, 50 μl of iron chelators or radical scavengers was added before the iron salt addition. The volume of each tube was made to 0.67 ml by the addition of Chelex-resin-treated distilled water and the mixtures incubated for 30 min at 37°. The pH of the final reaction mixture containing adriamycin was 7.1.

(b) Iron(III)superoxide-dependent peroxidation. In addition to the reactants described above in (a), 0.2 ml hypoxanthine (saturated aqueous solution) and 30 μl xanthine oxidase (0.7 units/ml) were added to each tube. The final volume was made to 0.67 ml as above and the mixtures incubated at 37° for 30 min.

Thiobarbituric acid reactivity (TBA) [11]. After incubation, 0.5 ml TBA (1% in 0.05 M NaOH) and 0.5 ml 25% v/v HCl were added to each tube, followed by heating at 100° for 15 min. When cool, each sample was extracted with 1.5 ml butan-1-ol and centrifuged at 3000 rpm for 5 min to separate the phases. The upper organic phase containing TBA-reactive material and adriamycin was read at 532 nm against appropriate blanks.

Table 1. Phospholipid peroxidation catalysed by an iron(III) salt and adriamycin

Inhibitors added	Rate of peroxidation as $A_{532\ nm}$/30 min (TBA reactivity)	% Inhibition
Blank (lipid only)	0.03	
Blank (adriamycin only)	0.13	
Blank (adriamycin + lipid)	0.17	
Blank [lipid + iron(III), 120 μM]	0.13	
Control [Lipid + iron(III) + adriamycin]	0.57	
Control + superoxide dismutase, 0.04 mg/ml	0.52	13
Control + catalase, 0.04 mg/ml	0.59	0
Control + alumin, 0.04 mg/ml	0.56	3
Control + mannitol, 7.5 mM	0.56	3
Control + thiourea, 0.75 mM	0.57	0
Control + urea, 0.75 mM	0.58	0
Control + EDTA, 0.3 mM	0.31	65
Control + DETAPAC,* 0.3 mM	0.34	58
Control + desferrioxamine, 0.3 mM	0.23	85
Control + propyl gallate, 0.15 mM	0.22	88

* DETAPAC = Diethylenetriaminopentaacetic acid.

Where indicated in the control and test samples, ferric chloride was added to give a final iron concentration of 120 μM. % Inhibition was calculated after subtraction of the lipid and adriamycin blank values. The results are a mean of three separate experiments which were reproducible to ±5%.

Table 2. Phospholipid peroxidation catalysed by an iron(III) salt and adriamycin in the presence of a superoxide-generating system

Inhibitors added	Rate of peroxidation as $A_{532\ nm}$/30 min (TBA reactivity)	% Inhibition
Blank (lipid only)	0.03	
Blank (adriamycin only)	0.13	
Blank [lipid + iron(III), 120 μM]	0.13	
Blank (lipid + xanthine oxidase and hypoxanthine [$O_2^{\cdot-}$])	0.04	
Blank (lipid + adriamycin + $O_2^{\cdot-}$)	0.24	
Blank (lipid + iron(III) + $O_2^{\cdot-}$)	0.51	
Blank (lipid + iron(III) + $O_2^{\cdot-}$) + superoxide dismutase, 0.04 mg/ml	0.08	(84)
Control (lipid + iron(III) + adriamycin + $O_2^{\cdot-}$)	1.22	
Control + superoxide dismutase, 0.04 mg/ml	0.67	52
Control + catalase, 0.04 mg/ml	1.10	11
Control + albumin, 0.04 mg/ml	1.07	14
Control + mannitol, 7.5 mM	1.13	9
Contol + thiourea, 0.75 mM	1.04	17
Control + urea, 0.75 mM	1.10	11
Control + EDTA, 0.30 mM	0.40	78
Control + DETAPAC,* 0.30 mM	0.34	84
Control + desferrioxamine, 0.3 mM	0.26	91
Control + propyl gallate, 0.15 mM	0.26	91

* DETAPAC = Diethylenetriaminopentaacetic acid.

