reactivity changes, which clearly show that insulin binds to lipid bilayers in a highly specific manner involving its dimer-forming surface.

ACKNOWLEDGMENTS

We gratefully acknowledge the advice and technical assistance of Dr. George Oda. We are grateful to Dr. A. Storer and Dr. D. Rose of the National Research Council, Ottawa, for the use of the computer graphics facility.

Registry No. Insulin, 9004-10-8.

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Inhibition of Protein Cross-Linking in Ca^{2+}-Enriched Human Erythrocytes and Activated Platelets†

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ABSTRACT: Treatment of human erythrocytes with Ca^{2+}, in the presence of ionophore A23187, causes the formation of high molecular weight ($>10^6$) membrane protein polymers. This phenomenon, known to involve cross-linking of essentially all of the band 4.1 and 2.1 (ankyrin) proteins, as well as some spectrin, band 3, and hemoglobin molecules, could be prevented by preincubating the cells with a noncompetitive inhibitor of intrinsic transglutaminase, 2-[3-(diallylamino)propionyl]benzothiophene, at concentrations of about (3-6) $\times 10^{-4}$ M. The compound also eliminated the proteolytic breakdown of the two major transmembrane proteins band 3 and glycophorin, which would otherwise occur during the Ca^{2+} loading of fresh human red cells. In addition, the inhibitor effectively blocked the formation of a cross-linked protein polymer in thrombin-activated human platelets.

Elevation of the intracellular concentration of Ca^{2+} ions leads to marked changes in the covalent structures of proteins in human erythrocytes, as is evident from the examination of the membranes of Ca^{2+}-treated cells. In general, such protein modifications fall into two categories: polymerization and proteolytic degradation. Polymerization might occur (i) through reaction of proteins with bifunctional cross-linking agents, e.g., malonedialdehyde, generated from lipid peroxidation (Hochstein & Jain, 1981; Allen et al., 1984), (ii) through production of disulfides (Liu et al., 1977; Kosower et al., 1981), or (iii) through N^{ϵ}-(γ-glutamyl)lysyl side-chain peptide bridges (Lorand et al., 1976, 1978, 1979b, 1980; Siefring et al., 1978; Bjerrum et al., 1981). The latter reaction of protein fusion is due to the activation of latent transglutaminase, and in the present work, we explore the possibility of preventing this type of protein polymerization in Ca^{2+}-loaded human red cells with a noncompetitive inhibitor of transglutaminase: 2-[3-(diallylamino)propionyl]benzo-

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thiophene or DAPBT.[1] Attention was also given to the blocking by this compound of the proteolytic degradation of band 3 and glycophorin, which is somehow coupled to the cross-linking reaction in fresh erythrocytes (Lorand et al., 1983; Lorand & Michalska, 1985). The inhibitory effect of DAPBT was also demonstrated on protein cross-linking occurring in human platelets (Bruner-Lorand et al., 1982) upon stimulation by thrombin.

Materials and Methods

Procedures for measuring the kinetics of incorporation of [1,4-$^{14}C_2$]putrescine into *N,N'*-dimethylcasein with a filter paper assay (Lorand et al., 1972) as catalyzed either by purified guinea pig liver transglutaminase (Lorand et al., 1979) or by human erythrocyte lysate (Siefring et al., 1978) followed exactly the details given in the latter two references. 2-[3-(Diallylamino)propionyl]benzothiophene or DAPBT was a generous gift from M. Maamer, Innothera, Paris, France.

Erythrocytes were obtained by centrifuging fresh citrated human blood (3500 rpm for 5 min) and by washing 4 times with a buffer solution containing 5 mM Tris-HCl (pH 7.4), 60 mM NaCl, 150 mM KCl, and 10 mM glucose. Loading of the cells (hematocrit 20%) with 1.5 mM Ca^{2+} was carried out in the presence of 20 μM ionophore A23187 at 37 °C for periods specified in the figure legends. Incubation with DAPBT (<1 mM) as required was commenced 30 min earlier. Following treatment with Ca^{2+} (or with 1.5 mM Mg^{2+} in the controls) for 1–12 h, the cells were washed in the above Tris–NaCl–KCl–glucose buffer, with 5 mM EDTA added, and then lysed in a 5 mM phosphate buffer (pH 8) containing several protease inhibitors (1 mM each of benzamidine and phenylmethanesulfonyl fluoride and 0.5 mM each of iodoacetamide and EDTA). Membranes were isolated and analyzed either by the methods of SDS–PAGE (Fairbanks et al., 1971) or by cross-immunoelectrophoresis as published previously (Bjerrum et al., 1983; Lorand et al., 1983). The polyspecific antibody to human erythrocyte membrane proteins (A104, lot 099A, Dako Corp., Santa Barbara, CA; absorbed with serum albumin) and the monospecific antibody to band 3 were the same as given in the latter reference.

