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Ca^{2+} release from platelet intracellular stores by thapsigargin and 2,5-di-(t-butyl)-1,4-benzohydroquinone: relationship to Ca^{2+} pools and relevance in platelet activation

Kalwant S. AUTHI,* Shailaja BOKKALA, Yatin PATEL, Vijay V. KAKKAR and Felix MUNKONGE
Platelet Section, Thrombosis Research Institute, Manresa Road, Chelsea, London SW3 6LR, U.K.

The effects of the Ca^{2+}-ATPase inhibitors thapsigargin (Tg) and 2,5-di-(t-butyl)-1,4-benzohydroquinone (tBuBHQ) were examined by using Ca^{2+}-regulatory systems of platelet mixed membranes, saponin-permeabilized and intact platelets. Both agents inhibit Ca^{2+}-ATPase activities of platelet mixed membranes, without any effect on the basal Mg^{2+}-ATPase activity. Tg is more effective (EC_{50} = 35 nM) than tBuBHQ (EC_{50} = 580 nM). The effect of the two inhibitors on $^{45}Ca^{2+}$ release from saponin-permeabilized platelets has also been characterized. $^{45}Ca^{2+}$ uptake into non-mitochondrial intracellular stores occurs via an ATP-dependent mechanism, and if added at equilibrium the second messenger Ins(1,4,5)P_3 releases 50 % of the accumulated $^{45}Ca^{2+}$. Maximally effective concentrations of Tg (1 μM) and tBuBHQ (50 μM) release 77 % and 68 % of the accumulated $^{45}Ca^{2+}$. Addition of Ins(1,4,5)P_3 together with either Tg or tBuBHQ resulted in a non-additive release which was the same as with either Tg or tBuBHQ alone, indicating that the Ins(1,4,5)P_3-sensitive Ca^{2+} pool was a subset of the pool that is sensitive to the Ca^{2+}-ATPase inhibitors. Release of $^{45}Ca^{2+}$ by either Tg or tBuBHQ was not affected by heparin, which totally blocked Ins(1,4,5)P_3-induced Ca^{2+} release, and Tg was found not to affect [^{32}P]Ins(1,4,5)P_3 binding to its receptor on mixed membranes. Thus both Tg and tBuBHQ release Ca^{2+} from a pool that totally overlaps the Ins(1,4,5)P_3-sensitive pool without affecting Ins(1,4,5)P_3 function. In intact indomethacin-treated Fura 2-loaded platelets, Tg and tBuBHQ cause Ca^{2+} elevation, arising from release from intracellular stores and influx from the outside. Both Tg and tBuBHQ elevated Ca^{2+} to similar levels, which were less and slower than those observed with thrombin. Addition of thrombin to cells already treated with Tg or tBuBHQ produced further elevation of Ca^{2+}, indicating agonist utilization of a Ca^{2+}-ATPase inhibitor-insensitive pool. In aggregation experiments Tg and tBuBHQ showed different functional effects. In indomethacin-treated cells Tg induces slow aggregation and secretion responses, whereas tBuBHQ only induces shape change. Both agents show synergistic secretory responses with the protein kinase C activator dioctanoylglycerol (DiC_8). Tg also showed greater ability than tBuBHQ to release [^{3}H]arachidonic acid (AA) from [^{3}H]AA-labelled platelets. Additionally, in [^{32}P]P_i-labelled platelets both Tg and tBuBHQ induced phosphorylation of myosin light chain, a 27 kDa protein and the 45 kDa protein pleckstrin, but Tg showed a greater ability than tBuBHQ to cause phosphorylation of pleckstrin. These studies indicate that Tg and tBuBHQ are effective in releasing the Ins(1,4,5)P_3-sensitive Ca^{2+} pool in platelets. The differences obtained in Tg- and tBuBHQ-induced functional responses may reflect additional effects of Tg on protein phosphorylation.

INTRODUCTION

Elevation of the cytosolic levels of Ca^{2+} is a fundamental process in the activation of platelets by most agonists. Ca^{2+} elevation occurs as a result of release from intracellular stores and influx from the outside. Ins(1,4,5)P_3 is the major intracellular second messenger thought to be responsible for Ca^{2+} release from intracellular stores [1]. However, like most electrically non-excitable cells, platelets contain Ins(1,4,5)P_3-sensitive and -insensitive intracellular pools, as the second messenger only releases a fraction of the total non-mitochondrial Ca^{2+} store [2–4]. When first described, the Ca^{2+} pool on which Ins(1,4,5)P_3 acts and the store that is insensitive to Ins(1,4,5)P_3 was broadly defined as an endoplasmic-reticulum-type pool [5], but since then studies on a number of tissues have suggested that the Ins(1,4,5)P_3-sensitive pool is a specialized organelle that may be distinct from the endoplasmic reticulum [6–9]. The nature of the Ca^{2+}-release process from the Ins(1,4,5)P_3-insensitive store is also largely unknown. GTP-dependent release [6] and uptake [10] processes have been described in organelles that are distinct from those that affect Ins(1,4,5)P_3, and more recently a large number of studies have described Ca^{2+}-induced Ca^{2+}-release mechanisms (using caffeine as a pharmacological probe) from the Ins(1,4,5)P_3-insensitive stores, and such mechanisms are thought to be important in the propagation of Ca^{2+} oscillations (e.g. [11–14]). In platelets the intracellular Ca^{2+} store has often been referred to as the dense tubular system [15,16] and is a membrane system that is analogous to the smooth sarcoplasmic reticulum of skeletal muscle. This membrane system has been purified by using high-voltage free-flow electrophoresis and is rich in Ca^{2+}-ATPase, giving Ca^{2+} sequestration, the binding site or receptor for Ins(1,4,5)P_3 through which Ca^{2+} is released, and all the enzymes necessary for prostanoid biosynthesis [17–21]. The separation of Ins(1,4,5)P_3-sensitive from -insensitive Ca^{2+} stores in these membranes has not been determined.

