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Modulation of Erythrocyte Acetylcholinesterase Activity and Its Association with G Protein-Band 3 Interactions

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Abstract Circulating acetylcholine, substrate of membrane acetylcholinesterase (AChE), is known to enhance the band 3 protein degree of phosphorylation. The purpose of this study was to verify whether the band 3 phosphorylation status is associated with a G protein and whether it is an influent factor on AChE enzyme activity. From blood samples of healthy donors, erythrocyte suspensions were prepared and incubated with AChE substrate (acetylcholine) and inhibitor (velnacrine), along with protein tyrosine kinase (PTK) and tyrosine phosphatase (PTP) inhibitors. AChE activity was determined by spectrophotometry and extract samples were analyzed by western blotting using primary antibodies to different G protein subunits. Our results with phosphorylated band 3 (PTP inhibitor) show an increase in erythrocyte AChE ($p < 0.0001$). A dephosphorylated band 3 state (PTK inhibitor) shows a significant decrease. We identified a potential linkage of protein subunits $G\alpha_{i1/2}$ and G_β with band 3 protein. $G\alpha_{i1/2}$ and G_β may be linked to the band 3 C-terminal site. $G\alpha_{i1/2}$ is associated with the band 3 N-terminal domain, except for the control and ACh aliquots. G_β is associated with both phosphorylated and dephosphorylated band 3 in the presence of velnacrine. We conclude that an erythrocyte G protein with subunits $G\alpha_{i1/2}$ and G_β is associated with band 3. AChE depends on the degree of band 3 phosphorylation and its association with $G\alpha_{i1/2}$ and G_β.

Keywords Acetylcholine · Acetylcholinesterase · Protein band 3 · $G\alpha_{i1/2}$ protein · G_β protein · Velnacrine

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There is knowledge that the cholinergic system is not confined to the nervous system, but is virtually ubiquitous. The non-neuronal cholinergic system (NNCS), widely expressed in human cells independently of a neural regulation, represents a local anti-inflammatory and regulatory system providing homeostasis at the cellular and organic levels (Wessler et al. 1998, 1999, 2003). Whereas acetylcholine (ACh) is commonly known as a classic neurotransmitter, evidence was gathered concerning its function as a global signaling player in nature, with a central role in the microvasculature (Sastry and Sadavongvivad 1979; Tracey 2002; Kawashima and Fujii 2000; Wessler and Kirkpatrick 2001).

Circulating ACh can be produced by T lymphocytes and endothelial cells. Red blood cells account for the blood elements with the highest expression of the membrane-bound enzyme acetylcholinesterase (AChE), that hydrolyzes ACh (Wright and Plummer 1973; Saldanha 1985). Additionally, recent studies have assigned to ACh a significant key role in several erythrocyte events, such as hemorrheological and oxygen-carrying properties, nitric oxide (NO) metabolism and mobilization of nitric oxide-derived metabolites, alongside relevant changes in plasma

ions and pH (Almeida et al. 2006a, b; Mesquita et al. 2001; Santos et al. 2003; Carvalho et al. 2004). Little is known, however, about the erythrocyte AChE other than its influence on limiting ACh bioactivity, though it has been investigated as a potential marker of cell membrane integrity (Zabala et al. 1999). On the other side, velnacrine maleate (VM) has been described in red cells as an AChE inhibitor and formerly assigned to strengthen cytoskeletal protein-protein interactions in erythrocyte membranes (Mesquita et al. 2000). In red cells, intracellular phosphorylated/dephosphorylated states assume a key role in the modulation of band 3 protein-mediated effects (Bordin et al. 1995). Src-like is the major family of nonreceptor tyrosine kinases (especially $p53/56^{lyn}$) with which $p72^{syk}$ counteracts the effect of protein tyrosine phosphatases (PTPs), thereby resulting in different degrees of band 3 phosphorylation (Wang et al. 1997). Syk is known to catalyze band 3 primary phosphorylation, while Lyn is responsible for secondary phosphorylation following activation by Syk (Bordin et al. 2005; Brunati et al. 1996, 2000; Almeida et al. 2006a).

Erythrocyte NO translocation is mediated by membrane protein band 3, which is dependent on its phosphorylation degree as verified by us previously (Carvalho et al. 2008). Furthermore, we recently hypothesized the potential involvement of a heterotrimeric G protein in signal transduction events mediated by AChE-band 3 interactions (Carvalho et al. 2004). Therefore, the present study has two main focuses. One is to verify whether the AChE enzyme activity in red blood cells is modified under the influence of intracellular band 3 phosphorylation status. The second focus relies on the identification of a G protein and its possible association with AChE activity and the degree of band 3 phosphorylation.