Platelets were prepared from fresh human blood (Massini & Luscher, 1974) and were suspended to a concentration of 10^9 cells/mL in the medium recommended by Phillips et al. (1980), containing 0.154 M NaCl, 0.01 M Tris-HCl of pH 7.6, and 1 mM EDTA. Activation of platelets was carried out at 37 °C by gentle addition of 1 unit of human thrombin (kindly provided by Dr. John W. Fenton II of the New York State Department of Health, Albany, NY) lasting for 2 min. Then, 5 units of hirudin (Sigma Chemical Co., St. Louis, MO) was admixed to quench thrombin activity, followed by 5-min standing at room temperature (~20 °C), and then addition of 1 mL of the above NaCl–Tris–EDTA buffer, with inclusion of 1 mM leupeptin (a gift from the United States-Japan Cooperative Cancer Research Program). Platelets were removed by centrifugation (Sorvall angle centrifuge, setting 5), and the pellet was stored at −20 °C prior to solubilization in 3% SDS, 10% glycerol, and 62.5 mM Tris-HCl (pH 6.8) and reduction by 5-min boiling with 40 mM dithiothreitol. SDS–PAGE analysis was performed according to Laemmli (1970), employing a 5–20% exponential gradient in the resolving gel with 3% stacking gel and 25-μL samples. Electrophoresis at 15 °C took 16 h at 50 V. Gels were fixed in 10% 2-propanol and were stained with 0.05% Coomassie Blue R (Calbiochem, La Jolla, CA).

[1] Abbreviations: DAPBT, 2-[3-(diallylamino)propionyl]benzothiophene; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; EDTA, ethylenediaminetetraacetate; Tris, tris(hydroxymethyl)aminomethane.

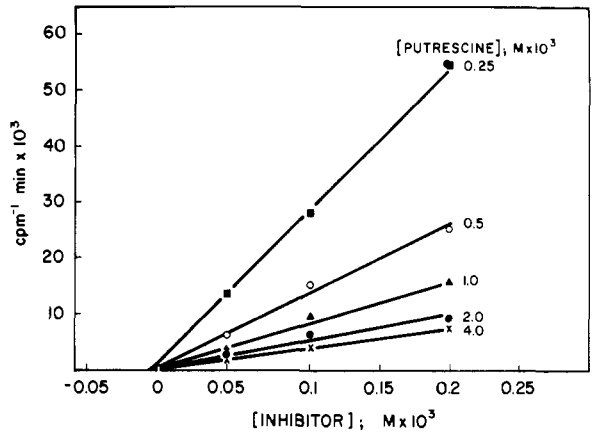


FIGURE 1: Kinetics of inhibition of transglutaminase by DAPBT (0.05, 0.1, and 0.2 mM; abscissa) on the reaction of incorporating [^{14}C]-putrescine into *N,N'*-dimethylcasein at various concentrations (0.25, 0.5, 1, 2, and 4 mM of the amine substrate). Reciprocal velocities for incorporation of radioactivity are given on the ordinate.

Results

Kinetics of Inhibition of Intracellular Transglutaminase Activities. Samama et al., (1979) showed that DAPBT was a noncompetitive inhibitor of activated human fibrin stabilizing factor (factor $XIII_a$), assaying this extracellular transglutaminase by incorporation of dansylcadaverine into casein (Lorand et al., 1969). Prior to examining the effect of DAPBT on whole cells, it was deemed important to show that in addition to factor $XIII_a$ the compound would also inhibit intracellular transglutaminases, enzymes of quite different molecular properties and kinetic specificities. Figure 1 presents Dixon plots for the incorporation of [^{14}C]putrescine (0.25–4 mM) into *N,N'*-dimethylcasein (Lorand et al., 1979a) at various concentrations (0.05–0.2 mM) of DAPBT, with purified guinea pig liver transglutaminase as enzyme. Results are consistent with a noncompetitive mode of inhibition by DAPBT, with an apparent K_i of about 10^{-5} M.