Recently two unrelated compounds, the sesquiterpene lactone tumour promoter thapsigargin (Tg) and 2,5-di-(t-butyl)-1,4-benzohydroquinone (tBuBHQ) have proved useful in the study of Ca^{2+} homoeostasis in a number of different cell types [22–27]. Both compounds elevate cytosolic Ca^{2+} levels via the release of

Abbreviations used: Tg, thapsigargin; tBuBHQ, 2,5-di-(t-butyl)-1,4-benzohydroquinone; AA, arachidonic acid; 5HT, 5-hydroxytryptamine creatine sulphate; Fura 2 AM, fura 2-acetoxymethyl ester; PGI_2, prostaglandin I_2 (prostacyclin); DiC_8, 1,2-dioctanoylglycerol.

* To whom correspondence should be addressed.

Ca^{2+} from intracellular stores, followed by influx from the extracellular medium. Also, their mechanism of action appears to involve not surface receptor occupancy, but an inhibition of the Ca^{2+}-ATPase [22,27]. In platelets, although both agents elevate intracellular Ca^{2+} levels they have differing functional effects. Tg has been shown to induce aggregation and secretion, but these responses are inhibited by cyclo-oxygenase inhibitors [28–30]. tBuBHQ appears not to give aggregation or secretion, but has been shown to inhibit the formation of thromboxane B_2 [30]. Additionally, the pools of intracellular Ca^{2+} mobilized by these agents have not been appropriately defined. In this study we report a comparison of the effects of these two agents on Ca^{2+} mobilization, using platelet membranes, saponin-permeabilized and intact platelets, examining the pools of Ca^{2+} that are mobilized, and their relevance in platelet activation.

MATERIALS AND METHODS

Materials

Dipotassium ATP and human thrombin were obtained from Sigma Chemical Co. (Poole, Dorset, U.K.). Adenosine 5′-[γ-^{32}P]triphosphate ([^{32}P]ATP) (30 Ci/mmol), [^{3}H]arachidonic acid ([^{3}H]AA; TRK 757), 5-hydroxy[^{14}C]tryptamine creatine sulphate (5HT) (CFA 170), D-*myo*-inositol 1,4,5-[5-^{32}P]trisphosphate (PB 500; 1000 Ci/mmol) ([^{32}P]Ins(1,4,5)P_3), unlabelled Ins(1,4,5)P_3 and $^{45}Ca^{2+}$ (5–50 mCi/mg; CES 3) were obtained from Amersham International (Amersham, Bucks., U.K.). Fura 2-acetoxymethyl ester (Fura 2 AM) was obtained from Molecular Probes (Cambridge Biosciences, Cambridge, U.K.). Tg was initially supplied by Dr. Ole Thastrup (Copenhagen, Denmark) and subsequently obtained from Calbiochem (Nottingham, U.K.), and tBuBHQ was obtained from Dr. S. Orrenius (Stockholm, Sweden). All other reagents were of analytical grade wherever possible, and solutions were prepared in analytical-grade water (BDH, Poole, U.K.).

Preparation of platelet mixed membrane fractions by differential centrifugation

Human platelet mixed membrane (intracellular and plasma-membrane) fractions were prepared as detailed previously [3]. Washed platelets were resuspended for sonication in 0.34 M sorbitol/10 mM Hepes (pH 7.2)/2 mM dithiothreitol and the protease inhibitors aprotinin (0.3 unit/ml), pepstatin A (20 μM) and phenylmethanesulphonyl fluoride (1 mM). After sonication, the platelet homogenates were layered on to 1–3.5 M sorbitol density gradients and centrifuged at 42000 *g* for 90 min. A mixed membrane fraction containing both surface and intracellular membranes, but well separated from the granule fraction, was isolated from the gradient, sedimented further at 100000 *g* for 60 min, resuspended in sonication buffer without proteolytic inhibitors, and used for Ca^{2+}-ATPase and [^{32}P]Ins(1,4,5)P_3-binding studies.

Determinations of Ca^{2+}-ATPase activities

Ca^{2+}-ATPase activities were estimated using [γ-^{32}P]ATP by the procedure of Chamberlain et al. [31], as described by Hack et al. [20]. Final incubations contained 120 mM KCl, 5 mM $MgSO_4$, 20 mM Hepes, pH 7.2, 1 mM MgATP containing [γ-^{32}P]ATP, 50 μM $CaCl_2$ and mixed membranes (100 μg of protein). Incubations were carried out for 15 min at 37 °C and stopped by addition of 2.5 ml of Norit A (activated charcoal, 25 mg/ml in 0.1 M H_3PO_4). After centrifugation (1500 *g* for 20 min), 1 ml of the supernatant was taken and the radioactivity representing released [^{32}P]P_i was measured by liquid-scintillation spectrometry. The basal Mg^{2+}-ATPase activity was estimated in the presence of 5 mM EGTA.

Binding of [^{32}P]Ins(1,4,5)P_3 to membranes was carried out by a modification of the procedure of Worley et al. [32]. The incubation mixtures contained 25 mM Na_2HPO_4, 100 mM KCl, 20 mM NaCl, 1 mM EGTA, 1 mg/ml BSA, pH 8.0, 0.1 nM [^{32}P]Ins(1,4,5)P_3, 200 μg of protein and various concentrations of Tg. Incubations were carried out for 30 min at 4 °C, and at the end a sample was taken out and rapidly filtered through a 0.45 μm-pore filter. The filter was then washed with 2 × 10 ml of a buffer containing 0.25 M sorbitol and 10 mM K_2HPO_4, pH 7.4, and the radioactivity on the filter was counted by liquid scintillation.

