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Effect of Membrane-Permeable Sulfhydryl Reagents and Depletion of Glutathione on Calcium Mobilisation in Human Platelets

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Abstract. Exposure to peroxides is known to increase the sensitivity of platelets towards activation by agonists. Similar platelet-activating effects are induced by sulfhydryl reagents that evoke Ca^{2+}-induced Ca^{2+} release (CICR) by stimulating the Ca^{2+}-releasing property of the inositol-1,4,5-trisphosphate receptor. We questioned whether these compounds may act by mobilising intracellular calcium in platelets by altering the intracellular glutathione redox state. Using FURA2-loaded, aspirin-treated platelets, Ca^{2+} signals were studied following exposure to the membrane-permeable sulfhydryl reagents, thimerosal and disulfiram, the glutathione peroxidase substrate, tert-butyl hydroperoxide, and the inhibitor of glutathione reductase, 1,3-bis(2-chloroethyl)-1-nitrosourea (BCNU). In single platelets monitored by fluorescence imaging techniques, thimerosal and disulfiram elicited repetitive spiking in $[Ca^{2+}]_i$ after variable lag times, indicating that these compounds stimulated CICR. BCNU caused $[Ca^{2+}]_i$ spiking of only low amplitude, whereas tert-butyl hydroperoxide was inactive. In platelets in suspension devoid of extracellular $CaCl_2$, the sulfhydryl reagents, at concentrations which decreased glutathione by 25%, strongly increased the Ca^{2+} responses of agonists that stimulated phospholipase C (thrombin) or acted independently of phospholipase C stimulation (thapsigargin). However, Ca^{2+} release was only slightly promoted by concentrations of BCNU that resulted in substantial depletion of the glutathione level. Tert-butyl hydroperoxide was without effect on glutathione, but partially inhibited Ca^{2+} mobilisation with these agonists. It is concluded that, in platelets, the potent CICR-promoting effects of sulfhydryl reagents are not solely due to their reaction with intracellular glutathione, but that extensive reduction in glutathione content is associated with Ca^{2+} mobilisation and CICR. BIOCHEM PHARMACOL **53**;10: 1533–1542, 1997. © 1997 Elsevier Science Inc.

KEY WORDS. platelet; Ca^{2+}-induced Ca^{2+} release; inositol 1,4,5-trisphosphate; FURA2; glutathione; sulfhydryl reagent

In non-electrically excitable cell types, mobilisation of Ca^{2+} from the intracellular stores is subjected to a Ca^{2+}-stimulated mechanism that has been denoted Ca^{2+}-induced Ca^{2+} release (CICR) or Ca^{2+} excitability of the endoplasmatic reticular membranes [1–3]. The mechanism underlying CICR, i.e., the potentiating effect of cytosolic Ca^{2+} concentration $[Ca^{2+}]_i$ on reticular Ca^{2+} release, is considered to be brought about by the inositol-1,4,5-trisphosphate ($InsP_3$) receptor function. It is envisioned that the Ca^{2+} channels that are opened by $InsP_3$ binding to the receptor have a bell-shaped sensitivity to the concentrations of both Ca^{2+} and $InsP_3$, in such a way that a moderate increase in either concentration promotes channel opening, while a higher than tenfold increase is inhibitory [2, 3]. The process of CICR is thought to underlie the oscillatory changes in $[Ca^{2+}]_i$ often observed in individual cells of several kinds stimulated by $InsP_3$-generating receptor agonists [1–3].

In various cell types, the sulfhydryl reagent thimerosal evokes oscillations in $[Ca^{2+}]_i$ that closely resemble those elicited by $InsP_3$-forming agonists [4–6]. There is good evidence that thimerosal acts in the absence of phospholipase C stimulation by sensitising the $InsP_3$ receptor and, thus, by stimulating CICR [4, 5, 7–10]. We have previously described that single platelets responded by steep increases in $[Ca^{2+}]_i$ followed by spiking or oscillatory changes in $[Ca^{2+}]_i$, regardless of whether the cells were stimulated with $InsP_3$-elevating agonists (e.g., ADP or thrombin) or with compounds acting independently of $InsP_3$ generation (e.g., the endomembrane Ca^{2+}-ATPase inhibitor, thapsigargin) [11, 12]. Since thimerosal potentiated the Ca^{2+}-mobilising effect of either type of agonist, it was concluded that it may

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Abbreviations: BCNU, 1,3-bis(2-chloroethyl)-1-nitrosourea; CDNB, 1-chloro-2,4-dinitrobenzene; CICR, Ca^{2+}-induced Ca^{2+} release; GSH, reduced glutathione; GSSG, oxidised glutathione; $InsP_3$, inositol 1,4,5-trisphosphate.

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have a stimulatory effect on CICR in platelets, similarly to its effect in other cells. This suggestion has basically been confirmed by others, who observed that such sulfhydryl reagents are capable of stimulating the release of Ca^{2+} from platelet internal membrane fractions [13]. Thimerosal also induces platelet aggregation, partially due to its Ca^{2+}-mobilising effect [14]. It is therefore to be expected that the CICR-promoting effect of thimerosal, and probably of other membrane-permeable sulfhydryl reagents as well, causes oscillatory or spiking Ca^{2+} responses in single platelets, although this has not yet been reported.

Reduced glutathione (GSH) is the most abundant non-proteinous sulfhydryl compound in many cell types, with intracellular concentrations ranging from 0.5 to 10 mM [15]. Since its oxidised dimer, oxidised glutathione (GSSG), once generated by glutathione peroxidase, is continuously converted back to the reduced form by glutathione reductase, GSH is by far the most abundant form in resting cells [15]. In permeabilised hepatocytes, evidence has been collected that oxidised GSSG, but not GSH, stimulates the Ca^{2+} channel function of the $InsP_3$ receptors by modifying the oxidation state of critical sulfhydryl groups regulating Ca^{2+} permeability [16, 17]. In general, lipid peroxides such as tert-butyl hydroperoxide appear to have a potent oxidising effect on intracellular GSH [18–21], which is accompanied by a stimulatory effect on Ca^{2+} mobilisation in endothelial cells [18]. In these cell systems, tert-butyl hydroperoxide is also known to deplete the Ca^{2+} content of $InsP_3$-sensitive stores [22] and sensitise the $InsP_3$ receptors for Ca^{2+} release [23]. Accordingly, the intracellular glutathione redox state may be one of the controlling elements of $InsP_3$ receptor functioning.