Inhibition of the incorporation of [^{14}C]putrescine into the casein substrate by DAPBT was also examined with human red cell lysate serving as enzyme (Siefring et al., 1978). Again, a noncompetitive type of inhibition was observed, but a less favorable K_i (about 10^{-3} M) was obtained. Nevertheless, given the appreciable water solubility of DAPBT (ca. 10^{-2} M at 37 °C), even with this reduced inhibitory potency it was possible to proceed with experiments in intact cells.

Inhibition of Protein Changes in Ca^{2+}-Treated Human Red Cells. Treatment of erythrocytes with Ca^{2+} in the presence of ionophore A23187 is known to produce significant alterations in the covalent structures of membrane proteins. In this study, we focused on two enzyme-mediated events: cross-linking by transglutaminase, which can be observed by conventional SDS–PAGE methods (Lorand et al., 1976; Siefring et al., 1978), and proteolysis, which can be best examined by crossed immunoelectrophoresis (Lorand et al., 1983; Lorand & Michalska, 1985). Both of these techniques were employed in this work.

Washed human red cells (20% hematocrit) were incubated for 0.5 h with various concentrations (0.1, 0.3, and 0.6 mM) of DAPBT prior to the addition of Ca^{2+} (2 mM) and ionophore A23187 (20 μM). Following 1–8 h of incubation, the cells were lysed, ghosts were isolated, and protein profiles were examined by SDS–PAGE (Fairbanks et al., 1971). Cells that

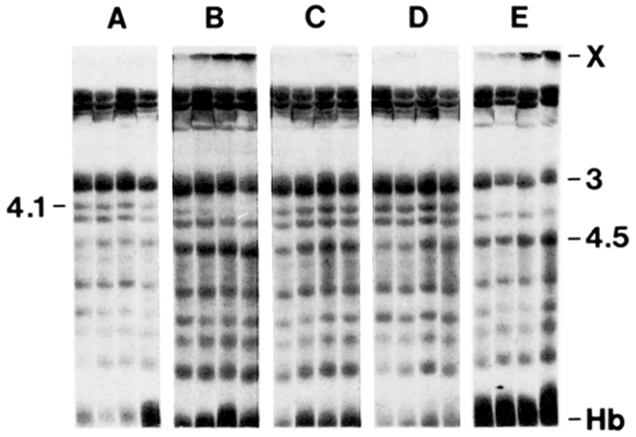


FIGURE 2: Inhibition of formation of cross-linked membrane protein polymers by DAPBT in Ca^{2+}-enriched human red cells. Washed erythrocytes were suspended (20% hematocrit, 37 °C) in a medium containing 100 mM KCl, 60 mM NaCl, 10 mM glucose, 5 mM Tris-HCl (pH 7.4), and various concentrations of the transglutaminase inhibitor DAPBT (0.1 mM in set B; 0.3 mM in set C; 0.6 mM in set D). Thirty minutes later, 20 μM ionophore A23187 and 2 mM $CaCl_2$ were added to allow Ca^{2+} loading to proceed for periods of 1, 2, 4, and 8 h (corresponding to the gels in each set from left to right, respectively). Neither set A nor set E contained DAPBT; set E, however, contained $CaCl_2$, whereas in the control A set 1.5 mM $MgCl_2$ was substituted for $CaCl_2$. Cells were lysed following the various incubation periods; membranes were prepared, then reduced, and analyzed by SDS–PAGE (Fairbanks et al., 1971). Positions of the cross-linked polymer (X), bands 3, 4.1, and 4.5, and hemoglobin (Hb) are marked on the Coomassie Blue stained protein profiles.

were treated with Ca^{2+} or Mg^{2+} in the presence of ionophore, but in the absence of DAPBT, served as controls. Results of these experiments are presented in Figure 2. Panel A shows the gels for the Mg^{2+}-treated controls for 1, 2, 4, and 8 h of incubation (left to right); panels B–D represent corresponding sets with Ca^{2+}, added after the cells were preincubated with 0.1, 0.3, and 0.6 mM DAPBT, respectively. Panel E is the Ca^{2+} control, without DAPBT. In agreement with previous work (Lorand et al., 1976; Siefring et al., 1978), the enrichment of cells by Ca^{2+} caused the disappearances of bands 2.1 and 4.1, diminutions of the relative staining intensities of band 3 and spectrin, and the appearance of high molecular weight cross-linked polymers (marked by X in Figure 2). Inclusion of 0.6 mM or even 0.3 mM DAPBT in the medium (panels D and C) offered nearly total protection against all the above changes brought about by the Ca^{2+} treatment of cells.