Studies of $^{45}Ca^{2+}$ uptake and release in saponin-permeabilized human platelets

The isolation and washing procedures were as described previously [33,34]. The platelets were resuspended in a buffer consisting of 1 mM glucose, 1 mM $MgCl_2$, 0.5 mM NaH_2PO_4, 6 mM $NaHCO_3$, 140 mM NaCl and 10 mM Hepes (pH 7.4) at a cell count of 1 × 10^9 platelets/ml. A 100 μl portion of this suspension was added to a reaction mixture (total 0.5 ml) which contained (final concentrations) 112 mM KCl, 27 mM NaCl, 0.8 mM glucose, 0.34 mM NaH_2PO_4, 4.8 mM $NaHCO_3$, 5.8 mM $MgCl_2$, 5 mM ATP, 50 μM EGTA, 48 μM $CaCl_2$ containing 2 μCi of $^{45}Ca^{2+}$ and 8 mM Hepes, pH 7.4. Antimycin (10 μM) and oligomycin (5 μM) were added to eliminate mitochondrial Ca^{2+} effects, as used by Streb et al. [5]. $^{45}Ca^{2+}$ uptake into intracellular stores was initiated by addition of saponin (20 μg/ml) or cells, and release of the cation was measured by adding appropriate agents after incubation for 20 min. Reactions were terminated by rapid vacuum filtration of 450 μl samples through pre-wetted Whatman nitrocellulose filters (0.45 μm pore size). The filters were subsequently washed with 3 × 5 ml of ice-cold buffer consisting of 120 mM KCl, 5 mM EGTA, 5 mM $MgCl_2$ and 20 mM Hepes (pH 7.4). Filters were removed and counted for radioactivity by liquid-scintillation spectrometry.

Fura 2 labelling of washed human platelets

Fura 2 labelling of platelets was carried out as described previously [35]. Briefly, platelets from 60 ml of human blood taken into 0.1 vol. of 3.8% (w/v) trisodium citrate were isolated and resuspended in 5 ml of a labelling buffer consisting of 10 mM Hepes, 140 mM NaCl, 5 mM KCl, 1 mM $MgSO_4$, 10 mM glucose and 1 μM prostacyclin (PGI_2), pH 7.4 (buffer A). Loading of the cells was carried out for 45 min at 37 °C with 2 μM Fura 2 AM. At the end a further 10 ml of buffer A was added and the cells were sedimented (1000 *g* for 15 min), washed once in a buffer consisting of 36 mM citric acid, 10 mM EDTA, 5 mM glucose, 5 mM KCl and 90 mM NaCl, pH 6.5, and after a further centrifugation the cells were resuspended in buffer A without PGI_2 at 3 × 10^8 cells/ml.

Samples of the platelet (0.5 ml) preparations were transferred into the spectrofluorimeter (Perkin–Elmer LS-5B) cuvette thermostatically maintained at 37 °C and magnetically stirred. The Fura 2 fluorescence was monitored at 340 nm excitation and 500 nm emission with 10 nm slit width. Ca^{2+} concentrations were calculated by the procedure of Grynkiewicz et al. [36]. The traces shown are typical of duplicate determinations within the same

batch of platelets; similar results were observed in at least six different experiments using platelets from different donors.

Preparation of human platelets for aggregation, secretion and protein-phosphorylation studies

Labelling of platelets with [^{14}C]5HT and [^{3}H]AA was carried out in platelet-rich plasma using 0.1 μCi of [^{14}C]5HT/ml and 1 μCi of [^{3}H]AA/ml for 20 min and 60 min respectively. At the end, the platelet-rich plasma was acidified to pH 6.5 with 0.1 M citric acid and centrifuged at 1200 *g* for 15 min. The platelet pellet was washed in a buffer consisting of 36 mM citric acid, 103 mM NaCl, 5 mM KCl and 5 mM glucose, pH 6.5, with 60 nM PGI_2, centrifuged as above and resuspended in a Hepes Tyrode buffer consisting of 10 mM Hepes, pH 7.4, 145 mM NaCl, 5 mM KCl, 1 mM $MgSO_4$, 1 mM $CaCl_2$ and 5 mM glucose at 3×10^8 cells/ml.

For protein-phosphorylation studies platelets were incubated in the wash buffer for 90 min at 37 °C with 0.25 mCi of carrier-free [^{32}P]P_i/ml and washed twice before resuspension in the Hepes Tyrode medium at 6×10^8 cells/ml. Incubations were carried out with 300 μl suspensions at 37 °C with stirring (1000 rev./min) and aggregation was monitored by measuring the extent of light transmission in an aggregometer (Aggrecorder II; Daiichi Kagaku Co., Kyoto, Japan). Secretion of [^{14}C]5HT from dense granules and [^{3}H]AA from phospholipids was measured by stopping the reactions with cold 16 mM EDTA containing 1% formaldehyde, followed by centrifugation at 10000 *g* for 3 min and measurement of the radioactivity in the supernatant by liquid-scintillation counting. Results are expressed as percentage release compared with the total radioactivity present in the incubations. For protein phosphorylation the incubations were stopped after 3 min with 150 μl of sample buffer, containing 9% SDS, 6% β-mercaptoethanol, 15% glycerol, 0.186 M Tris, pH 6.8, and 0.03% Bromophenol Blue. After boiling for 3 min at 100 °C, polyacrylamide-gel electrophoresis was carried out with 40–60 μg of protein, followed by staining and autoradiography to observe the phosphorylated proteins.

RESULTS

Inhibition of Ca^{2+}-ATPase by Tg and tBuBHQ

Studies were initially carried out to determine the effects of Tg and tBuBHQ on Ca^{2+}-ATPase activities associated with Ca^{2+} translocation. Platelet membranes exhibit a basal Mg^{2+}-ATPase activity (estimated in the presence of EGTA), which is further stimulated in the presence of Ca^{2+} [20]. Figures 1(a) and 1(b) show typical results of the effects of addition of various concentrations of Tg and tBuBHQ on the Ca^{2+}- and Mg^{2+}-ATPase activities of different platelet mixed membranes (hence different control total activities, repeated on at least three other membrane preparations). Both Tg and tBuBHQ led to total inhibition of Ca^{2+}-ATPase activity, without any effect on the basal Mg^{2+}-ATPase activity. Tg is more potent, with $EC_{50} = 35$ nM and total inhibition at concentrations greater than 1 μM. tBuBHQ is less potent, with $EC_{50} = 580$ nM and total inhibition at concentrations greater than 1 μM.