It is well known that hydrogen peroxide and lipid peroxides [24, 25], as do sulfhydryl reagents [14, 26], stimulate the activation and aggregation properties of platelets. Since all these compounds in principle are capable of reducing intracellular GSH concentration [22, 27], it can be hypothesised that their platelet-activating effect may involve a common pathway: stimulation of $InsP_3$ receptor-mediated mobilisation of Ca^{2+} due to depletion and/or oxidation of GSH. In this paper, we investigated whether modification of the GSH concentration in platelets can influence Ca^{2+} release, and whether the Ca^{2+}-mobilising effects of sulfhydryl reagents are indeed secondary to GSH depletion. We thus compared the effects on Ca^{2+} signalling and glutathione levels of two membrane-permeable sulfhydryl reagents, thimerosal and disulfiram [26], of the substrate of glutathione peroxidase, tert-butyl hydroperoxide [18, 28], and of the specific inhibitor of glutathione reductase [29], 1,3-bis(2-chloroethyl)-1-nitrosourea (BCNU).

MATERIALS AND METHODS

Materials

BCNU was a gift from Bristol-Myers Squibb (Evansville, IN, USA). Thimerosal (ethylmercuri-thiosalicylic acid, sodium salt) was purchased from Janssen, Beersse, Belgium; disulfiram and tert-butyl hydroperoxide were obtained from Aldrich (Milwaukee, Wisc, USA); 5,5′-dithio-bis(2-nitrobenzoic acid) was purchased from Across (Geel, Belgium). Glutathione reductase, NADPH, reduced glutathione, thapsigargin, 1-chloro-2,4-dinitrobenzene (CDNB), bovine serum albumin (BSA) and bovine α-thrombin were purchased from Sigma (St. Louis, MO, USA), and FURA2 acetoxymethyl ester was supplied by Molecular Probes (Eugene, OR, USA). All other reagents were of analytical grade.

Platelet Isolation and Activation

Human platelets were isolated from healthy volunteers, as described previously [11]. Briefly, the cells were treated with 100 μM aspirin (acetyl salicylic acid) and incubated with 1 μM FURA2 acetoxymethyl ester at 37°C for 45 min, except where indicated otherwise. After two wash steps, the platelets were suspended in HEPES buffer (pH 7.45) of the following composition (in mM): NaCl 145, HEPES 10, D-glucose 10, KCl 5, $MgSO_4$ 1, BSA 0.1% (w/v) and apyrase (0.1 U ADPase/mL) at a concentration of 1×10^8 platelets/mL. Platelets were activated with thrombin (0.5 nM) or thapsigargin (100 nM) in the presence of either EGTA (1 mM) or $CaCl_2$ (1 mM), under stirring at 37°C. Where indicated, the FURA2-loaded cells were incubated (37°C) with tert-butyl hydroperoxide, thimerosal or disulfiram for 5 min, or with BCNU for 30 min before the addition of other agonists.

Measurements of Cytosolic $[Ca^{2+}]_i$

Fluorescence from suspensions of aspirin-treated, FURA2-loaded platelets was recorded with an SLM/AMINCO (Urbana, IL, USA) spectrofluorometer. For determination of $[Ca^{2+}]_i$, the ratio of fluorescence at excitation wavelengths of 340 and 380 nm was calibrated according to Grynkiewics et al. [30], as described previously [31]. All fluorescent signals were corrected for background fluorescence of buffer and agonists. Levels of $[Ca^{2+}]_i$ were always corrected for the changes in FURA2 fluorescence that occurred in the absence of sulfhydryl or glutathione reagents.

For single cell measurements, aspirin-treated, FURA2-loaded platelets were suspended in HEPES buffer supplemented with apyrase (0.2 U ADPase/mL), 1% (w/v) BSA and 10 μM H-Arg-Gly-Asp-Ser-OH. The platelets were allowed to adhere to a coverslip coated with fibrinogen, which was mounted in an open perfusion chamber placed on a Nikon Diaphot inverted microscope (Nikon, Tokyo, Japan) [32]. After 10 min of adhesion, unattached cells were washed away and the immobilised platelets were bathed in 0.5 mL HEPES buffer supplemented with H-Arg-Gly-Asp-Ser-OH (to prevent spreading of the platelets) in the presence or absence of 1 mM $CaCl_2$. Experiments were carried out at 37°C or at room temperature, as indicated.

Changes in FURA2 fluorescence were measured using a Quanticell fluorometric system (Applied Imaging, Sunderland, UK), basically following previously described procedures [30]. The excitation light was passed alternately through 340 and 380 nm band pass filters, and fluorescent light above 505 nm was detected by a charge-coupled device camera from Photonic Sciences (Robertsbridge, UK), working at standard video rate. Fluorescence images were digitised, background-substracted and averaged 4 times, ratios were calculated, and the resulting averaged ratio images (obtained every 2 sec) were stored on a 650 MByte read/write optical disc. As before [11], data of single cell measurements are expressed as changes in the 340/380 nm fluorescence ratio instead of changes in $[Ca^{2+}]_i$, because tentative calibration measurements revealed considerable cell to cell variability in the calibration parameters.

Determination of Total and Reduced Glutathione

Aspirin-treated platelets (2×10^9/4 mL) were suspended in HEPES buffer pH 7.45 in the presence of 1 mM EGTA. After incubation with glutathione reagent or sulfhydryl reagent under stirring at 37°C, the platelets were centrifuged at 9000 × g for 1 min. Pellets were resuspended in 200 μL of 100 mM phosphate buffer pH 7.4, after which an equal volume of 8% (w/v) trichloric acid containing 4 mM EDTA was added. The resulting mixture was immediately centrifuged at 9000 × g for 5 min, and the supernatant was used for measurements of total and reduced glutathione.

Reduced glutathione (GSH) was determined using a spectrophotometric assay based on the reaction of free thiol groups with 5,5′-dithio-*bis*(2-nitrobenzoic acid), DTNB, which was detected at a wavelength of 412 nm, essentially as described by Anderson [33]. Briefly, 200 μL supernatant was mixed with 225 μL of 500 mM Tris/HCl (pH 8.9). Subsequently, 400 μL of this mixture was added to a 1 mL cuvette containing 600 μL of a freshly prepared solution of 1 mM 5,5′-dithio-*bis*(2-nitrobenzoic acid) in phosphate buffer. Absorbance at 412 nm was then measured after 10 min when the reaction was complete. These procedures were carried out in a nitrogen atmosphere.