Upon Ca^{2+} loading, there was also an increase in the relative intensity of band 4.5 (e.g., compare gels in panel A with those in panel E in Figure 2), which was in accord with earlier findings that fresh red cells respond to Ca^{2+} treatment also by fragmentation of band 3, generating band 4.5 (Lorand et al., 1983; Lorand & Michalska, 1985). The experiments with DAPBT suggested that, at 0.6 mM concentration, this compound offered significant protection against the hydrolytic breakdown of band 3 (see gels in panel D, Figure 2). Therefore, it was of interest to further analyze this question by the method of crossed immunoelectrophoresis, which would also reveal the degree of fragmentation of the other major transmembrane protein: glycophorin.

Results obtained with crossed immunoelectrophoresis, either against a polyspecific antibody to human erythrocyte membrane proteins (Figure 3, set A) or against a monospecific anti-band 3 antibody (Figure 3, set B), showed that the degradation of the band 3 protein to 3′ (considered to be the equivalent of band 4.5 in SDS–PAGE) could be inhibited by DAPBT. The effect of the inhibitor was even more remarkable with regard to glycophorin. In the absence of DAPBT, this transmembrane protein underwent extensive degradation during 3 h of incubation of the cells with Ca^{2+} (see bracketed area in top row, left panel, set A in Figure 3), and hardly any immunoprecipitate of glycophorin was detectable following 12 h of Ca^{2+} treatment (second row, left panel, set A). The DAPBT-containing samples (center panels: top row, 3 h; second row, 12 h), however, showed as strong glycophorin peaks as the Mg^{2+} controls (right hand panels: top row, 3 h; second row, 12 h; set A).

Inhibition of Protein Cross-Linking in Thrombin-Activated Platelets by DAPBT. An interesting feature of activating human platelets by thrombin is the formation of high molecular weight protein polymers that cannot be reduced in size by treatment with dithiothreitol in SDS, indicating that cross-links other than disulfides are important (Bruner-Lorand et al., 1982; Cohen et al., 1985). With a 5–20% exponential gradient for SDS–PAGE, the polymeric material (marked X in the right-hand lane of Figure 4) produced during 2 min of thrombin stimulation remained on top of the gel. If, however, 1 mM of DAPBT was added prior to the addition of thrombin, no polymer formation was observed (left lane, Figure 4).

Discussion

Transamidating enzymes of the endo-γ-glutamine:ϵ-lysine transferase type, commonly called transglutaminases, can catalyze a nucleophilic reaction between ϵ-amino groups of some endo lysine residues of one protein, as electron donor, and the γ-carbonylamide functionalities of specific endo glutamines of another protein, as acceptors. Accordingly, N^{ϵ}-(γ-glutamyl)lysine bridge formation is a characteristic feature of the reaction, leading to covalently fused oligomeric or polymeric products. Their size varies from one biological system to another ranging, for example, from the relatively small ($M_r \sim 55\,000$) cross-linked dimers of β-crystallin subunits in Ca^{2+}-treated rabbit lens (Lorand et al., 1985) to very high molecular weight ($>10^6$) heteropolymers found in the membranes of Ca^{2+}-loaded human erythrocytes (Siefring et al., 1978). Transglutaminases can also catalyze the incorporation of small primary amines into the acceptor γ-glutamine sites of protein substrates, and amine incorporation competes against protein cross-linking. In fact, apart from measuring the frequency of N^{ϵ}-(γ-glutamyl)lysine, inhibition of cross-linking by extraneously added amines (e.g., dansylcadaverine, histamine) constitutes the main strategic approach for probing transglutaminase-mediated reactions in biological systems (Lorand & Conrad, 1984). This enzyme-directed and site-specific labeling of acceptor glutamine residues by the amine tracers can be of particular advantage. Nevertheless, even with the best amines, there are obvious limitations for inhibiting cellular reactions. Metabolism of the amine (e.g., oxidation, reactions with aldehydes, etc.) might alter its character; difficulties in penetration may prevent raising its concentration to high enough levels in terms of the K_i value (approximately ca. 10^{-4} M for specific amines; Lorand et al., 1979a). Also, because of the competitive nature of the reaction, inhibition of protein cross-linking by amines might be of relatively short duration.