Studies on $^{45}Ca^{2+}$ uptake and release in saponin-permeabilized platelets

Tg and tBuBHQ have been shown to cause intracellular Ca^{2+} release from a number of different cell types. In order to characterize the Ca^{2+} release by Tg and tBuBHQ from platelet intracellular stores, $^{45}Ca^{2+}$-uptake and -release studies were carried out in saponin-permeabilized platelets. We have previously demonstrated that Ca^{2+} uptake occurs via an ATP-dependent mechanism which reaches equilibrium after 15 min, occurs into an endoplasmic-reticulum-type store, and is prevented by addition of the cationophore A23187 (10 μM) [34]. In this system the effects of Tg and tBuBHQ were determined and compared with the results obtained with Ins(1,4,5)P_3. The second messenger Ins(1,4,5)P_3 (10 μM), added after 20 min equilibration, produced release of 50% of the accumulated $^{45}Ca^{2+}$ (Figure 2a) within 2–5 min of addition of the agent. Addition of tBuBHQ (50 μM) and Tg (1 μM), concentrations that will totally block Ca^{2+}-ATPase activities, at 20 min resulted in a $^{45}Ca^{2+}$ release of 68% and 77% in 5 min respectively (Figures 2a and 2b). In experiments examining the effects of adding at equilibrium Ins(1,4,5)P_3 and Tg together and measurement after 5 min, there was non-additive $^{45}Ca^{2+}$ release which was the same as that by Tg alone, indicating that Ca^{2+} was released from overlapping pools. When tBuBHQ and Ins(1,4,5)P_3 were added at 20 min, the release obtained was again non-additive and the same as with tBuBHQ alone, again indicating that inhibition of the Ca^{2+}-ATPase released Ca^{2+} from the Ins(1,4,5)P_3-sensitive pool. The effect of heparin on $^{45}Ca^{2+}$ release induced by Ins(1,4,5)P_3, Tg and tBuBHQ was also tested. In the presence of 20 μg/ml heparin added at the start of the incubations, Ins(1,4,5)P_3-induced Ca^{2+} release was totally inhibited, in contrast with that by Tg and tBuBHQ, which remained unaffected.

A binding site for Ins(1,4,5)P_3 which probably reflects the receptor through which InsP_3 acts to release Ca^{2+} has been shown

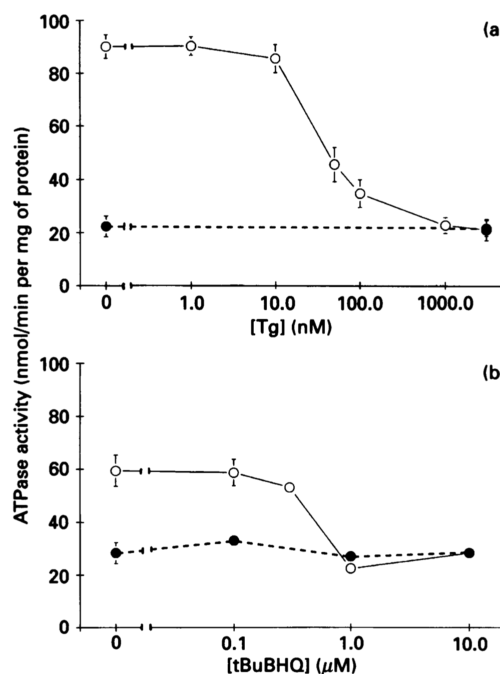

Figure 1 Effect of Tg (a) and tBuBHQ (b) on Ca^{2+}- and Mg^{2+}-ATPase activities of human platelet membranes

Ca^{2+}-ATPase activities (○) were measured in the presence of 50 μM Ca^{2+} and Mg^{2+}-ATPase activities (●) were measured in the presence of 5 mM EGTA. Points are means ± S.E.M. of triplicate or means of duplicate determinations, carried out on at least three other membrane preparations. (**a**) and (**b**) represent data from different membrane preparations and hence with different total activities.

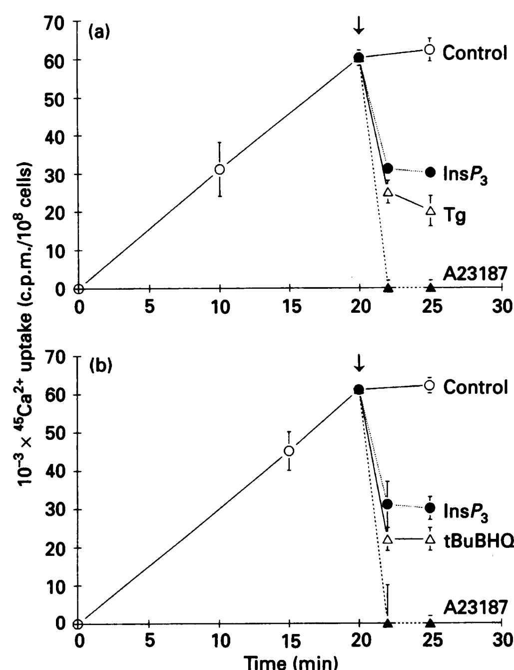

Figure 2 Comparison of $^{45}Ca^{2+}$ release from intracellular stores of saponin-permeabilized platelets by InsP_3, Tg (a) and tBuBHQ (b)

Saponin-permeabilized platelets were allowed to accumulate $^{45}Ca^{2+}$ for 20 min and then challenged (arrow) with 10 μM Ins(1,4,5)P_3 (●) or 1 μM Tg (△; **a**), or 50 μM tBuBHQ (△; **b**). Incubations were stopped at various times by rapid filtration. Also shown is the release pattern obtained with A23187 (▲). Points are means ± S.E.M. of triplicate determinations, with similar results obtained on three other platelet preparations.

Table 1 Effect of Tg and heparin on [^{32}P]InsP_3 binding to platelet mixed membranes

Agents were added at zero time with 0.1 nM InsP_3 and incubations were stopped after 30 min at 4 °C. Values are means ± S.E.M. of triplicate determinations.