Total glutathione was measured by a cyclic assay based on Tietze [34]. Briefly, 200 μL supernatant was diluted three times with phosphate buffer, and 200 μL of the dilution was added to a 1 mL cuvette containing 800 μL of a freshly prepared solution of 0.26 mM NADPH, 5.5 mM EDTA and 0.75 mM 5,5′-dithio-*bis*(2-nitrobenzoic acid) in phosphate buffer. After 2.5 min of equilibration, 50 μL of a solution of glutathione reductase (21 U/mL in phosphate buffer containing 6.3 mM EDTA) was added. Exactly 30 sec after this addition, the change in absorbance at 412 nm was recorded during a 1-min period. Concentrations of total and reduced glutathione were calculated using reference solutions of commercially available GSH. Apparent mean platelet volume was determined using a Coulter counter. Intracellular glutathione concentrations in platelets were calculated by assuming an averaged volume of platelet cytosol, taken as 85% of the apparent mean volume [35], of 6 ± 0.3 fl. Platelet treatment with sulfhydryl or glutathione reagents influenced the measured apparent volume by an average of only 7%.

Statistics

Statistical significance was determined by two-sided Student's t-test for paired observations.

RESULTS

Effects of Sulfhydryl and Glutathione Reagents on $[Ca^{2+}]_i$ in Suspended and Single, Immobilised Platelets

Human platelets were loaded with the Ca^{2+} probe FURA2 in the presence of aspirin to inhibit cyclooxygenase activity and block the formation of platelet-activating thromboxane A_2. In suspensions of these platelets, levels of resting $[Ca^{2+}]_i$ were 44 ± 3 nM. The membrane-permeable sulfhydryl reagents, thimerosal and disulfiram, and the glutathione reagent BCNU caused gradual, time- and concentration-dependent increases in $[Ca^{2+}]_i$ (data not shown, but see below). After 5 min of incubation, 10 μM thimerosal, 20 μM disulfiram and 30 μM BCNU raised $[Ca^{2+}]_i$ in platelets by 41 ± 9, 22 ± 9 and 10 ± 1 nM, respectively (mean values ± SEM, n = 7–8). In contrast, the glutathione peroxidase substrate, tert-butyl hydroperoxide (10 μM), had no appreciable Ca^{2+}-elevating effect under these conditions.

Studies with single platelets immobilised on fibrinogen without activation revealed that both thimerosal (10 μM) and disulfiram (20 μM) elicited repetitive spiking in $[Ca^{2+}]_i$ in 59% and 68% of the observed cells, respectively (Fig. 1A, C). With either sulfhydryl reagent, Ca^{2+} spiking started only after a lag phase of 3.5–7 min, and reached a frequency of 1–2/min (n = 20–22 platelets). Averaging of the Ca^{2+} signals from 20 cells that were spiking in response to thimerosal or disulfiram resulted in a gradual increase in $[Ca^{2+}]_i$, which resembled the Ca^{2+} response of a population of platelets in suspension (Fig. 1B, D). In single platelets, BCNU (30 μM) also evoked repetitive increases in $[Ca^{2+}]_i$ which, however, were preceded by a lag time of about 15 min and were of much lower amplitude than the spikes elicited by the sulfhydryl reagents (Fig. 2A–B). Typically, whereas Ca^{2+} spikes in response to thimerosal and disulfiram could be observed at temperatures of 20°C and 37°C, those with BCNU were only detectable at 37°C. Tert-butyl hydroperoxide (10 μM) evoked no or hardly detectable changes in $[Ca^{2+}]_i$ in single platelets (Fig. 2C–D).

Modulation of Agonist-induced Ca^{2+} Responses by Sulfhydryl Reagents

To further investigate the effects of the membrane-permeable sulfhydryl reagents on Ca^{2+} signalling, suspensions of aspirin-treated platelets were activated with various ago-