Materials and Methods

Chemicals

ACh chloride plus protein tyrosine kinase (PTK) inhibitor (syk inhibitor) and aminogenistein ($p53/56^{lyn}$ inhibitor) were purchased, respectively, from Calbiochem (Darmstadt, Germany) and Sigma (St. Louis, MO, USA), and calpeptin (PTP inhibitor) was purchased from Calbiochem (Darmstadt, Germany). Hoechst Marion Roussel Pharmaceuticals (Sommerville, NJ, USA) provided the velnacrine maleate compound. Polyclonal rabbit antibodies against protein G_{β}, protein $G\alpha_{i1/2}$, protein $G\alpha_{i3}$, protein $G\alpha_{i3}/G\alpha_{0}$, protein $G\alpha_{S}$, and protein $G\alpha_{q/11}$, all at a dilution of 1/1000, recognizing a C-terminal sequence specific peptide conjugated to KLH, were purchased from BIOMOL International LP (Exeter, UK). Two polyclonal rabbit antibodies against protein band 3, one recognizing an epitope in the N-terminus of human origin and the other recognizing an epitope in the C-terminus of human origin, were both purchased from Santa Cruz Biotechnology Inc. Pertussis toxin from *Ordetella pertussis* was purchased from Sigma (Aldrich, Inc., USA).

Preparation of Erythrocyte Suspensions

Human venous blood samples were collected from the forearm vein of 15 healthy Caucasian men after informed consent. The blood container tubes were prepared with 10 IU ml^{-1} of sodium heparin (anticoagulant). Blood was centrifuged at 1040 *g* for 10 min in a Sorvall TC6 centrifuge. Afterward, plasma and buffy coat (leukocytes and platelets) were discarded. Erythrocyte suspensions were performed, after previous passage through an Imugard IG 500 column, with the addition of sodium chloride (0.9% at pH 7.4; AnalaR; BDH Laboratory, Poole, UK), in order to reconstitute the initial hematocrit (Ht; $\sim$45%).

Experimental Design

Erythrocyte suspensions ($n = 15$) were incubated for 30 min at 37°C, with AChE effectors (with either 10 μM ACh or 10 μM VM), in the presence and absence of $p72^{syk}$ inhibitor (10 μM), $p53/56^{lyn}$ inhibitor (10 μM AMGT), and PTP inhibitor (10 μM calpeptin). All erythrocyte suspensions were incubated after slight agitation. Another set of aliquots of equal composition was prepared in the presence of pertussis toxin.

Acetylcholinesterase Enzyme Activity

AChE enzyme activity was measured using a Beckman 35 UV/vis spectrophotometer (Fullerton, CA) at 37°C, using the colorimetric method proposed by Ellman et al. (1961) as modified by Kaplan et al. (1964). Measurements were performed at 25°C in erythrocyte suspensions diluted in 0.1 M phosphate buffer, pH 8.0 (1/2500), containing the final concentrations of all effectors present in the above erythrocyte suspensions. The substrate used was acetylthiocholine. The reaction between the thiol group of acetylthiocholine and the Ellman's reagent (2-nitrobenzoic acid) yields a yellow photochromic compound which can be read by spectrophotometry at 412 nm. Enzyme activity data points were calculated as the average absorbance of two identical aliquots, corrected by blank subtraction.

The color intensity is directly proportional to the erythrocyte AChE activity. One unit of enzyme activity (IU) is defined as the amount of enzyme that degrades 1 μmol of ACh per hour at 25°C. The concentration of

hemoglobin is determined and the results of enzyme activity are expressed as IU/(min × mg Hb).

Preparation of Membrane Skeletons by Detergent Extraction of Whole Erythrocytes

Preparation of membrane skeletons was based on the method described by others (Minetti and Ciana 2003; Bordin et al. 2002). Purified erythrocytes were washed with buffer A (5 mM Hepes, 2 mM $MgCl_2$, 150 mM NaCl, and 5 mM glucose, pH 7.4, 300–305 mosmol/kg water) and resuspended to 45% Ht in the same buffer. After centrifugation, packed cells were resuspended at the same Ht with buffer A containing 2 mM EGTA. Cells were sedimented and the supernatants discarded. The mixture was added to the pellet of ice-cold buffer A containing a 2% Triton X-100, 0.5 mM DTT, and protease inhibitor cocktail, followed by aliquot incubation for 30 min on ice. All samples, with a final Ht of 3%, were layered on a 35% sucrose (w/v) solution in an ultracentrifuge tube and spun at 98,000 *g* for 90 min (at 4°C) using an L8-M ultracentrifuge (Beckman, USA). Samples (supernatants) were analyzed for protein content using the CBQCA protein quantification kit (Molecular Probes) and stored at –80°C until analysis.

