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EFFECTS OF TOLUENE ON PLATELET MEMBRANE GLYCOPROTEIN Ib AND ACTIN-BINDING PROTEIN*

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Abstract—Effects of the organic solvent toluene on the platelet membrane receptor glycoprotein Ib (GP Ib) and the cytoskeletal protein, actin-binding protein (ABP), were studied and related to the effects of the local anesthetic dibucaine. The glycocalicin-region of GP Ib contains the binding site for von Willebrand factor; intracellularly GP Ib is linked to the cytoskeleton via ABP. Both GP Ib and ABP are substrates for a calcium-dependent protease, calpain. Washed platelets were incubated with toluene or dibucaine. The toluene concentration in the platelet suspension was analysed by gas chromatography. Using 1.5–2.8 mmol/L toluene, calpain was activated, leading to degradation of ABP and release of glycocalicin from GP Ib. The latter phenomenon was paralleled by a reduced von Willebrand factor-induced platelet agglutination. At lower toluene concentrations (0.3–1.4 mmol/L), degradation of ABP was not detected but an initial increased agglutination that declined to the control level with time was observed. These effects of toluene on the GP Ib–ABP complex are similar to those observed with 1 mmol/L dibucaine. The lowest toluene concentrations used correspond to those that have been found in blood from toluene abusers ("sniffers").

Long-term abuse of toluene-containing solvents as well as occupational exposure to toluene may lead to chronic neurological toxicity, such as cerebellar disease and chronic encephalopathy [1]. The biochemical mechanisms involved in the development of injuries like these are not, at present, fully understood. However, neuronal membranes are rich in lipid and, as a highly lipophilic substance, the organic solvent toluene partitions easily into such membranes [2] and acts possibly by binding to hydrophobic pockets on integral proteins [3]. It is therefore of considerble interest to study how toluene affects membrane proteins.

Despite functional and structural differences, human blood platelets and neurons have structural similarities that have made the easily available platelets interesting as models for neurons [4]. Thus, the platelets are, in several aspects, relevant for studies of the effects of organic solvents on membranes. Like toluene, the tertiary amine local anesthetics are lipophilic substances. In addition to their well-known inhibitory effect on neuronal responses, local anesthetics modify several non-neuronal activities and have been shown to induce platelet membrane alterations [5, 6]. Previous studies have shown that the local anesthetic dibucaine [7, 8] exerts specific effects on the platelet membrane glycoprotein Ib (GP Ib§) and the cytoskeletal protein ABP. These effects are mediated via activation of calpain, a calcium-dependent neutral thiol protease present in platelets.

GP Ib is a transmembrane protein consisting of two disulfide-linked polypeptide chains, the α-chain of 145 kDa and the β-chain of 25 kDa [9]. The highly glycosylated extracellular end of the GP Ib α-chain is called glycocalicin. This region functions as a binding site for von Willebrand factor, a plasma protein which links platelets to a damaged vessel wall [10–13]. GP Ib is associated with the submembraneous actin cytoskeleton via ABP [7, 14–16]. ABP is a dimer consisting of two identical M_r = 250,000 subunits that are proteolysed by activated calpain into two main degradation products of M_r = 190,000 and 90,000 [17–19].

In this paper, we present data showing that the organic solvent toluene, in concentrations relevant to the level found in toluene abusers ("sniffers") [20], has an effect on the human platelet GP Ib–ABP complex comparable to that of dibucaine. This demonstrates a marked effect of toluene on certain membrane-associated proteins.