	[^{32}P]InsP_3 bound	
	(c.p.m.)	(%)
Control [^{32}P]InsP_3	5240 ± 242	100
+ 10 μM InsP_3	522 ± 64	10
+ Tg (0.1 μM)	5135 ± 340	98
+ Tg (1 μM)	5030 ± 242	96
+ Tg (10 μM)	5554 ± 402	106
+ heparin (20 μg/ml)	628 ± 60	12

to be present on intracellular membranes of human platelets prepared by high-voltage free-flow electrophoresis [21]. Using mixed membranes, a study was carried out to determine if Tg interferes with Ins(1,4,5)P_3 binding. Table 1 shows that addition of Tg (0.1–10 μM) did not affect the binding of [^{32}P]Ins(1,4,5)P_3 to platelet membranes. Under the same conditions, heparin, which has been shown to inhibit binding of Ins(1,4,5)P_3 in a number of tissues, was able to displace the binding totally. Taken together, these results indicate that the Ca^{2+}-ATPase inhibitors Tg and tBuBHQ released more Ca^{2+} than did Ins(1,4,5)P_3 from intracellular stores of saponized platelets, and from a pool that overlapped the Ins(1,4,5)P_3-sensitive pool, but without interference with the Ins(1,4,5)P_3 receptor.

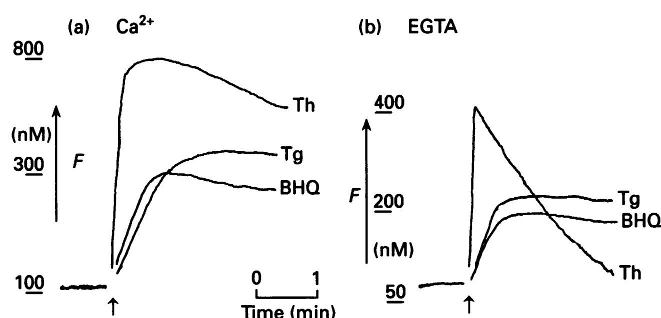

Figure 3 Effects of tBuBHQ, Tg and thrombin on Ca^{2+} elevation in Fura 2-labelled human platelets

(**a**) Typical traces, repeated on at least six other preparations, using indomethacin-treated Fura 2-labelled platelets in the presence of 1 mM Ca^{2+}; (**b**) shows corresponding results obtained in the presence of 1 mM EGTA. Approximate cytosolic [Ca^{2+}] are shown as indicated. Small arrows indicate point of addition of agent. Concentrations used were thrombin (Th) 1 unit/ml, Tg 3 μM and tBuBHQ (BHQ) 50 μM. F = fluorescence.

Effects of Tg and tBuBHQ on Ca^{2+} mobilization in Fura 2-loaded platelets

The effects of the two Ca^{2+}-ATPase inhibitors were also tested on Fura 2-labelled platelets. It is well accepted that agonists such as thrombin and thromboxane A_2 induce elevation of cytosolic Ca^{2+} levels arising from release of intracellular stores and influx from the outside. In these studies the platelets have been treated with 10 μM indomethacin, to avoid the formation of thromboxane A_2. Addition of Tg (3 μM) or tBuBHQ (50 μM) resulted in the elevation of cytosolic Ca^{2+} levels (Figures 3a and 3b). In confirmation of the results of Brune and Ullrich [30], maximally effective concentrations of the ATPase inhibitors produced Ca^{2+} elevations that were slower and considerably less than that obtained by thrombin (Figure 3) or the thromboxane mimetic U46619 (results not shown). In the presence of extracellular Ca^{2+}, Tg and tBuBHQ elevated Ca^{2+} to approx. 300 nM compared with much higher levels obtained with 1 unit/ml thrombin. In the absence of extracellular Ca^{2+} (i.e. with 1 mM EGTA), Tg and tBuBHQ again produced less mobilization of Ca^{2+}, to approx. 200 nM compared with values of 400 nM and greater with thrombin. In further experiments to examine the intracellular pools mobilized in intact cells, in the presence of 1 mM EGTA the addition of tBuBHQ (50 μM) 3 min after Tg (1 μM) produced no further change in the Ca^{2+} transients, indicating mobilization of Ca^{2+} from overlapping intracellular pools (similar results were obtained in the presence of extracellular Ca^{2+} with the additional component of Ca^{2+} influx). In some experiments the addition of Tg 3 min after tBuBHQ only slightly further elevated Ca^{2+} levels, indicating that Tg was able to release marginally more Ca^{2+} than tBuBHQ. These experiments indicate that both agents effectively released Ca^{2+} from the same intracellular pool of Ca^{2+} which overlapped the InsP_3-sensitive stores as determined with saponin-permeabilized platelets. Experiments were also carried out to determine the effect of agonist addition to Tg- or tBuBHQ-treated platelets. In the absence of extracellular Ca^{2+}, addition of thrombin (1 unit/ml) or U46619 (5 μM) 3 min after Tg or tBuBHQ treatment of Fura 2-loaded platelets resulted in a substantial further release of Ca^{2+} from the intracellular stores (Figures 4a and 4b). These increases

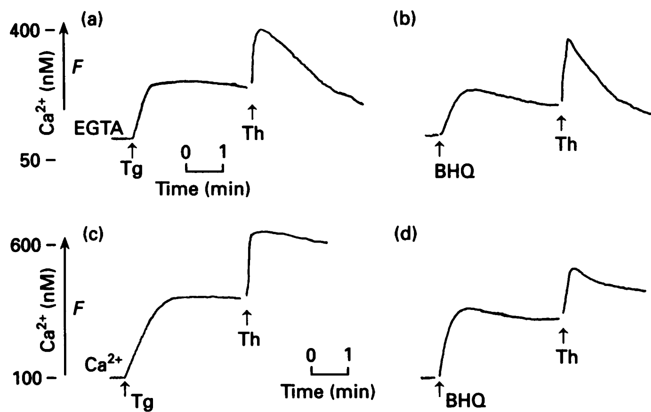

Figure 4 Effects of thrombin on Ca^{2+} elevation in platelets with Tg or tBuBHQ

Experiments were carried out in the presence of 1 mM EGTA (**a** and **b**) or 1 mM Ca^{2+} (**c** and **d**): 1 μM Tg or 50 μM tBuBHQ (BHQ) was added, followed by thrombin (Th; 1 unit/ml) 3 min later. Traces are typical of those obtained with at least six other platelet preparations.