Western Blotting Analysis of Proteins G_β, $G\alpha_{i1}/G\alpha_{i2}$, $G\alpha_{i3}$, $G\alpha_{i3}/G\alpha_0$, $G\alpha_S$, and $G\alpha_{q/11}$

Extract samples solubilized from erythrocyte membrane skeletons (60 μg of total protein for each lane) were treated with 80 mM Tris/HCl, pH 6.8, buffer containing 16% (v/v) glycerol, 4.5% (w/v) sodium dodecyl sulfate (SDS), 150 mM dithiothreitol, 2-mercaptoethanol (100 μl/ml sample buffer), and 0.01% bromophenol blue, by heating the mixture at 95°C for 10 min.

Samples were loaded onto a 7.5% polyacrylamide gel with 0.5% SDS (SDS-PAGE) and a mixture of protein markers (Precision Plus Protein Standards, 10–250 kDa; BioRad, Richmond, CA, USA) for estimation of protein molecular mass. The gel runs were at 50 mA for ∼ 90 min in 1.5 M Tris–HCl, pH 8.8, buffer for the resolving gel and in a 0.5 M Tris–HCl, pH 6.8, buffer for the stacking gel.

For western blotting analysis, SDS-PAGE gels were transferred to a nitrocellulose membrane (Protan BA 85 Cellulonitrat[e]; Schleicher and Schuell, Dassel, Germany) using a vertical wet transfer system at 100 V, for 1 h. Following the transfer, membranes were stained with 0.5% Ponceau S in 5% (w/v) trichloroacetic acid solution for 2 min, to control for protein transfer. After washing out the Ponceau S staining with PBS, blots were blocked using PBS with 5% (w/v) nonfat milk and 0.1% Tween 20, for 1 h at room temperature. Blots were subsequently incubated with polyclonal rabbit antibodies against anti-protein G_β, anti-protein $G\alpha_{i1/2}$, anti-protein $G\alpha_{i3}$, anti-protein $G\alpha_{i3}/G\alpha_0$, anti-protein $G\alpha_S$, and anti-protein $G\alpha_{q/11}$, all at a dilution of 1/1000, recognizing a C-terminal sequence-specific peptide conjugated to KLH (from BIOMOL International LP, Exeter, UK). All blots were incubated in PBS with 5% (w/v) nonfat milk and 0.1% Tween 20 with slight shaking at 4°C overnight. The next day blots were washed three times using PBS with 0.1% Tween 20 and then incubated with horseradish peroxidase-linked secondary antibodies (bovine anti-rabbit IgG; Santa Cruz Biotechnology) at a dilution of 1/5000, for 1 h at room temperature, in PBS with 5% (w/v) nonfat milk and 0.1% Tween 20. Finally, blots were washed twice with PBS with 0.1% Tween 20 and once with PBS. Results were visualized by enhanced chemiluminescence (Super-signal West Pico trial kit; Pierce, Rockford, IL), followed by exposure to Super RX Fugi Medical X-ray film (Fugifilm; Tokyo) and subsequent development.

Immunoprecipitation with Proteins G_β and $G\alpha_{i1/2}$

Erythrocyte membrane-soluble extracts were immunoprecipitated with either anti-protein G_β or protein $G\alpha_{i1/2}$ antibody and immunoprecipitates were analyzed by western blotting for band 3, C-terminus, and for band 3, N-terminus. For immunoprecipitation of protein G_β or protein $G\alpha_{i1/2}$, an aliquot of 60 μl of membrane extract, containing 500 μg of protein in 240 μl of buffer A, was mixed with 10 μg of protein G Sepharose 4B (Sigma Aldrich, New Road, UK). After gentle agitation at 4°C for 1 h, supernatants were used and polyclonal rabbit antibodies against protein G_β or protein $G\alpha_{i1/2}$ (dilution 1/100) were added. Samples were incubated overnight at 4°C, with slight agitation. The mixture was then centrifuged at 13,000 rpm and the pellet was washed three times (once with NaCl 500 mM buffer and then twice with buffer A). Aliquots were solubilized in sample buffer and electrophoresed. Identification of coprecipitated protein G_β or protein $G\alpha_{i1/2}$ was carried out by immunoblotting using the rabbit anti-protein band 3 antibody (C-terminus or N-terminus at a dilution of 1/500) as explained above.