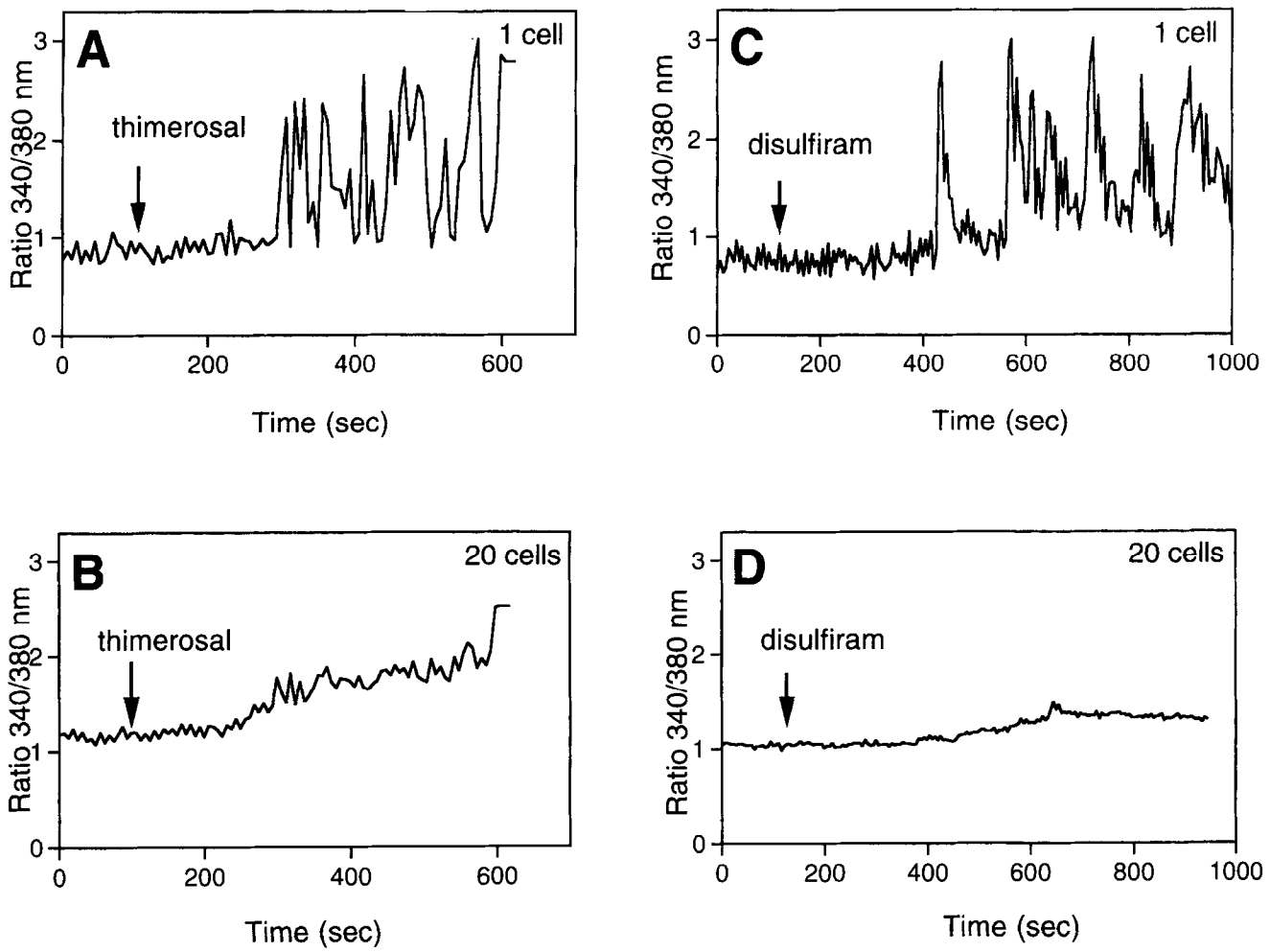

FIG. 1. Ca^{2+} responses of single, immobilised platelets to membrane-permeable sulfhydryl reagents. Platelets on fibrinogen were activated with 10 μM thimerosal (A, B) or 20 μM disulfiram (C, D) in the presence of 1 mM $CaCl_2$ at 20°C. Shown are representative traces of Ca^{2+} responses of individual cells (A, C), and integrated traces of Ca^{2+} responses of 20 cells (B, D). Changes in $[Ca^{2+}]_i$ are expressed as changes in the fluorescence ratio at 340/380 nm excitation.

nists in the presence or absence of thimerosal or disulfiram. These experiments were carried out in the presence of 1 mM EGTA to exclude Ca^{2+} signals due to the influx of external Ca^{2+}. Preincubation of platelets with 10 μM thimerosal or 20 μM disulfiram strongly potentiated the Ca^{2+} release induced by a sub-optimal (0.5 nM) concentration of thrombin (Fig. 3A–B), a phospholipase C-activating agonist. With higher doses of thrombin (4 nM), sufficient for maximal phospholipase C activation, $[Ca^{2+}]_i$ increases were no longer potentiated by these sulfhydryl reagents (data not shown). As observed before, the Ca^{2+}-ATPase inhibitor thapsigargin (100 nM), which acts independently of phospholipase C activation in aspirin-treated platelets [10], caused a gradual increase in $[Ca^{2+}]_i$ (Fig. 3C–D). Preincubation of the platelets with 10 μM thimerosal or 20 μM disulfiram potentiated these thapsigargin-evoked Ca^{2+} responses. Quantitative analysis of the data revealed that 10 μM thimerosal had a stronger potentiating effect than 20 μM disulfiram on the responses with both thrombin and thapsigargin (Fig. 4), although statistical significance was not reached. Five-fold higher concentrations of either sulfhydryl reagent mobilised more Ca^{2+} in the absence of co-agonist, but attenuated the subsequent thrombin-induced Ca^{2+} signals. The higher concentration of thimerosal reduced the thapsigargin-induced Ca^{2+} mobilisation, whereas that of disulfiram still stimulated the thapsigargin response (Fig. 4).

Modulation of Agonist-induced Ca^{2+} Responses by Glutathione Reagents

Tert-butyl hydroperoxide and BCNU caused marked reduction in intracellular GSH content in a number of cell types [19–21]. We first investigated the effects of these agents on platelet Ca^{2+} signalling. At a concentration of 10 μM, tert-butyl hydroperoxide by itself had only a minor Ca^{2+}-mobilising effect, whereas it inhibited the Ca^{2+} mobilisation induced by thrombin or thapsigargin (Fig. 5A–B). Quantitatively similar effects were found with higher doses of tert-butyl hydroperoxide, up to 100 μM (data not