Where indicated in the control and test samples, ferric chloride was added to give a final iron concentration of 120 μM. % Inhibition was calculated after subtraction of the lipid and adriamycin blank values. The results are a mean of three separate experiments which were reproducible to ±5%.

Results

When phospholipid liposomes were incubated with an iron(III) salt, little TBA-reactive material was detected (Table 1). Adriamycin alone added to phospholipid liposomes was slightly more stimulatory, but like the iron(III) salt this stimulation could be inhibited by addition of desferrioxamine (data not shown). Addition of both an iron(III) salt and adriamycin could enhance iron(III)-dependent lipid peroxidation (Fig. 1). This reaction was inhibited by the iron chelators EDTA, DETAPAC and desferrioxamine, and the organic oxygen radical scavenger propyl gallate (Table 1). Hydroxyl radical scavengers, such as mannitol and thiourea, and the enzymes catalase and superoxide dismutase were without significant effect. Albumin and urea served as controls for the specificity of these reactions.

In the presence of a xanthine oxidase/hypoxanthine superoxide-generating system, iron(III) salt and adriamycin–iron(III) dependent lipid peroxidation was increased over the range of iron concentrations (Fig. 1). This reaction was partly inhibited by superoxide dismutase and more markedly inhibited by iron chelators and the antioxidant propyl gallate (Table 2).

Discussion

Adriamycin has been shown to induce the peroxidation of cardiac lipids in mice [5]. Several recent reports have also shown that adriamycin can stimulate NADPH-dependent microsomal lipid peroxidation [7, 12, 13]. In these systems it is proposed that adriamycin, following a single electron reduction to the semiquinone-free radical, can under aerobic conditions reduce dioxygen with the formation of superoxide and hydroxyl radicals [14]. Evidence for the formation of hydroxyl radicals in such systems has been presented [7]. In contrast, the iron-catalysed peroxidation of phospholipid liposomes does not involve hydroxyl radicals [9] even when enhanced by adriamycin.

Adriamycin can complex ferric ions [15] with a stoichiometry of three adriamycin molecules to one ferric ion [16] and the adriamycin-stimulated lipid peroxidation described here is entirely dependent on the presence of an iron salt. The propagation reactions of lipid peroxidation, measured as thiobarbituric acid reactivity, takes place following a metal-catalysed decomposition of lipid peroxides, traces of which are present in the initial preparations of phospholipid. Although peroxidation was greatly increased in the presence of a superoxide-generating system, stimulation by iron–adriamycin (at a molar ratio of approximately 2:1) was only partly inhibited by superoxide dismutase. Since the major role of superoxide radicals in iron–catalysed phospholipid peroxidation appears to be the reduction of ferric ions to the ferrous state [9], our results suggest that part of the iron present in our reaction was not being reduced by superoxide but was nevertheless stimulating lipid peroxidation.

These findings imply that adriamycin does not necessarily have to undergo a reductive cycle in order to catalyse peroxidative damage to lipids. Since both lipid peroxides and non-protein-bound iron are normally present in biological systems [17, 18] it is conceivable that in situations where normal antioxidant protection is overwhelmed, adriamycin would greatly amplify iron-catalysed damage to biological membranes.

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Stereoselective inhibition of prostaglandin-induced calcium release from platelet membranes by trimetoquinol

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An elevation of free intracellular calcium levels and binding to calmodulin serves to link membrane-initiated events with cellular effects in biological systems [1]. Studies with platelets have examined relationships between prostaglandin endoperoxides, thromboxane A_2 (TXA_2), cyclic AMP, and calcium in the modulation of platelet activation [2–7]. Prostaglandin-mediated aggregation involves an interaction of inducers at a surface membrane to trigger the release of arachidonic acid (AA) from membrane phospholipids. Released AA is converted to the bioactive metabolites, prostaglandin endoperoxides (PGG_2 and PGH_2) and TXA_2, by the dense tubular system [8, 9]. TXA_2 is thought to increase the mobilization of calcium from the dense tubular system and the activation of platelet contractile proteins, whereas cyclic AMP promotes uptake of calcium into the dense tubular system or facilitates extrusion of calcium outside the cell [6, 7].