Statistical Analysis

Data are expressed as mean ± SD. Statistical analysis was conducted using the Statistical Package for the Social Sciences (SPSS) software, version 16.0. Result comparisons were analyzed by one-way ANOVA or Student's *t*-tests for paired observations. Statistical significance was set at $p < 0.05$.

Results

Erythrocyte Acetylcholinesterase Activity

One-way ANOVA revealed a statistically significant effect of the phosphorylation status effectors on enzyme activity (Fig. 1). Control erythrocyte samples showed an average enzyme activity of 277.45 ± 14.15 IU/(min × mg Hb).

The presence of ACh (the natural substrate of AChE and its enzyme activator) significantly enhanced AChE activity (294.82 ± 12.38 IU/[min × mg Hb] versus control; $p < 0.03$). On the contrary, the presence of AChE's competitive inhibitor, velnacrine maleate, decreased enzyme activity (40.00 ± 6.51 versus control; $p < 0.0001$).

In the presence of calpeptin (PTP inhibitor), AChE activity was significantly reduced (158.64 ± 9.08 IU/[min × mg Hb] versus control; $p < 0.0001$). ACh did not alter this effect mediated by calpeptin, while velnacrine effectively decreased AChE activity, compared with the control (37.45 ± 4.72 IU/[min × mg Hb]; $p < 10^{-10}$) and samples with calpeptin ($p < 0.0001$).

The presence of Syk inhibitor induced a significant decrease in AChE activity values (158.36 ± 11.94 IU/[min × mg Hb] versus control; $p < 10^{-10}$), and this effect was enhanced by VM (49.27 ± 8.05 IU/[min × mg Hb] versus control; $p < 10^{-10}$), as opposed to Ach, whose levels were significantly higher (226.09 ± 10.63 IU/[min × mg Hb] versus control; $p < 0.0001$). The presence of aminogenistein (AMGT) triggered a similar decrease in AChE activity (149.09 ± 12.87 IU/[min × mg Hb] versus control; $p < 10^{-10}$) and this effect was statistically significantly influenced by AChE effectors (ACh, VM), as happened with Syk inhibitor-incubated aliquots.

The presence of both PTK inhibitors decreased the values of AChE activity compared to the control ($p < 10^{-10}$), Syk_i-treated ($p < 0.0001$) and AMGT-treated ($p < 0.0001$) samples. In this case, ACh does effect higher AChE activity (183.18 ± 10.33 IU/[min × mg Hb] versus Syk_i + AMGT (119.27 ± 8.06); $p < 0.0001$), as opposed to velnacrine, which triggers lower AChE activity (47.36 ± 5.22 IU/[min × mg Hb] versus Syk_i + AMGT; $p < 0.0001$).

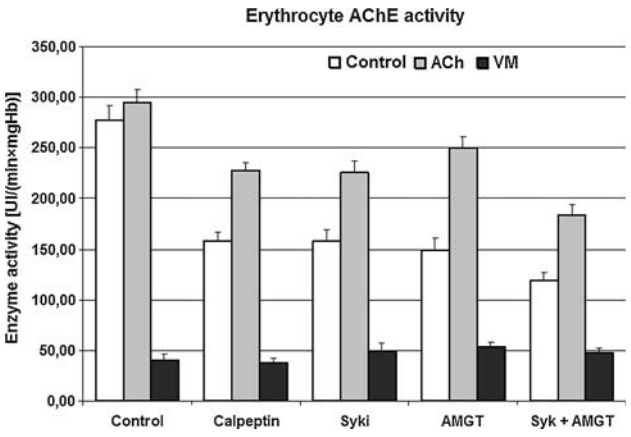

Fig. 1 Changes of in vitro AChE enzyme activity after incubation of erythrocyte suspensions with 10 μM ACh and 10 μM VM, in the presence and absence of $p72^{syk}$ inhibitor (10 μM), $p53/56^{lyn}$ inhibitor (10 μM AMGT), and PTP inhibitor (10 μM calpeptin) Values are mean ± SD; $n = 15$

Detection of Proteins $G\alpha_{i1}$, G_β, $G\alpha_{i/2}$, $G\alpha_{i3}$, $G\alpha_{i3}/G\alpha_0$, $G_{\alpha S}$, and $_{G\alpha q/11}$

Erythrocyte membrane-soluble extracts (control sample aliquots) were submitted to western blotting analysis, followed by immunoblotting with specific antibodies against the G protein subunits, and we verified that human erythrocytes contain the following G proteins: proteins $G\alpha_{i1}$, G_β, $G\alpha_{i1}/\alpha_{i/2}$, $G\alpha_{i3}$, $G\alpha_{i3}/G\alpha_0$, and $G_{\alpha S}$. Protein $G_{\alpha q/11}$ was not observed (Fig. 2; black arrows).