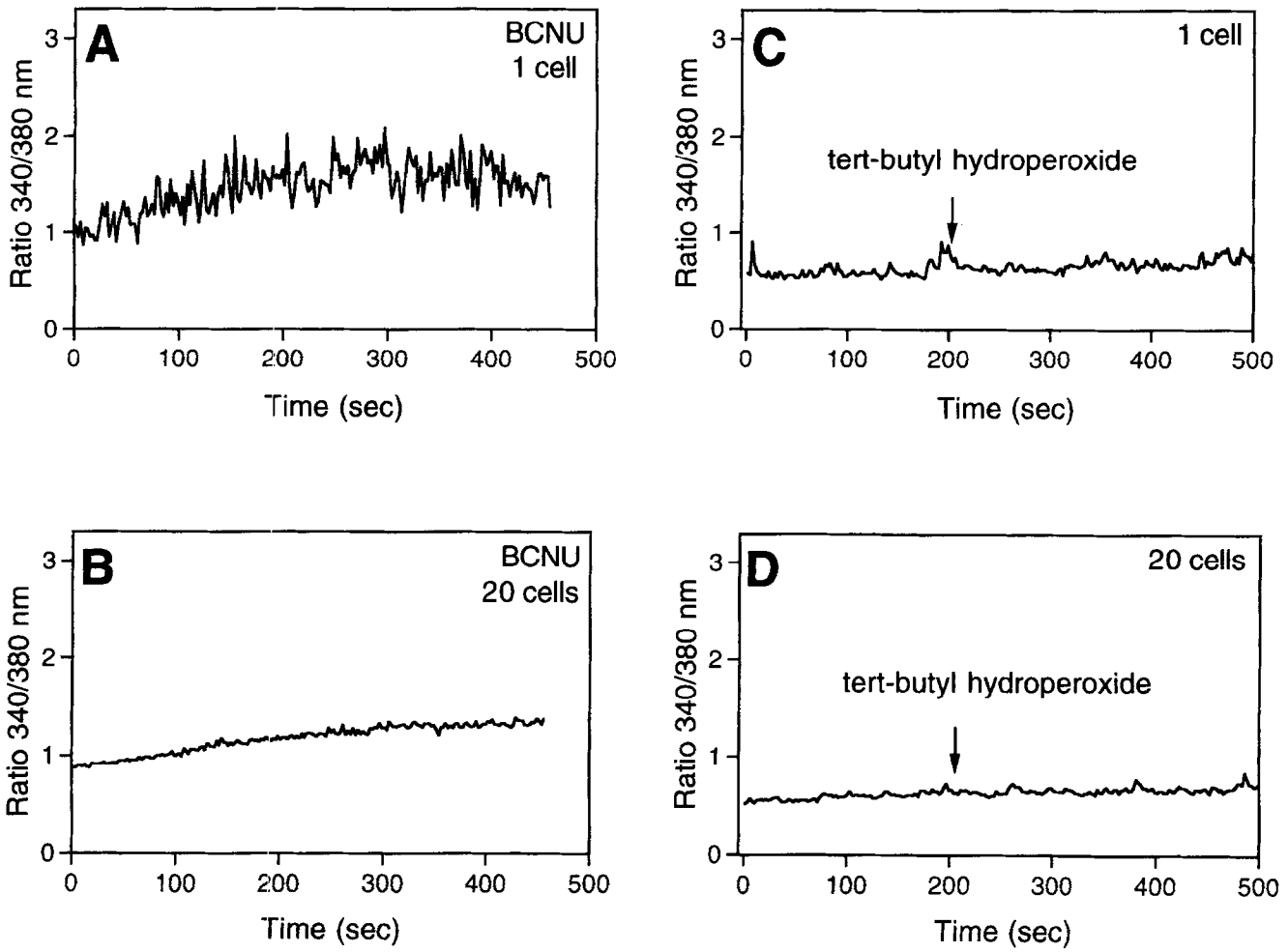

FIG. 2. Ca^{2+} responses of single, immobilised platelets to glutathione reagents. Platelets on fibrinogen were incubated with 30 μM BCNU (A, B) or 10 μM tert-butyl hydroperoxide (C, D) at a temperature of 37°C. BCNU was added 15 min before the start of the measurement. Shown are representative traces of Ca^{2+} responses of individual cells (A, C), and integrated traces of Ca^{2+} responses of 20 cells (B, D). Changes in $[Ca^{2+}]_i$ are expressed as changes in the fluorescence ratio at 340/380 nm excitation. Fluorescent values of resting cells were about 0.6.

shown). In contrast, BCNU, at an optimal dose of 30 μM, slightly stimulated the thapsigargin-induced Ca^{2+} response (Fig. 5C). Unfortunately, the effect of BCNU on thrombin-induced Ca^{2+} signalling could not be measured, since this reagent inhibited the proteolytic activity of thrombin (data not shown). Statistical analysis showed that the effects of both tert-butyl hydroperoxide and BCNU reached significance (Fig. 6).

Effects of Sulfhydryl and Glutathione Reagents on the Reduced and Total Glutathione Content in Platelets

Concentrations of reduced and total glutathione were measured under conditions similar to those of the $[Ca^{2+}]_i$ measurements. Incubation of the platelets with 10 μM thimerosal or 20 μM disulfiram resulted in a moderate decrease in the levels of both reduced and total glutathione of 20 to 30% (Table 1). Five-fold higher concentrations of these sulfhydryl reagents depleted platelet GSH levels by 55 to 70%. Typically, under the latter conditions, levels of measured total glutathione were equal to or sometimes even smaller than those of GSH. This was due to the fact that disulfiram and, to a lesser degree, thimerosal influenced the assay of the determination of total glutathione (data not shown). Indeed, disulfiram has been reported to have an inhibitory effect on the activity of glutathione reductase [36], the enzyme used in the assay for measuring total glutathione.

Preincubation of platelets with 10 or 25 μM tert-butyl hydroperoxide did not result in significant changes in levels of total or reduced glutathione (Table 1). In contrast, incubation with 30 μM BCNU resulted in a reduction of GSH by as much as 80%. Since BCNU is a potent inhibitor of glutathione reductase [29], the level of total glutathione could not be determined in the BCNU-treated platelets.

DISCUSSION

We have investigated the effects of membrane-permeable sulfhydryl and glutathione reagents on Ca^{2+} signalling in