MATERIALS AND METHODS

Commercial materials. Acrylamide, *N,N'*-dimethylene-bis-acrylamide, molecular weight standards (high), nitrocellulose membrane (0.45 μm) and *N,N,N,N*-tetramethyl ethylenediamine (TEMED) were from Bio Rad Laboratories (Richmond, CA, U.S.A.); and dimethylsulfoxide (DMSO) was from BDH Chemicals Ltd (Poole, U.K.). Coomassie Brilliant Blue G and R, dibucaine (cinchocaine hydrochloride), Nonidet P-40 (NP-40), *o*-dianisidine,

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§ Abbreviations: ABP, actin-binding protein; GP Ib, platelet membrane glycoprotein Ib, NP-40, Nonidet P-40; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; TTG buffer, Tris–glycine buffer containing 1% Triton X-100; TS buffer, Tris–saline buffer.

pyronin Y, sodium dodecyl sulfate (SDS) and Tris were from the Sigma Chemical Co. (St Louis, MO, U.S.A.). Na_2-EDTA, H_2O_2, methanol, toluene (p.a. 99.5% purity) and Triton X-100 were from Merck (Darmstadt, F.R.G.). Gelatine was from Difco Laboratories (Detroit, MI, U.S.A.); agarose (grade HSA) from Litex (Copenhagen, Denmark) and leupeptin from Peptide Institute Inc. (Hyogo-Ken, Japan). Horseradish peroxidase-conjugated rabbit anti-mouse antiserum was from Dako Immunoglobulins (Copenhagen, Denmark). Calpeptin was a kind gift from Dr Tsujinaka (Osaka, Japan). Calpeptin was dissolved in DMSO at 10 mg/mL.

Dibucaine was dissolved at 10 mmol/L in Tris–saline (TS) buffer, consisting of 20 mmol/L Tris and 150 mmol/L NaCl (pH 7.4), by the addition of the minimal amount of HCl required and readjustment to neutral pH. One tenth dilution in the same buffer gave pH 7.4.

Bovine von Willebrand factor (bovine anti-hemophilic globulin factor VIII, Speywood Laboratories Ltd, Nottingham, U.K.) was dissolved in TS buffer and extensively dialysed against the same buffer.

Polyclonal rabbit antiserum. Polyclonal rabbit antiserum against the GP Ib split product glycocalicin was prepared as described previously [10, 14].

Monoclonal antiserum. Monoclonal antiserum against the 90 kDa degradation product of ABP (TI10) was a generous gift from Dr J. M. Wilkinson, Department of Biochemistry and Cell Biology, Royal College of Surgeons of England (London, U.K.).

Platelet isolation. After discarding the first 2 mL of blood, nine parts of blood were drawn by venepuncture into one part of anticoagulant, consisting of 45 mmol/L Na_2-EDTA in 120 mmol/L NaCl (pH 4.6), and centrifuged at 320 *g* for 10 min. Platelets were isolated from the resulting platelet rich plasma by repeated centrifugations at 2000 *g* and resuspensions in washing solution consisting of 148 mmol/L NaCl, 20 mmol/L Tris, 5 mmol/L glucose and 0.6 mmol/L Na_2-EDTA (pH 7.4), as described previously [12]. The platelets were counted in a Thrombocounter model C (Coulter Electronics, Harpenden, U.K.). After the third washing, the platelet pellet was resuspended in TS buffer to the desired platelet concentration.

Experimental design. Effects of toluene were studied in two toluene concentration ranges designated low (<1.5 mmol/L) and high (>1.5 mmol/L), and compared to the effects of 1 mmol/L dibucaine. Incubations were carried out in 10 or 50 mL glass flasks with leak-tight Mininert Teflon® push-button valves (Pierce, Rockford, IL, U.S.A.). Toluene or dibucaine was added to the incubation flask containing TS buffer, making up maximally 10% of the total flask volume. The solutions were allowed to equilibrate for at least 30 min in a shaking incubator at 37°. The experiments were started with the addition of a small volume of concentrated platelet suspension that represented maximally 2% of the total flask volume. Thus, the toluene concentration changed only slightly due to the addition of the lipid containing cells. Samples were drawn from the incubation mixture with a syringe needle and used for electrophoretic analysis or platelet agglutination studies, and for determination of the toluene concentration. In some experiments, the platelet suspension was pre-incubated for 30 min at 37° with the membrane-permeable calpain inhibitor, calpeptin, before incubation with toluene or dibucaine.