Thus, we have been searching for noncompetitive inhibitors of the transglutaminase-mediated cross-linking, with relatively low toxicity. This work deals with the compound 2-[3-(diallylamino)propionyl]benzothiophene or DAPBT, which has been reported to inhibit platelet aggregation as well as the activity of coagulation factor $XIII_a$ and to show little toxicity in mice (D_{50} = 2 g/kg) or in rats (Samama et al., 1979).

Liver translutaminase (Clark et al., 1959) is perhaps the most thoroughly investigated intracellular transamidase in this

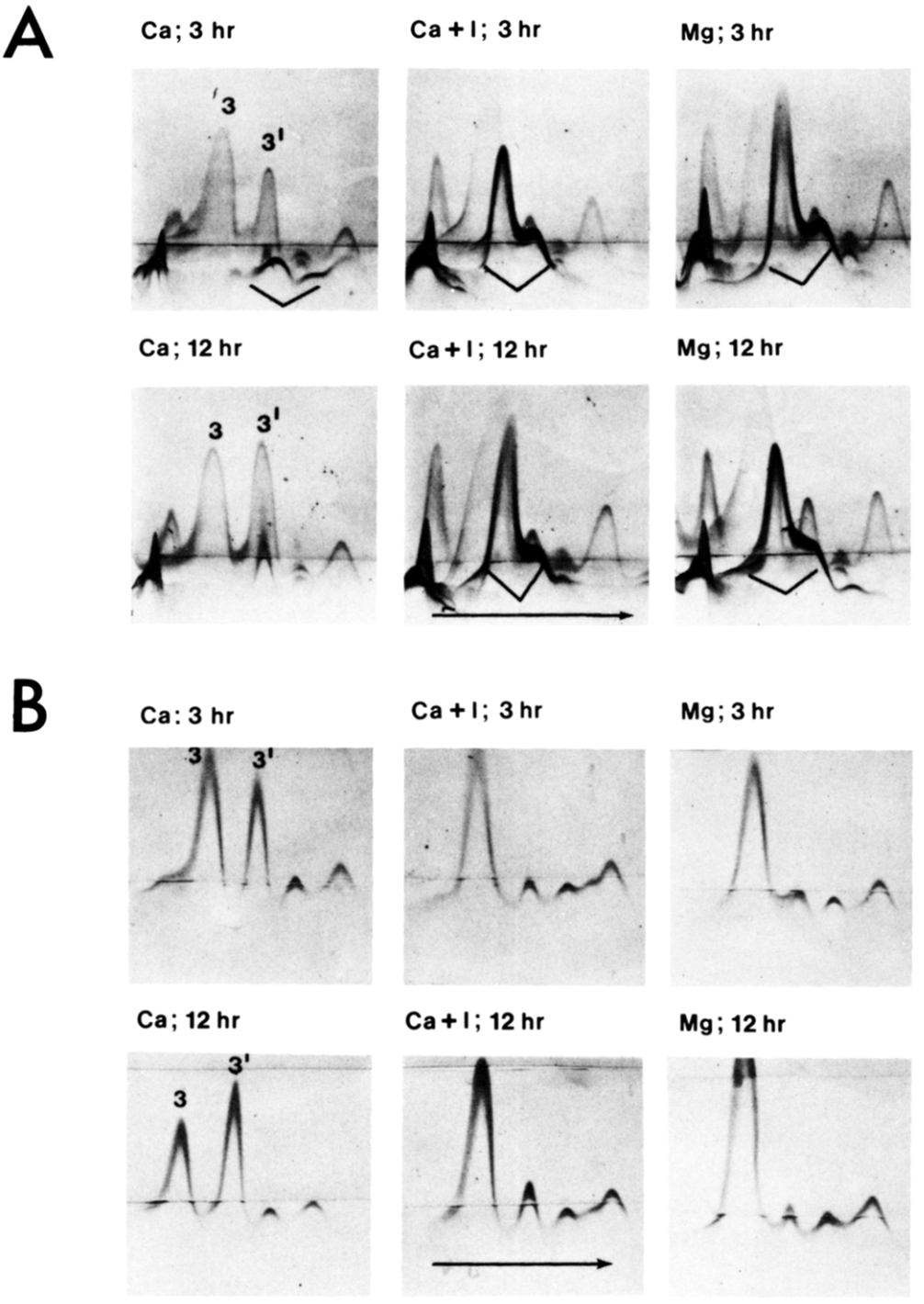


FIGURE 3: DAPBT inhibits the breakdown of transmembrane proteins in Ca^{2+}-loaded erythrocytes, as demonstrated by cross-immunoelectrophoresis against polyspecific antibody to human red cell ghost (panel A) and against monospecific antibody to band 3 (panel B). Cells were incubated with 1.5 mM $CaCl_2$ (or $MgCl_2$ in controls) and 20 μM ionophore A23187, in the absence and presence of 1 mM DAPBT (with 30 min of preincubation added for this inhibitor; I). Membranes were isolated after 3- and 12-h treatment of the cells, and cross-immunoelectrophoresis was carried out as described previously (Lorand et al., 1983). Migration in SDS–PAGE was toward the anode on the right (arrow, first dimension); for immunoelectrophoresis using 1.5 mM thick agarose gels, the anode was on top. Antibody concentrations were 10 and 24 $\mu L/cm^2$ in A and B, respectively. Band 3 and its major, immunocross-reactive degradation product are labeled as 3 and 3′. Positions for the glycophorin-related immunoprecipitates are shown between V-shaped brackets.