Trimetoquinol (TMQ) inhibits platelet activation induced by collagen, epinephrine, ADP, stable PGH_2 analogs (U46619 and U44069), AA and TXA_2 [10, 11], as well as by low dose thrombin and phospholipase C, from *Clostridium perfringens* [12]. TMQ exists as two stereoisomers, of which the $R(+)$-isomer is more potent as an inhibitor of platelet aggregation mediated by the prostaglandin-dependent pathway [10, 11]. We have proposed that the TMQ isomers act as antagonists of endoperoxide/TXA_2 action at the receptor level [11]. Other workers have indicated that TMQ does not interfere with prostaglandin biosynthesis [13, 14]. Thus, TMQ represents a unique chemical entity which is an antagonist of TXA_2 action and may be a novel agent for the treatment of thromboembolic disorders.

It may be suggested that TMQ modulates platelet function by interference with an event in the prostaglandin-dependent pathway that is common to the action of endoperoxides and TXA_2. TXA_2 has been envisioned as a calcium ionophore [15] and is capable of increasing the release of calcium from isolated membranes of the dense tubular system preloaded with radioactive calcium [16]. Previous reports have suggested that TMQ does not modify platelet adenylate cyclase activity or cyclic AMP levels [10]; thus, this agent would not be expected to alter uptake of calcium into the dense tubular system. Our experiments were initiated to determine whether the stereoisomers of TMQ block the release of calcium induced by prostaglandins in isolated platelet membranes in a concentration range which is similar to their blockade of aggregation.

In this report we have evaluated (a) the comparative actions of the stable PGH_2 analogs (U46619 and U44069) on the time-course release of calcium, and (b) the stereodependent ability of the TMQ isomers to block U46619-induced calcium release from isolated membranes of the platelet dense tubular system. U46619 has been characterized recently as a TXA_2 mimetic [17] in many biological tissues and is used as an experimental tool to assess the interaction of the TMQ isomers with TXA_2 sensitive sites for calcium release in this particulate fraction.

Methods

Chemicals. $^{45}Ca^{2+}$ (specific activity 38.9 μCi/mg), as the chloride salt, was obtained from the New England Nuclear Corp. (Boston, MA) and diluted for use. A23187, a cationic ionophore, and the epoxymethanoPGH_2 analogs [U46619: 15*S*-hydroxy-9α, 11α-(epoxymethano)prosta-5Z-13E-dienoic acid; and U44069: 15*S*-hydroxy-11α, 9α-(epoxymethano)-prosta-5Z-13E-dienoic acid] were received from the Eli Lilly Co. (Indianapolis, IN) and the UpJohn Laboratories (Kalamazoo, MI) respectively. The TMQ Isomers were a gift from the Tanabe Seiyaku Co., Ltd. (Saitama, Japan), and racemic TMQ [(+/−)-1-(3′,4′,5′-trimethoxybenzyl)-6,7-dihydroxy-1,2,3,4-tetrahydroisoquinoline] was synthesized in our laboratory [18]. U44069 and U46619 were prepared in ethanolic solutions and diluted in 0.05 M potassium phosphate buffer, pH 7.4. All compounds were freshly prepared in the buffer for use in the experiments.

Isolation of platelet membranes. Membranes were isolated by using the method of Robblee, *et al.* [19]. Human platelet-rich plasma was obtained from the American Red Cross or local blood banks and was centrifuged at 150 *g* for 3 min to obtain a pellet. The 150 *g* supernatant fraction was recentrifuged at 1800 *g* for 20 min, and the pellet was resuspended in citrated saline and the procedure repeated. The pellet was resuspended in a solution containing 30 mM KCl, 5 mM $MgCl_2$, 50 mM potassium oxalate, and 20 mM Tris–HCl, pH 7.0. This suspension was sonicated using a Sonic 300 dismembrator (Artek Systems Corp., Farmingdale, NY) at intermittant 30-sec periods for a total time of