Analysis of toluene. At least two samples of each incubation mixture were taken during the incubation period, to determine the toluene concentration using external standards. The external standards were made in TS buffer containing platelets in a concentration similar to the concentration of platelets in the incubation mixture, the procedure being described elsewhere [20]. Aliquots from the incubation mixtures (or standards) were transferred into ice-cold glass vials containing distilled water and sealed with teflon lined septa. Toluene was measured by the head-space technique using a Sigma 2000-HS 100 (Perkin-Elmer, Norwalk, CT, U.S.A.) gas chromatograph, allowing equal heating conditions for all samples (40 min at 62°). A stainless steel column (2 m × 3.2 mm) packed with 5% Carbowax 20M on carbopack B, 60/80 mesh (Supelco, Gland, Switzerland) was employed. Column temperature was 150°, flame ionization detection 200° and gas flow (N_2) 20 mL/min.

SDS–polyacrylamide gel electrophoresis (SDS–PAGE). SDS–PAGE of reduced samples (3% 2-mercaptoethanol) on 7% polyacrylamide gel was performed by the method of Laemmli [21], essentially as modified by LeStourgeon and Beyer [22]. The Mini Protean apparatus of Bio Rad was used for electrophoresis.

During incubation of 3×10^8 platelets/mL with toluene, dibucaine or TS buffer only, samples were transferred to Eppendorf tubes and centrifuged for 1 min at 8000 *g*. The resulting platelet pellets were solubilized in one part SDS-sample buffer (2% SDS, 500 mmol/L sucrose, 3.8 mmol/L NaH_2PO_4, 16.2 mmol/L Na_2HPO_4, 20 μL/mL saturated solution of pyronin Y) and one part NaCl (154 mmol/L) to 5×10^9 platelets/mL. In some experiments, the platelet pellets were extracted in TTG buffer (1% Triton X-100, 38 mmol/L Tris, 100 mmol/L glycine, pH 8.7) containing 4.2 mmol/L leupeptin and centrifuged for 4 min at 8000 *g*. The supernatant was added to an equal volume of SDS-sample buffer, to a final concentration of 2.5×10^9 platelets/mL. All samples were reduced with 3% 2-mercaptoethanol for 5 min at 100° prior to electrophoresis.

Western blotting. Western blotting was performed essentially as described by Towbin *et al.* [23]. After SDS–PAGE, the proteins were transferred electrophoretically to a nitrocellulose membrane. Unbound sites were blocked with 1% gelatine and the membrane was further incubated with a monoclonal antibody against an epitope in the 90 kDa fragment of ABP (TI10) (personal communication). After washing, the membrane was incubated with horseradish peroxidase-conjugated rabbit anti-mouse antiserum. Bound antibodies were detected using 0.05% *o*-dianisidine and 0.015% H_2O_2.

Agglutination of whole platelets induced by bovine von Willebrand factor. Agglutination was followed