family of enzymes, present also in the human red cell (Brenner & Wold, 1978). We have shown that the purified transglutaminase was noncompetitively inhibited by DAPBT with an apparent K_i of about 10^{-5} M (Figure 1). Even though this value rose to about 10^{-3} M when tested against whole human erythrocyte lysate as enzyme (suggesting that DAPBT may bind to cell components other than transglutaminase) the (3–6) $\times 10^{-4}$ M concentration of DAPBT found to inhibit membrane protein alterations in the Ca^{2+}-treated human red cell (Figure 2) compares favorably with the 2×10^{-3} M requirement for inhibiting fibrin cross-linking in plasma and with the 10^{-4} M concentration needed for 50% inhibition of platelet aggregation (Samama et al., 1979). Though the mechanism of DAPBT inhibition is unknown, it is possible that the α,β-unsaturation site of the compound may be functionally significant in this regard. It could be postulated (and we are grateful to our colleague Dr. K. N. Parameswaran for this suggestion) that following a binding step between transglutaminase (E) and

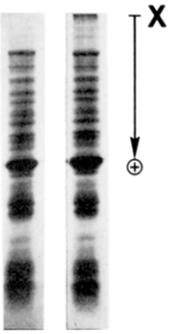
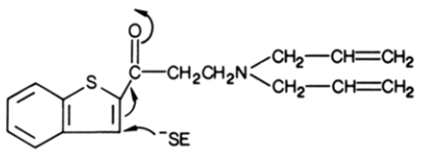


FIGURE 4: Reduced protein profile of human platelets following activation by thrombin, in the presence (left lane) and absence (right lane) of the transglutaminase inhibitor DAPBT (1 mM). Coomassie Blue stained patterns are shown for Laemmli (1970) gels, with a 5–20% exponential gradient in the resolving phase and a 3% stacking gel. The polymeric material formed upon platelet activation in the absence of DAPBT from the medium is marked by X.

the inhibitor (I), represented by dissociation constant K, the inhibitor reacts with a nucleophilic group in the enzyme (possibly the active center cysteine SH) with a rate constant k, leading to irreversible inactivation:



The pathway of inactivation would thus be as follows: $E + I \overset{K}{\rightleftharpoons} E; I \overset{k}{\rightarrow} E\text{–}I$.