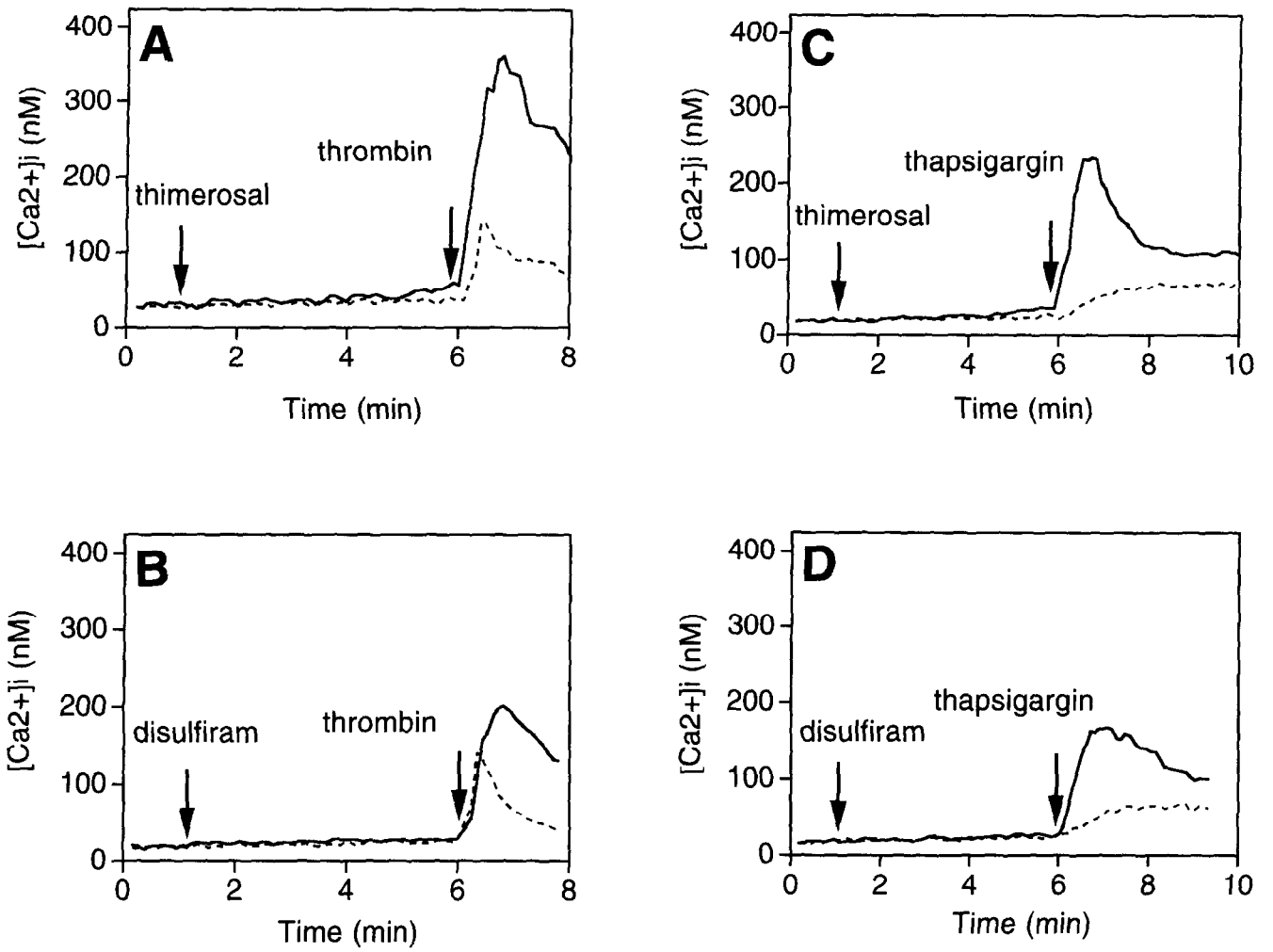

FIG. 3. Ca^{2+} responses of platelets in suspension to sulfhydryl reagents. Platelets were incubated for 5 min with EGTA-containing buffer alone (dashed lines) or with one of the following substances (solid lines): 10 μM thimerosal (A, C) or 20 μM disulfiram (B, D). The cells were then activated with 0.5 nM thrombin or 100 nM thapsigargin, as indicated. Traces of changes in $[Ca^{2+}]_i$ are given, representative of 6–8 experiments.

human platelets under conditions where secondary ADP- and thromboxane A_2-mediated effects were inhibited and influx of Ca^{2+} was not allowed to occur. It appeared that the sulfhydryl reagents thimerosal and disulfiram stimulated the release of Ca^{2+} and potentiated the Ca^{2+} signals evoked by other Ca^{2+}-mobilising agonists such as thrombin and thapsigargin. These Ca^{2+}-stimulating effects were accompanied by only moderate decreases in the intracellular levels of total and reduced glutathione. On the other hand, the potent glutathione reductase inhibitor BCNU stimulated the Ca^{2+} release in platelets only slightly, whereas it caused extensive depletion of glutathione. Tert-butyl hydroperoxide, a proposed substrate of glutathione peroxidase in endothelial cells [18, 19, 28], was inhibitory to the agonist-induced Ca^{2+} release in platelets under conditions which hardly influenced the intracellular glutathione levels. This lack of correlation between the effects of these compounds on Ca^{2+} mobilisation and glutathione content thus indicates that glutathione is not a major factor in determining the degree of Ca^{2+} store depletion. Accordingly, the potent stimulatory effects of the sulfhydryl reagents on platelet Ca^{2+} signalling can only be explained to a limited extent by their potential to reduce intracellular GSH.

Previous investigations have shown that the Ca^{2+} release process in platelets is subjected to CICR [11, 12], which is considered to be a crucial mechanism in the initiation and prolongation of Ca^{2+} oscillations and waves [2–5, 7, 8]. We report that sulfhydryl reagents such as thimerosal and disulfiram can induce repetitive spiking in $[Ca^{2+}]_i$ in platelets. Whereas oscillations in $[Ca^{2+}]_i$ in response to thimerosal have also been observed in other cell types [4–6, 37], those in platelets are characteristic with respect to their latency (3 to 6 min) and the high frequency of the Ca^{2+} spikes (1–2/min). Similar spiking effects were observed in single platelets stimulated with other sulfhydryl reagents, such as N-ethyl maleimide and high doses of its derivative, U73122, both of which completely suppress phospholipase C activity and $InsP_3$ generation. Since thimerosal, similarly, does not lead to the formation of

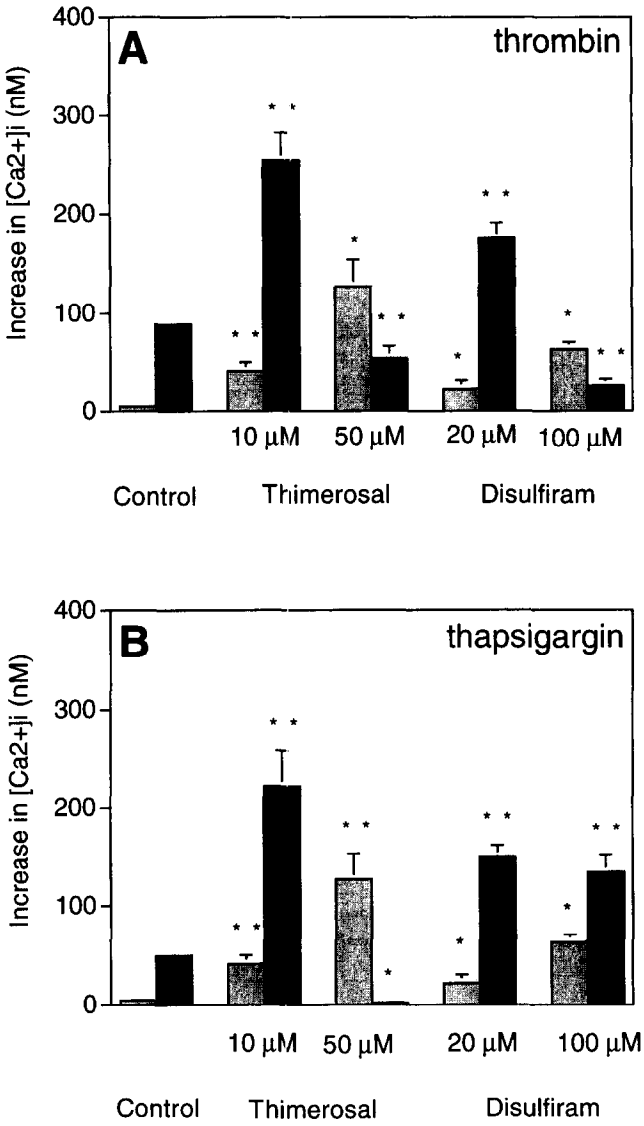

FIG. 4. Quantitative effects of membrane-permeable sulfhydryl reagents on agonist-induced changes in $[Ca^{2+}]_i$. Platelets in suspension were incubated for 5 min with EGTA-containing buffer alone (control), or in the presence of 20 and 100 μM disulfiram or 10 and 50 μM thimerosal. The cells were then activated with 0.5 nM thrombin (A) or 100 nM thapsigargin (B). Grey bars represent the measured increases in $[Ca^{2+}]_i$ during 5 min of incubation with buffer or sulfhydryl reagent; black bars represent maximal agonist-induced increases in $[Ca^{2+}]_i$. Data are mean values ± SEM of 6–8 independent experiments. Values significantly different from control conditions are marked as: * $p < 0.05$, ** $p < 0.01$ (two-sided Student's t-test for paired observations).