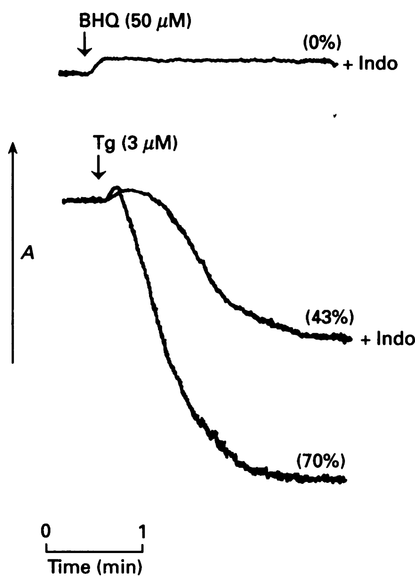

Figure 5 Aggregation of human platelets induced by Tg or tBuBHQ

Traces shown are typical aggregation profiles of platelet suspensions treated with tBuBHQ (BHQ; 50 μM) or Tg. Indo represents 10 μM indomethacin. Values after the aggregation traces indicate % secretion of [^{14}C]5HT into the medium.

were transient and returned to basal levels within 5 min. Thus both thrombin and U46619 were able to cause further release of Ca^{2+} from a pool that was insensitive to the Ca^{2+}-ATPase inhibitors. With Ca^{2+} in the extracellular media a similar pattern of Ca^{2+} elevations was obtained, but the return to basal levels was slower (over 15 min), reflecting the Ca^{2+}-influx component maintaining Ca^{2+} levels in the cytosol (Figures 4c and 4d).

Functional responses induced by Tg and tBuBHQ and their relationship to Ca^{2+} elevation

The previous studies indicated that in indomethacin-treated platelets both Tg and tBuBHQ elevated Ca^{2+} levels, but that

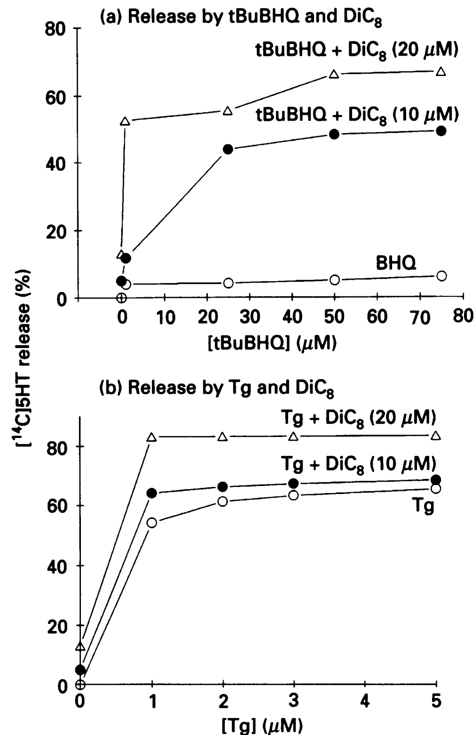

Figure 6 Effects on [^{14}C]5HT secretion induced by various concentrations of tBuBHQ and Tg in combination with DiC_8

Platelet suspensions were incubated with stirring in aggregometer tubes with (**a**) various concentrations of tBuBHQ and either 10 μM (●) or 20 μM (△) DiC_8, and secretion of dense granules was measured after 3 min. In (**b**) studies were carried out with Tg (○) or in combination with 10 μM (●) or 20 μM (△) DiC_8. Points are means of duplicate determinations repeated on two other platelet preparations.

these signals are less than those obtained with thrombin and U46619. Experiments were carried out to examine the extent of functional responses obtained in indomethacin-treated [^{14}C]5HT-labelled platelets. Addition of Tg (0.5–5 μM) produced shape change and a slow aggregation and secretion response within 5 min incubation (Figure 5). Addition of tBuBHQ (1–50 μM) produced only shape change, with no aggregation or secretion of [^{14}C]5HT. Under these conditions, both thrombin (0.1 unit/ml) and U46619 (5 μM) produced good aggregation responses (traces not shown). Addition of tBuBHQ and Tg together did not result in any additivity of responses (aggregation or secretion), but reflected the response produced by Tg alone. As these agents induced modest elevations of intracellular [Ca^{2+}], experiments were also carried out to investigate possible synergistic effects, measuring dense-granule secretion with the membrane-permeant protein kinase C activator 1,2-dioctanoylglycerol (DiC_8). This diacylglycerol is known to activate protein kinase C, causing a small secretory response without elevation of the cytosolic Ca^{2+} levels [37]. Figure 6 shows that addition of either Tg or tBuBHQ with DiC_8 resulted in a synergistic [^{14}C]5HT-secretory response in indomethacin-treated platelets. This effect was particularly prominent with tBuBHQ, which itself did not give any secretion of [^{14}C]5HT.

The ability of Tg to cause aggregation alone in indomethacin-treated platelets indicated that it may have additional stimulatory actions, as it mobilized similar amounts of Ca^{2+} to tBuBHQ. Studies were then carried out to determine the ability of Tg and tBuBHQ to release AA from [^{3}H]AA-labelled cells. In these

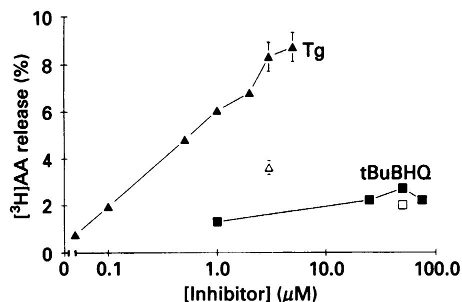

Figure 7 Release of [^{3}H]AA by Tg and tBuBHQ from human platelets

Dose–response relationship of Tg- and tBuBHQ-induced [^{3}H]AA release measured over 3 min. The effect of 1 μM staurosporine is also shown when 3 μM Tg (△) and 50 μM tBuBHQ (□) were used. Points are means ± S.E.M. of triplicate determinations, or means of duplicates with similar results obtained in two other preparations.