The position of protein G_β, and $G\alpha_{i1}/\alpha_{i/2}$ subunits, in a complex aggregate form, appeared to be similar to that of the protein band 3 lane (Fig. 2; gray arrows).

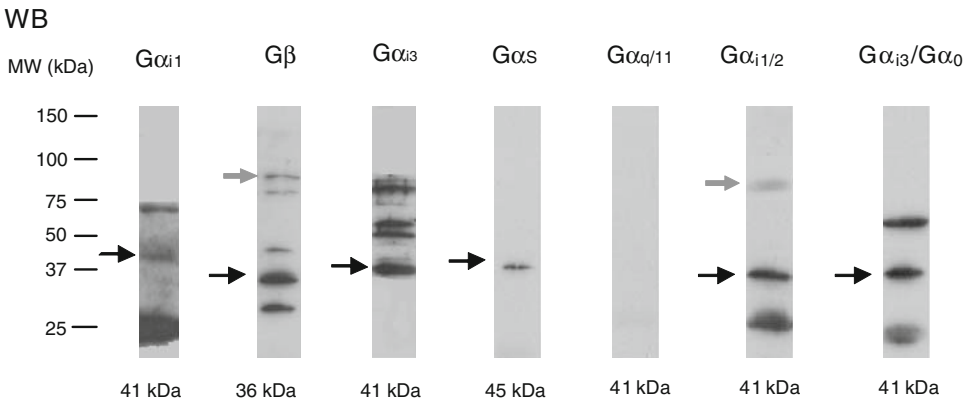

Fig. 2 Detection of proteins Gαi1, Gβ, Gαi1/Gαi2, Gαi3, Gαi3/Gα0, GαS, and Gαq/11 on erythrocyte membrane-soluble extracts (control) by immunoblotting

Detection of Protein Band 3 Levels in the Presence and Absence of AChE effectors and PTK/PTP Inhibitors

Erythrocyte membrane-soluble extracts were immunoprecipitated with either G_β or $G\alpha_{i1}/\alpha_{i/2}$ antibodies and electrophoresed as described above. Figures 3 and 4 show the identification of protein band 3 at its N- and C-terminus, respectively, obtained with band 3 antibodies in all erythrocyte suspensions previously incubated with control (lane 1), 10 μM ACh (lane 2), 10 μM VM (lane 3), 10 μM p72syk inhibitor (lane 4) with ACh (lane 5) or with VM (lane 6), and 10 μM p53/56lyn inhibitor (lane 7) with ACh (lane 8) or with VM (lane 9). Band 3 is visualized in the N domain mapped with antibody, in the erythrocyte suspensions aliquots previously treated with calpeptin (lane 4) and $p72^{syk}$ and $p53/56^{lyn}$ inhibitors assembled with VM (lane 9). In the remaining aliquots the N-specific domain is not seen. No band 3 lanes were visualized for the N domain in any of the pertussis toxin-treated aliquots (Fig. 5). However, anti $G\alpha_{i1}/\alpha_{i/2}$ revealed the band 3 N domain in all samples, with the exception of the control (lane 1) and ACh-treated (lane 2) aliquots. Band 3 is visualized at the C-terminus in all pretreated aliquots immunoprecipitated with anti $G\alpha_{i1}/\alpha_{i/2}$ (Fig. 4). The same results were obtained in aliquots incubated with AChE effectors and PTP/PTK inhibitors, assembled with the pertussis toxin (Fig. 6).