Among the currently available inhibitors of membrane protein cross-linking in Ca^{2+}-loaded human red cells, DAPBT appears to be one of the most potent. On the basis of earlier work (Lorand et al., 1978), we estimate that generating an inhibitory effect comparable to that seen with 0.6 mM DAPBT (in panel D of Figure 2) would have necessitated the use of about 30 mM cystamine or 60 mM histamine. Moreover, the effect of DAPBT was of quite long duration, lasting more than 12 h in the face of Ca^{2+} loading (2 mM) of the cells at 37 °C. It completely blocked the disappearance of band 2.1 (ankyrin) and of band 4.1. The latter, in particular, is a most sensitive sign of protein cross-linking by transglutaminase in human erythrocytes, involving also some spectrin and band 3 molecules.

In Ca^{2+}-loaded fresh human cells, the anion transporting band 3 protein and glycophorin both undergo extensive proteolytic fragmentation. This can be prevented selectively by pepstatin (Lorand et al., 1983; Lorand & Michalska, 1985), which indicates that cross-linking and proteolysis are controlled by different sets of enzymes.[2] It is interesting to note that DAPBT not only blocked the formation of cross-linked membrane protein polymers but also eliminated the Ca^{2+}-related degradation of the two transmembrane proteins. It is possible that DAPBT might inhibit the two related reactions independently. However, it might also be suggested that the formation of cross-linked membrane protein clusters might impose some conformational change on band 3 and glycophorin that would render them more vulnerable to intrinsic proteolytic degradation. It remains to be seen whether these two events are, in fact, coupled in this sense.

Shortly after the transglutaminase-catalyzed reaction in Ca^{2+}-loaded human erythrocytes was recognized (Lorand et al., 1976, 1978), research was initiated in this laboratory to examine the possibility that similar protein cross-linking might also occur in platelets that are known to contain various types of transglutaminases (Schwartz et al., 1973; Puszkin & Raghuraman, 1985) and that can be activated by the influx of Ca^{2+} from the outside or by the release of Ca^{2+} from intracellular stores (Murer & Holme, 1970; Massini & Luscher, 1976; Massini et al., 1978; Charo et al., 1976; Rink et al., 1982). Indeed, it was found that activation of washed human platelets by ionophore A23187 (Cohen et al., 1981) or by thrombin (Bruner-Lorand et al., 1982; Cohen et al., 1985) was shown to cause the formation of nonreducible polymers, typical of transglutaminase action. Significant amounts of the polymer were found within 2 min of activation by thrombin (Figure 4, right lane) whereas preincubation with 1 mM DAPBT for 2 min prior to addition of thrombin prevented formation of the polymer (Figure 4, left lane).

One of the early observations with DAPBT was the inhibitory effect of this compound on platelet aggregation (Samama et al., 1979). It is not yet clear how this general action of DAPBT might relate to the inhibition of protein polymerization as presented in Figure 4. However, it is tempting to hypothesize that protein cross-linking in platelets might be a necessary prerequisite for cell aggregation, either by virtue of transmembrane rearrangements or by the covalent organization of critical membrane–cytoskeletal protein relationships.

Polymer formation in thrombin-activated platelets can be prevented not only by DAPBT but also by amines (e.g., 2 mM dansylcadaverine), which are known to compete against the ϵ-amino donor groups of lysines in transglutaminase-catalyzed cross-linking reactions (Lorand & Conrad, 1984). The amines become covalently incorporated into certain platelet proteins (Bruner-Lorand et al., 1982), which, by inference, are the building blocks contributing the γ-glutamine residues to forming the polymer (Lorand & Conrad, 1984). Use of anti-dansyl antibody, as recently introduced into this field (Lorand et al., 1986), should greatly aid in defining the cellular location and help in identifying and isolating these dansylcadaverine-labeled monomeric units. Immunological cross-matching of shared epitopes between the polymer and individual platelet proteins must also be explored, along the lines employed in the compositional analysis of cross-linked membrane proteins in human erythrocytes (Bjerrum et al., 1981).

Registry No. DAPBT, 61508-29-0; Ca, 7440-70-2; transglutaminase, 80146-85-6; thrombin, 9002-04-4.

[2] Ca^{2+} treatment of rat erythrocytes gives rise to such rapid proteolysis that fragmentation of membrane proteins, rather than cross-linking by N^{ϵ}-(γ-glutamyl)lysine, is the predominating feature (Kosower et al., 1983).

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