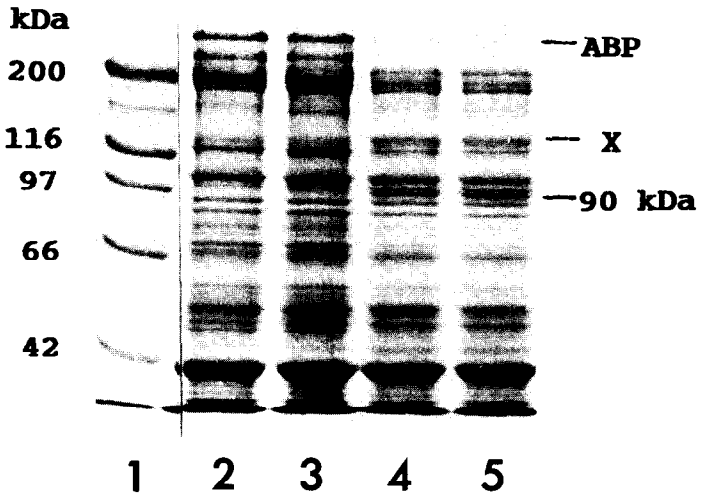

Fig. 1. Degradation of actin-binding protein (250 kDa) during incubation of platelets (3×10^8 cells/mL) with dibucaine at 37°, as demonstrated by SDS–PAGE and staining by Coomassie Brilliant Blue G. After incubation, aliquots were centrifuged at 8000 *g*; the platelet pellet solubilized in an equal volume of one part SDS-sample buffer and one part NaCl (154 mmol/L), to 5×10^9 platelets/mL, and reduced with 3% 2-mercaptoethanol prior to electrophoresis on a 7% gel. The lanes represent: molecular weight standards (lane 1), platelets incubated with TS buffer for 60 min (lane 2), platelets incubated with 1 mmol/L dibucaine for 3 (lane 3), 30 (lane 4) and 60 min (lane 5). To each lane 10 μL were applied. ABP, actin-binding protein; X, 140 kDa uncharacterized proteolysis product; 90 kDa, degradation product of ABP.

in a Chronolog aggregometer (Coulter Electronics) attached to a W + W recorder 600 (W + W Electronic Inc., Basel, Switzerland). Calibration was such that 0% transmission was set with 3×10^8 platelets/mL and 100% transmission with 1.5×10^8 platelets/mL. During incubation of 3×10^8 platelets/mL with toluene or dibucaine at 37°, aliquots were transferred to an aggregometer cuvette. The base line was established over 30 sec before the addition of bovine von Willebrand factor. A control curve obtained from platelets incubated with TS buffer only was run in parallel. Agglutination response was calculated as the change in light transmission (chart divisions) for the platelet suspension incubated with toluene or dibucaine during the first 2 min of agglutination and presented as a percentage of the parallel TS buffer control.

Rocket immunoelectrophoresis. Rocket immunoelectrophoresis was performed essentially as described by Laurell [24] and modified by Weeke [25]. Platelets (5×10^9 cells/mL) were incubated and centrifuged as described for SDS–PAGE. The supernatants (extracellular phase) were electrophoresed against antiserum to glycocalicin. The maximum amount of glycocalicin that was split off from GP Ib was calculated from the total platelet lysis caused by freezing and thawing of the platelets. The height of the immunoprecipitate peak was a measure of the amount of glycocalicin split off from GP Ib during the incubation.

RESULTS

Analysis of toluene

For reasons shown below, the experiments were divided into two groups related to the final toluene concentrations in the platelet suspensions, i.e. those below and those above 1.5 mmol/L (representing the interval 1.5–2.8 mmol/L). The toluene concentration in the first group (<1.5 mmol/L) was 1.1 ± 0.4 mmol/L (mean ± SD) with a range of 0.4 to 1.5 mmol/L (five experiments designed for the study of ABP degradation). The corresponding value in the second group (>1.5 mmol/L) was 2.1 ± 0.5 mmol/L with a range of 1.6 to 2.7 mmol/L (12 experiments designed for the study of ABP degradation). The variation in toluene concentration, based on two determinations in each sample during the incubation period, was negligible.

SDS–PAGE

SDS–PAGE of platelets solubilized in SDS-sample buffer demonstrated a triplet of bands in the M_r range of 250,000 to 200,000. These bands have been recognized previously as the ABP-subunit at 250 kDa [19], talin at 235 kDa [26, 27] and myosin at 200 kDa [8] (Fig. 1, lane 2).

SDS–PAGE of platelets solubilized in SDS-sample buffer, after incubation with 1 mmol/L dibucaine at 37° for 30 min or more, clearly demonstrated a degradation of ABP and talin (Fig. 1, lanes 4 and 5) and the appearance of bands at 90 and 190 kDa related to ABP, as previously described [8, 12, 18]. A band (X) always appeared in the 140 kDa area concomitantly with this degradation process (Fig. 1, lanes 4 and 5). The 190 kDa band appeared as a broadening and splitting of the 200 kDA band, and the 90 kDa band was observed close to another protein, around 100 kDa, regularly present in SDS-solubilized platelets.