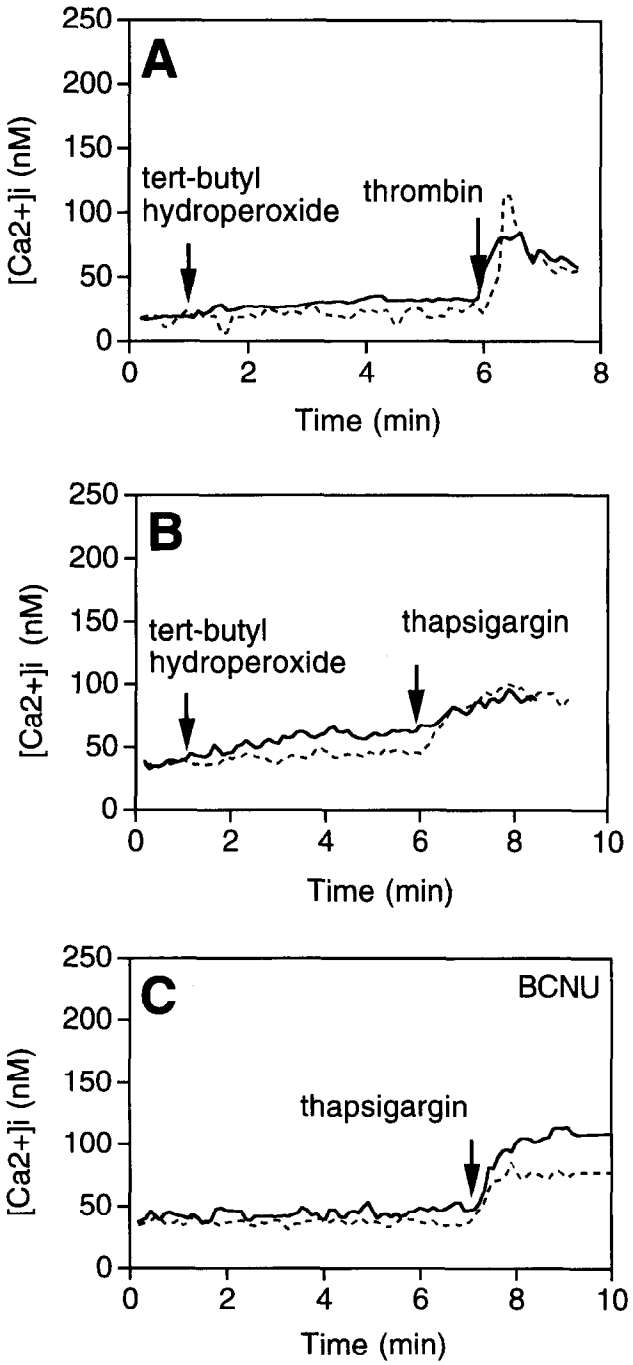

FIG. 5. Effects of glutathione reagents on agonist-induced Ca^{2+} responses of platelets in suspension. Platelets were incubated with EGTA-containing buffer alone (dashed lines) or in the presence of: (A, B) 10 μM tert-butyl hydroperoxide for 5 min or (C) 30 μM BCNU for 30 min (solid lines). The cells were then activated with 0.5 nM thrombin or 100 nM thapsigargin, as indicated. Traces of changes in $[Ca^{2+}]_i$ are given, representative of 6–8 experiments.

$InsP_3$ [14], its Ca^{2+}-mobilising effect can only be explained by either $InsP_3$ receptor sensitisation or inhibition of endomembrane Ca^{2+}-ATPase. However, the observation that the thimerosal-treated platelets continuously spike in $[Ca^{2+}]_i$ strongly argues against inhibition of the Ca^{2+}-ATPases (Fig. 1A and C), since the activity of these Ca^{2+} pumps is required for continuation of the oscillatory process [12, 38]. Inhibition of the Ca^{2+}-ATPases would prevent refilling of the stores and, thereby, Ca^{2+} spiking, such as was indeed observed in single platelets stimulated with thapsigargin [11]. Consequently, thimerosal and other sulfhydryl reagents most likely act on platelets by $InsP_3$ receptor sensitisation, as has been demonstrated for other cell types [4, 5, 7–10]. There is indeed good evidence that the $InsP_3$ receptors in platelets contain essential sulfhydryl