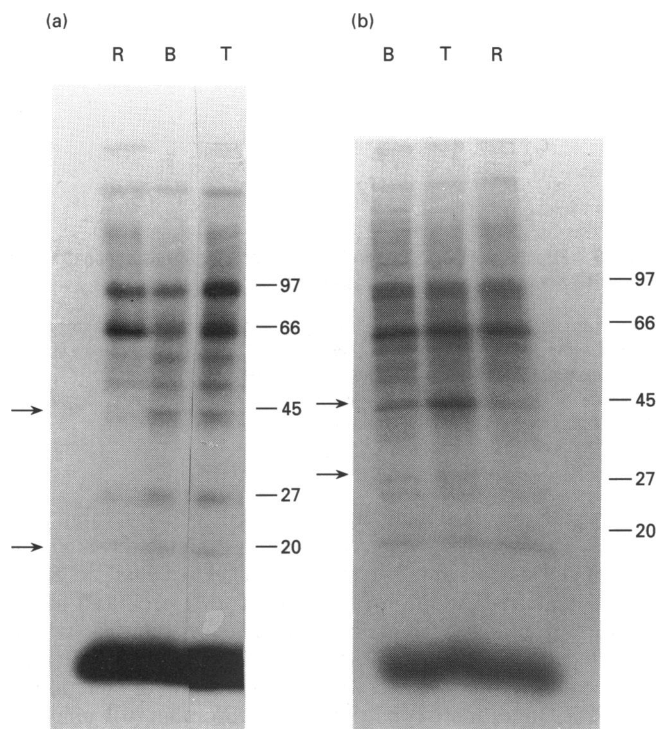

Figure 8 Effects of Tg and tBuBHQ on protein phosphorylation in human platelets

Two typical experiments are shown. [^{32}P]P_i-labelled platelets were incubated with 50 μM tBuBHQ (B) or 3 μM Tg (T) for 3 min, and protein phosphorylation was analysed by SDS/PAGE, followed by autoradiography. Values on the right represent molecular masses (kDa) of standard proteins. Arrows indicate the positions of 20 kDa myosin light chain and the 45 kDa protein pleckstrin. Lanes: R, resting platelets; T, Tg-treated platelets; B, tBuBHQ-treated platelets.

studies both indomethacin (10 μM) and phenidone (250 μM) were present to inhibit cyclo-oxygenase and lipoxygenase activities totally. Figure 7 shows that both Tg and tBuBHQ induced a dose-related release of [^{3}H]AA from human platelets, which was maximal at 50 μM for tBuBHQ and at 3 μM for Tg. Tg caused the release of much more [^{3}H]AA than did tBuBHQ (8 % and 3 % respectively), and compared well with thrombin (1 unit/ml releasing 10.7 ± 2 %). In further studies it was found that the protein kinase inhibitor staurosporine (1 μM) and the aggregation inhibitor peptide Arg-Gly-Asp-Ser (RGDS; 300 μM) inhibited Tg-induced [^{3}H]AA release to 3.6 % and 3.1 % respectively. Both staurosporine (Figure 7) and RGDS did not affect tBuBHQ-induced [^{3}H]AA release, indicating that this reflected an activity that was probably related to Ca^{2+} elevation but not to protein kinase activation or aggregation.

The effects of Tg and tBuBHQ on protein phosphorylation in [^{32}P]P_i-labelled cells under conditions similar to those of aggregation experiments were also examined. Figures 8(a) and 8(b) show autoradiograms of SDS/PAGE profiles of two typical experiments (repeated on at least three other preparations). Both Tg and tBuBHQ increased ^{32}P incorporation into myosin light chain (20 kDa peptide), the 45 kDa protein kinase C substrate pleckstrin and a 27–29 kDa protein. In a number of experiments Tg produced larger increases in phosphorylation of the 45 kDa protein compared with tBuBHQ (Figure 8b).

DISCUSSION

Recently the Ca^{2+}-ATPase inhibitors tBuBHQ and Tg have been shown to cause an increase in cytosolic Ca^{2+} in a large number of different cell types [22–29]. Their mechanism of action does not involve the formation of Ins(1,4,5)P_3 or the direct opening of a Ca^{2+} channel, as the rate of Ca^{2+} release is slower than in agonist stimulation or with Ca^{2+} ionophores, but was identified as initially inhibition of the Ca^{2+}-sequestration mechanism [22,24] and recently the Ca^{2+}-ATPase [22,38–41]. Our studies with platelet membrane Ca^{2+}-ATPase measurements confirmed these observations and indicated that both agents are effective inhibitors, with Tg being approx. 16 times more potent than tBuBHQ. Both agents also did not affect the basal Mg^{2+}-ATPase activity, and thus affected only the activity associated with Ca^{2+} translocation. Their mechanism of action is thought to involve inhibition of the formation of the Ca^{2+}-ATPase phosphoenzyme intermediate, which is a pre-requisite for Ca^{2+} translocation [41]. In this regard Tg is now known to be effective against all sarco- and endo-plasmic-reticulum-type Ca^{2+}-ATPases ('SERCA'), with no effect on the Ca^{2+}-ATPase present on the plasma membrane [40].

Our studies of $^{45}Ca^{2+}$ uptake and release in saponin-permeabilized platelets examined the properties of the two compounds on the major platelet Ca^{2+}-regulatory machinery, i.e. that of the intracellular sequestering organelles. In saponin-permeabilized platelets Ins(1,4,5)P_3 only released a fraction of the total non-mitochondrial Ca^{2+} pool. Maximally effective concentrations of both Tg and tBuBHQ when added to stores that had already sequestered Ca^{2+}, a condition that would be operative in resting intact platelets, did not totally release the stores, but released a fraction which was greater and which totally overlapped the Ins(1,4,5)P_3-sensitive fraction. This was confirmed in experiments where co-administration of Ins(1,4,5)P_3 with Tg or tBuBHQ to saponin-permeabilized platelets resulted in a non-additive Ca^{2+} release, and in fact was the same as with the Ca^{2+}-ATPase inhibitors alone. These findings indicated that the Ca^{2+}-ATPase inhibitors would be useful to deplete the Ins(1,4,5)P_3-sensitive pool in intact cells without the complexity of other signals generated as a consequence of surface receptor occupancy. Our studies also showed that Tg did not interfere with the Ins(1,4,5)P_3 receptor, as it was unable to affect the binding of [^{32}P]Ins(1,4,5)P_3 to platelet membranes. Whether Ca^{2+} release by Tg or tBuBHQ occurs via the Ca^{2+}-ATPase itself or at another distinct site is not known, but a study on AR42J cells indicated that tBuBHQ may release Ca^{2+} via a passive channel which could be inhibited by manoalide [42].