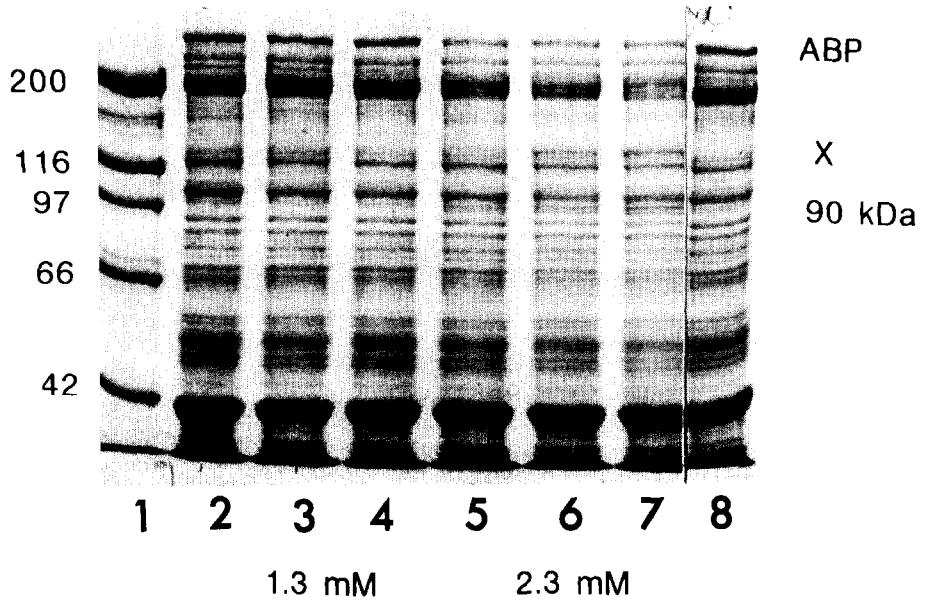

Fig. 2. Degradation of actin-binding protein during incubation of platelets (3×10^8 cells/mL) with toluene at 37°, as demonstrated by SDS–PAGE and staining by Coomassie Brilliant Blue G. For details and abbreviations see legend to Fig. 1. The lanes represent: molecular weight standards (lane 1); platelets incubated with 1.3 mmol/L toluene for 3, 30 and 60 min (lanes 2, 3 and 4, respectively); platelets incubated with 2.3 mmol/L toluene for 3, 30 and 60 min (lanes 5, 6 and 7, respectively); and platelets incubated with TS buffer for 60 min (lane 8).

SDS–PAGE of platelets incubated with toluene demonstrated a time and concentration dependent degradation of ABP (Fig. 2). In these experiments, a threshold level at a final toluene concentration of approximately 1.5 mmol/L was established. Toluene concentrations below 1.5 mmol/L (low concentration) (Fig. 2, lanes 2–4) did not induce a detectable degradation of ABP during the incubation periods used in these experiments. However, toluene concentrations above 1.5 mmol/L (high concentration) (Fig. 2, lanes 5–7) did induce degradation of ABP. This was observed as a partial disappearance of the ABP-band at 250 kDa and talin at 235 kDa, and the appearance of bands at 140 kDa (X) and 90 kDa (Fig. 2, lanes 6 and 7). The appearance of these bands after incubation of platelets with toluene in concentrations above 1.5 mmol/L was observed in all of 12 experiments. Clearly, there was a dose–response relationship. At 1.5–2.0 mmol/L, the 90 kDa fragment appeared only after long term incubation (30 min); at approximately 2.5 mmol/L, this fragment appeared almost immediately. At concentrations around 3.0 mmol/L and above, all bonds faded indicating that toluene at such high concentrations may have lead to lysis of some of the platelets with the results that fever platelets were sedimented prior to solubilization in SDS (data not shown). Also, the induction of a more extensive proteolysis under these conditions may have been possible.