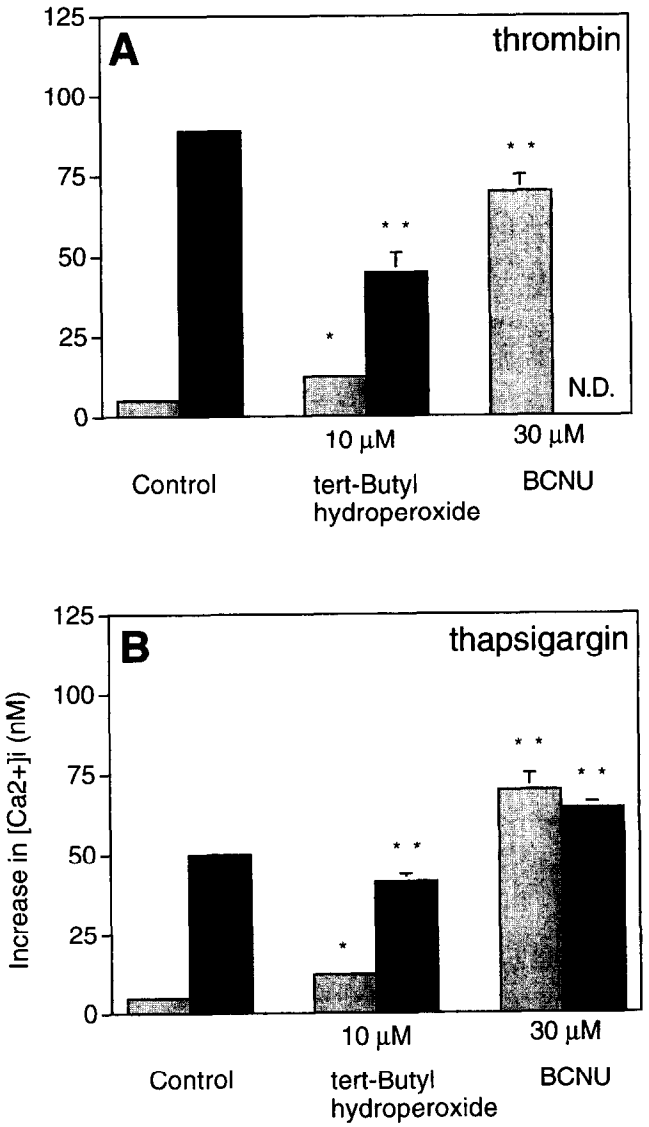

FIG. 6. Quantitative effects of glutathione reagents on agonist-induced changes in $[Ca^{2+}]_i$. Platelets in suspension were incubated during 5 min with EGTA-containing buffer alone (control) or in the presence of 10 μM tert-butyl hydroperoxide. Other platelets were incubated with 30 μM BCNU during 30 min. The cells were then activated with 0.5 nM thrombin (A) or 100 nM thapsigargin (B). Grey bars represent the measured rises in $[Ca^{2+}]_i$ during incubation with buffer or glutathione reagent; black bars represent maximal agonist-induced rises in $[Ca^{2+}]_i$. Data are mean values ± SEM of 6–8 independent experiments. Values significantly different from control conditions are marked as: * $p < 0.05$, ** $p < 0.01$ (two-sided Student's t-test for paired observations).

groups [13], and that disulfiram increases the receptor binding of $InsP_3$ [26].

Little is known of the glutathione concentration in platelets and its importance for the activation of these cells. Our data indicate that platelets contain a relatively low GSH level of about 2 mM, and that this level can be decreased without much influence on the Ca^{2+} signalling systems. Only at a reduced level of about 0.3 mM, i.e., such as that reached by prolonged incubation with BCNU, could distortions from normal Ca^{2+} homeostasis be observed. We can thus conclude that the millimolar concentration of glutathione present in resting platelets is a sufficient buffer to effectively prevent thiol oxidation of $InsP_3$ receptors and Ca^{2+}-ATPases. In single platelets, such prolonged treatment with BCNU resulted in repetitive Ca^{2+} spikes of rather low amplitude, which appeared only after prolonged lag times (15 min) and in experiments conducted at 37°C (Fig. 2). This contrasted with the spikes induced by thimerosal and disulfiram, which were detectable after much shorter lag times and at temperatures of 20°C and 37°C. This suggests that the effect of BCNU is due to enzymatic activity unrelated to the handling of intracellular Ca^{2+} which, indeed, is consistent with its known inhibitory effect on glutathione reductase [29]. In other cell systems, BCNU was reported to promote the formation of GSSG [22], and GSSG was shown to increase the binding of $InsP_3$ to its receptor [8, 16, 17]. Our data thus suggest that GSSG generated during prolonged incubation of platelets with BCNU may stimulate the Ca^{2+}-releasing property of the $InsP_3$ receptor. Evidence that the formation of GSSG, and not the depletion of GSH, causes Ca^{2+} release, was obtained by incubating platelets with the glutathione-S-transferase substrate, CDNB, which depletes intracellular GSH without GSSG formation [39]. In single, CDNB-treated platelets, no changes in $[Ca^{2+}]_i$ were observed (results not shown).

As far as we are aware, this is the first report with intact cells in which compounds inducing severe reduction of intracellular glutathione, such as BCNU, evoke spiking in $[Ca^{2+}]_i$. Typically, these Ca^{2+} signals in platelets were of much lower amplitude than those evoked by receptor agonists or by sulfhydryl reagents (Fig. 2A). Small, repetitive increases in $[Ca^{2+}]_i$ of low amplitude have also been observed in single cells of other types that were close to or in resting conditions, and these are usually referred to as 'Ca^{2+}-puffs' (reviewed in Ref. 3) or 'Ca^{2+}-blips' [40]. These elementary Ca^{2+} signals may result from the activation of single Ca^{2+}-release units or even individual $InsP_3$ receptors in the cell in response to threshold elevations of $InsP_3$ [3, 40]. Although we have no direct proof for this, our data concerning glutathione depletion and $[Ca^{2+}]_i$ spikes suggest that the BCNU-induced conversion of GSH into GSSG may also result in unitary Ca^{2+} release, possibly by weak sensitisation of the $InsP_3$ receptors.

Incubation of platelets with tert-butyl hydroperoxide resulted in attenuation of both the thrombin- and thapsigargin-induced Ca^{2+} responses, and in no more than small decreases (about 10%) in the concentrations of total and reduced glutathione. Regardless of the way of action of tert-butyl hydroperoxide, the Ca^{2+}-modifying effects are unlikely to be due to oxidation of GSH.

Taken together, our results suggest that $InsP_3$ receptor-mediated release of Ca^{2+} in platelets is potentiated by membrane-permeable sulfhydryl reagents such as thimerosal and disulfiram, and that this potentiation is not a direct consequence of reduction in intracellular GSH concentra-

TABLE 1. Effects of various reagents on reduced and total glutathione content in platelets. Platelets were incubated with thimerosal (5 min), disulfiram (5 min), *tert*-butyl hydroperoxide (5 min) or BCNU (30 min) in the presence of 1 mM EGTA at 37°C

Incubation condition		Reduced glutathione (mM)	Total glutathione (mM)
Control*		1.64 ± 0.12	1.97 ± 0.12
Thimerosal	10 μM	1.16 ± 0.17 (70.7%)	1.46 ± 0.28 (74.1%)
	50 μM	0.48 ± 0.12 (29.3%)	0.47 ± 0.19 (23.8%)
Disulfiram	20 μM	1.40 ± 0.17 (85.4%)	1.46 ± 0.21 (74.1%)
	100 μM	0.73 ± 0.16 (44.5%)	0.43 ± 0.08 (22.1%)
tert-Butyl hydroperoxide	10 μM	1.44 ± 0.22 (87.8%)	1.81 ± 0.12 (91.9%)
	25 μM	1.59 ± 0.53 (96.9%)	1.87 ± 0.32 (94.9%)
BCNU	30 μM	0.28 ± 0.04 (17.1%)	N.D.**

Total and reduced glutathione were determined as described in Materials and Methods. Data are expressed as intracellular concentrations (mean values ± SEM of 3–5 independent experiments).

* N = 14; ** N.D., not determined because of the inhibitory effect of BCNU on the glutathione reductase used in the assay.

tion. On the other hand, the data also indicate that extensive oxidation of GSH into GSSG, as accomplished by BCNU, results in only moderate sensitisation of Ca^{2+} release. Thus, under physiological conditions, the glutathione redox state is unlikely to be a major factor regulating the process of CICR.

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