In indomethacin-treated Fura 2-labelled platelets, both agents

raised cytosolic Ca^{2+} levels by similar amounts and from a similar pool. The peak levels raised are much less than those seen with thrombin (or U46619; results not shown). Earlier studies by others have reported higher levels raised by thapsigargin [28,29]; however, in those studies inhibitors of cyclo-oxygenase activity were not included. Indeed, Tg has been shown to be a good stimulator of thromboxane production [30,43], and thus the large Ca^{2+} elevation seen in the earlier studies was due to secondary thromboxane production. Interestingly, tBuBHQ has been shown to inhibit formation of thromboxane B_2 [30], and thus the Ca^{2+} elevation seen reflects its activity on the Ca^{2+}-ATPase alone.

It is important to determine whether agonists such as thrombin or thromboxane are able to release Ca^{2+} from only the InsP_3-sensitive stores or whether the additional stores are also utilized during platelet activation. As membrane-penetrating Ins(1,4,5)P_3 analogues are not available, this cannot be tested directly. In the presence of EGTA, the release of Ca^{2+} by Tg or tBuBHQ reflects release from a pool which totally overlaps the InsP_3-sensitive pool as determined by the experiments on saponin-permeabilized platelets. Under these conditions re-uptake into the stores by the Ca^{2+}-ATPase would also be inhibited. The addition of thrombin to Tg- or tBuBHQ-treated platelets led to a further transient elevation of cytosolic Ca^{2+}, reflecting usage of Ca^{2+} pools insensitive to the Ca^{2+}-ATPase inhibitors (Figure 4). Qualitatively similar results were obtained if Ca^{2+} is present on the outside, leading to more sustained levels of intracellular [Ca^{2+}] reflecting the additional component of Ca^{2+} entry. Similar results have been reported by Brune and Ullrich [43], who interpret these findings by suggesting two pools: one which is sensitive to the Ca^{2+}-ATPase inhibitors and one which is agonist [Ins(1,4,5)P_3]-sensitive. However, our studies with saponin-permeabilized platelets indicated that the InsP_3-sensitive pool was a subset of the Ca^{2+}-ATPase-inhibitors-sensitive pool, implying that thrombin is able to mobilize both the Ins(1,4,5)P_3-sensitive and -insensitive pools. The depletion of the InsP_3-sensitive pools by Tg and tBuBHQ in intact platelets does not affect the state of the Ca^{2+} pool that is insensitive to these inhibitors. The mechanism associated with Ca^{2+} release from the Ca^{2+}-ATPase-inhibitors-insensitive pool by agonists needs to be elucidated. Currently, Ins(1,4,5)P_3 is the only identified physiologically important intracellular Ca^{2+} messenger, and it has been suggested that agonists may utilize Ins(1,4,5)P_3-insensitive pools by either a linkage process or a Ca^{2+}-induced Ca^{2+}-release mechanism [14,44]. In other cell types, candidates that may serve a linkage role include GTP [44], perhaps mediated by a small G-protein [45], and Ins(1,3,4,5)P_4, which has been shown to stimulate resequestration [46] and release [47]. In saponin-permeabilized platelets GTP alone has little action on Ca^{2+} sequestration or release [48]. A Ca^{2+}-induced Ca^{2+}-release mechanism which may be important and activated by agonists is clearly not activated by Tg or tBuBHQ.

The effects of the two agents on intact cells reveal the extent of functional responses expressed when the Ins(1,4,5)P_3-sensitive pool was discharged by Tg and tBuBHQ. In the presence of indomethacin, tBuBHQ induced shape change, but no aggregation or secretion responses under conditions where thrombin and U46619 gave good responses. This reflected that the intracellular [Ca^{2+}] achieved by tBuBHQ was only sufficient to cause shape change, but not enough to give aggregation or secretion. However, Tg did produce a slow aggregation and secretory response, which implied additional actions of Tg over tBuBHQ. Both compounds showed good synergistic dense-granule secretory responses when added with a protein kinase C activator, DiC_8, indicating that the pool of Ca^{2+} mobilized was functionally relevant, and also confirmed the synergistic action of protein kinase C activation and Ca^{2+} elevation in promoting secretion. The additional actions of Tg to that of tBuBHQ were also reflected when release of [^{3}H]AA and protein phosphorylation were measured in intact platelets. The ability of Tg to release [^{3}H]AA compared well with that of thrombin, and was found to consist of at least two components. The aggregation inhibitor RGDS and the protein kinase inhibitor staurosporine inhibited Tg-induced [^{3}H]AA release to the levels seen with tBuBHQ, but neither agent was effective at inhibiting tBuBHQ-induced [^{3}H]AA release. This indicated that [^{3}H]AA release by tBuBHQ was due to its effects on Ca^{2+} mobilization, leading to phospholipase A_2 activation, but with Tg an additional component due to activation of protein kinases and/or aggregation also contributed to [^{3}H]AA release. Our studies on Tg- and tBuBHQ-induced protein phosphorylation in [^{32}P]P_i-labelled cells indicated increased incorporation of phosphate into myosin light chain (giving rise to shape change) and also into the 45 kDa protein pleckstrin. In a number of experiments, Tg stimulated phosphorylation of the 45 kDa protein to a higher extent than did tBuBHQ. This may account for the differences obtained in aggregation, secretion and [^{3}H]AA release shown by Tg and tBuBHQ. Recently Tg has been shown to phosphorylate a number of proteins on tyrosine residues [49]. A 130 kDa protein has been suggested to be important in Ca^{2+} fluxes, because its phosphorylation state appears to be antagonistically controlled by Ca^{2+} in the storage organelle and in the cytosol [49]. It would be of interest to determine the differences between the tyrosine-phosphorylation patterns of Tg and tBuBHQ which may also contribute to the different functional responses obtained.

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