Incubation of the platelet suspension with the membrane-permeable calpain inhibitor, calpeptin, prior to incubation with toluene or dibucaine inhibited the degradation of ABP (data not shown).

Western blotting

The 90 kDa band appearing during the incubation of platelets with toluene (above 1.5 mmol/L) or dibucaine was confirmed to be a degradation product of ABP by the use of a monoclonal antibody to the 90 kDa fragment of ABP (TI10), in Western blotting (Fig. 3, lanes 2 and 3). The bands visualized at higher molecular weights were considered to represent undegraded ABP, as well as uncharacterized degradation products containing the same binding epitope.

Platelet agglutination

As ABP was degraded, a pronounced effect was observed on the platelet agglutination induced by bovine von Willebrand factor. Incubation of platelets with 1 mmol/L dibucaine for 2 min led to an increased agglutination (153%) as compared to the control incubated with TS buffer alone (Table 1). After incubation for 10 min or more, a reduced agglutination response compared to the control was observed.

Incubation of platelets with as low a concentration of toluene as 0.3 mmol/L for 2 min led to an increased agglutination (163% of control) (Table 1). The agglutination response declined with time, approaching the response in the control platelet suspension after 60 min of incubation. Incubation with 1.3 mmol/L toluene (low concentration) increased initially the agglutination response, being, 164% of the control after 2 min of incubation, but after 30 min it declined towards the control level. A high concentration of toluene (2.0 mmol/L) reduced

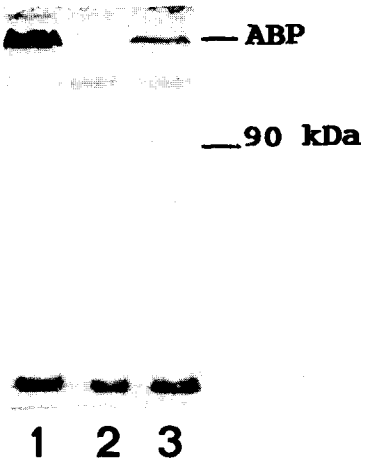

Fig. 3. Degradation of actin-binding protein and the appearance of its 90 kDa degradation product during incubation of platelets with toluene or dibucaine, as demonstrated by Western blotting with a monoclonal antibody against the 90 kDa fragment of actin-binding protein (TI10) after SDS–PAGE (7% gel). After incubation at 37°, aliquots were centrifuged and the pellets extracted to 5×10^9 platelets/mL in 1% Triton X-100 buffer containing 4.2 mmol/L leupeptin as calpain inhibitor. Prior to SDS–PAGE, samples were mixed with an equal volume of one part SDS-sample buffer and one part NaCl (154 mmol/L), and reduced with 3% 2-mercaptoethanol. The lanes represent: platelets incubated for 60 min with TS buffer (lane 1) or 1 mmol/L dibucaine (lane 2) or 2.5 mmol/L toluene (lane 3). To each lane 10 μL were applied. The nitrocellulose membrane was incubated with TI10 as primary antibody and rabbit anti-mouse antibody conjugated with horseradish peroxidase as secondary antibody. Bound antibodies were visualized by 0.05% *o*-dianisidine and 0.015% H_2O_2. Abbreviations as in legend to Fig. 1.

the agglutination response (84%) after only 2 min of incubation. In this case, a fall in the aggregometer base line prior to the addition of bovine von Willebrand factor was also observed (data not shown).

Rocket immunoelectrophoresis

In correspondence with the reduced von Willebrand factor-induced agglutination response, the GP Ib split product glycocalicin appeared in the extracellular medium in a time and concentration dependent manner. In a representative experiment, using a toluene concentration of 1.7 mmol/L, the release of glycocalicin increased from 10% after 3 min of incubation to 27% after 60 min (Fig. 4, well 2 compared with well 6). A higher level of toluene (2.9 mmol/L) led to the more substantial release of 24% after 3 min increasing to 81% after 60 min (Fig. 4, well 3 compared with well 7). Dibucaine (1 mmol/L) induced a release of 13% after 3 min that increased to 86% after 60 min (Fig. 4, well 4 compared with well 8). For comparison, the maximal amount of glycocalicin split off from GP Ib during total platelet lysis (100%) is presented in well 9.

DISCUSSION

Knowledge of the biochemical effects of the organic solvent toluene is scarce, even though toluene exposure may lead to severe brain damage. In the present paper we have studied effects of toluene on the human platelet receptor GP Ib, linked to the cytoskeleton via ABP. Three aspects have been investigated specifically: the ability of von Willebrand factor to induce GP Ib-related platelet agglutination; release of the extracellular von Willebrand factor binding area, glycocalicin, from GP Ib; and degradation of ABP. These processes were all affected by toluene, but at different

Table 1. Effect of toluene and dibucaine on von Willebrand factor-induced platelet agglutination

Period of incubation (min)	Agglutination response (%)			
	Dibucaine	Toluene		
	1.0 (mmol/L)	0.3	1.3 ± 0.2 (mmol/L)	2.0 ± 0.4
2	153 ± 54	163	164 ± 13	84 ± 15
5	196	155	157 ± 23	71 ± 31
10	78	148	134 ± 26	36
30	22 ± 10	146	100 ± 20	24 ± 16
60	ND	106	82 ± 12	29

The agglutination was performed as described in Materials and Methods, applying final concentrations of toluene or dibucaine as listed above.

Agglutination response was calculated as the change in light transmission (chart divisions) in the platelet suspension 2 min after the addition of bovine von Willebrand factor, and was presented as a percentage of that of the corresponding TS buffer control (100%).

Calculation of mean ± SD was based on 3–5 experiments, means only are of 2 experiments.

ND, not determined.

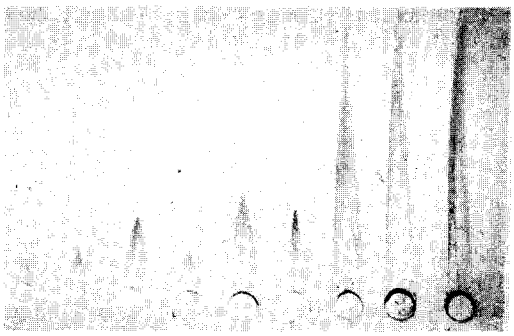

Fig. 4. Appearance of the GP Ib split product glycocalicin in the extracellular medium during incubation of platelets with toluene or dibucaine, as demonstrated by rocket immunoelectrophoresis with antiserum against glycocalicin in the gel, and staining with Coomassie Brilliant Blue R. Platelets (3×10^8 cells/mL) were incubated at 37° and centrifuged for 1 min at 8000 *g*. Supernatant (10 μL) was applied to the wells as follows: platelets incubated with TS buffer (wells 1 and 5), 1.7 mmol/L toluene (wells 2 and 6), 2.9 mmol/L toluene (wells 3 and 7), 1 mmol/L dibucaine (wells 4 and 8). Incubation periods were 3 min (wells 1, 2, 3 and 4) or 60 min (wells 5, 6, 7 and 8). Supernatant from a platelet suspension that had been lysed by freezing and thawing represents the maximal amount (100%) of releasable glycocalicin (well 9). Amounts of released glycocalicin relative to this were 5, 10, 24, 13, 36, 27, 81 and 86% for lanes 1 to 8, respectively.

concentrations of the solvent. The responses were compared to known effects of the local anesthetic dibucaine on the same system.

In a study of the influence of toluene on vehicle driving [20], the mean blood toluene concentration in abusers was reported to be 0.1 mmol/L and the single highest was 0.3 mmol/L. Considering the time course between toluene intake, blood sampling and toluene analyses, and the complex pharmacokinetics of toluene [28], these were regarded as being minimum concentrations [20]. From this point of view, the toluene concentrations used in the present paper may be considered as relevant to those in toluene abusers. However, care should be taken when attempting to relate the *in vitro* observations in this study to clinical symptoms.

Exposure of platelets to toluene concentrations above 1.5 mmol/L led to a degradation of ABP and talin, as observed on SDS–PAGE. A 90 kDa band appearing during this degradation process was verified as representing the 90 kDa fragment of ABP by using a monoclonal antibody (TI10) against this fragment. The observation of this band serves as a sensitive and specific detector of proteolysis induced by the calcium dependent protease calpain.

Calpain can use several of the platelet proteins as substrates in addition to ABP, such as talin [26, 27], GP Ib [7, 29] and aggregin [30]. The observed degradation of ABP and talin and the release of glycocalicin from GP Ib indicate that calpain was activated during the incubation of platelets with toluene, as was also observed to be the case with the local anesthetic dibucaine. It was found previously that in platelets treated with dibucaine, the intracellular calcium concentration rose sufficiently to activate calpain [6]. Also, it has been shown that toluene induces an increased intracellular calcium level in rat brain synaptosomes [31]. In these previous experiments, however, the toluene concentration in the cell suspension was not controlled. Incubation of platelets with the membrane-permeable calpain inhibitor, calpeptin, prior to exposure of platelets to toluene, inhibited degradation of ABP. This supports the hypothesis that toluene activates calpain. However, whether the activation is a direct effect of toluene or, as is more probable, an indirect effect via membrane alterations and the release of membrane bound, Ca^{2+}-bound stores is not known.

The bovine von Willebrand factor-induced platelet agglutination was reduced as a consequence of the release of glycocalicin from GP Ib. Theoretically, a surface exposure of calpain is a prerequisite for the release of glycocalicin. A fall in the aggregometer base line after prolonged incubation represents a change in platelet shape, probably accompanying some cell lysis. However, previous studies [6, 32] have reported a significant release of the cytoplasmic enzyme lactate dehydrogenase only after long term incubation with 1 mmol/L dibucaine. In addition, calpain was reported recently as becoming exposed on the extracellular surface of activated platelets by a process not solely due to platelet lysis [32]. In any case, the constant intensity of the Coomassie Blue staining after SDS–PAGE for almost all of the protein bands, other than those of ABP and talin, shows that the degradation of these proteins represents a specific effect.

At lower toluene concentrations (below 1.5 mmol/L) an increased agglutination response was observed where no proteolytic action of calpain could be demonstrated by the present techniques. Consequently, there is not a direct relationship between increased platelet agglutination and the degradation of ABP by calpain, at least as far as the total pool of ABP is concerned. The reason for the increased agglutination is unknown, though possibly a local degradation of membrane-associated ABP leading to a release of GP Ib from the cytoskeleton is involved. Also, perturbation of the phospholipid bilayer when toluene partitions into the plasma membrane may lead to a more favorable exposure of GP Ib for the binding of von Willebrand factor. It has been demonstrated that toluene alters neuronal phospholipid content [33, 34] and also affects several membrane-associated events in rat brain synaptosomes, such as membrane fluidity [2, 33, 35].

Calpains are cysteine proteases, distributed in different cells varying from neurons [36] to platelets. Lynch and Baudry [37] observed an activation of calpain and a degradation of the actin-binding protein fodrin in forebrain synaptic membranes, as well as an increased number of receptors for glutamate. They postulated that these processes may be involved in memory storage. There are, apparently, some parallels here to the events observed on the platelet receptor–cytoskeleton complex during toluene incubation.

We have demonstrated defined, biochemical

effects of the organic solvent toluene on the platelet receptor–cytoskeleton complex, GP Ib–ABP, as studied *in vitro*. To our knowledge, the biochemical mechanisms involved in the development of toluene-induced injuries in man are only partly understood. Effects on membrane receptor complexes should be considered with this in mind.

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