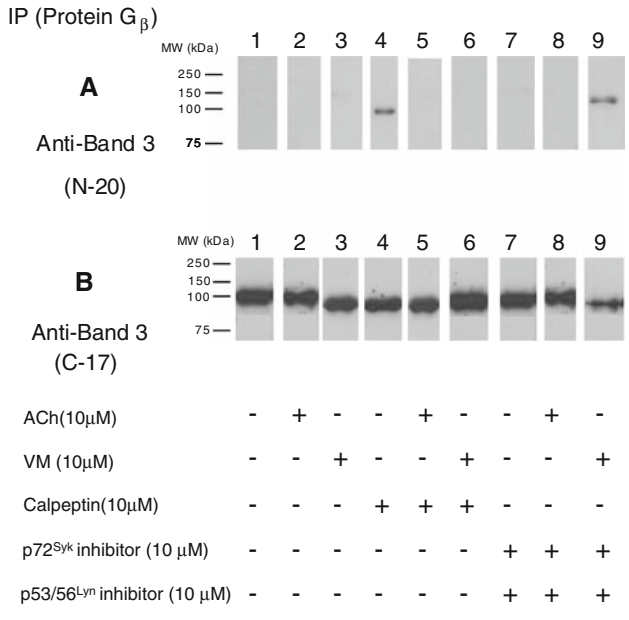

Fig. 3 Immunoprecipitation of protein Gβ on erythrocyte membrane-soluble extracts previously incubated with control (lane 1), 10 μM ACh (lane 2), 10 μM VM (lane 3), 10 μM $p72^{syk}$ inhibitor (lane 4) with ACh (lane 5) or with VM (lane 6), and 10 μM $p53/56^{lyn}$ inhibitor (lane 7) with ACh (lane 8) or with VM (lane 9). The immunoprecipitate was electrophoresed and immunoblotted with anti-band 3 antibodies for the C-terminal and N-terminal

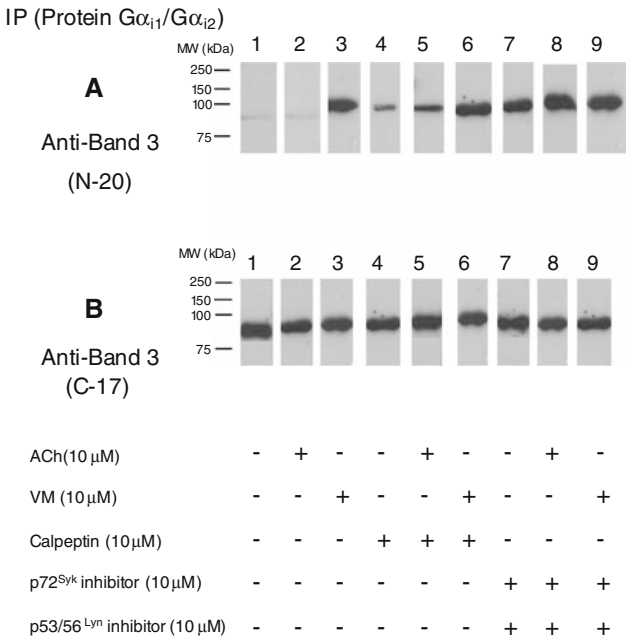

Fig. 4 Immunoprecipitation of protein Gαi1/Gαi2 on erythrocyte membrane-soluble extracts previously incubated with control (lane 1), 10 μM ACh (lane 2), 10 μM VM (lane 3), 10 μM $p72^{syk}$ inhibitor (lane 4) with ACh (lane 5) or with VM (lane 6), and 10 μM $p53/56^{lyn}$ inhibitor (lane 7) with ACh (lane 8) or with VM (lane 9). The immunoprecipitate was electrophoresed and immunoblotted with anti-band 3 antibodies for the C-terminal and N-terminal

Discussion

In this study, we report for the first time, to the best of our knowledge, the changes in erythrocyte AChE activity under the influence of band 3 protein phosphotyrosine status. The highest values of AChE activity were obtained in aliquots where the degree of basal phosphorylation was not manipulated, which encompassed control and ACh aliquots (Fig. 1). A "steady-state" balance between band 3 phosphorylated and dephosphorylated states has already been described for erythrocytes under physiological conditions (Wang et al. 1997; Bordin et al. 2002). This physiological behavior may influence the erythrocyte functions as a whole and the AChE conformational shape in particular, which consequently explains the changes in enzyme activity. ACh is the natural substrate of AChE and it was added to the erythrocyte suspensions at a lower concentration than the enzyme K_m, thereby explaining the slight increase in its activity ($p < 0.03$). The presence of velnacrine induces the same degree of AChE inhibition in all manipulated erythrocyte suspensions, as depicted in Fig. 1, independently of the band 3 phosphorylation degree. The same reduced activity is also obtained with lower velnacrine concentrations (results not shown). Nevertheless, the utilization of DMSO as solvent for preparation of PTK and PTP inhibitor solutions does not

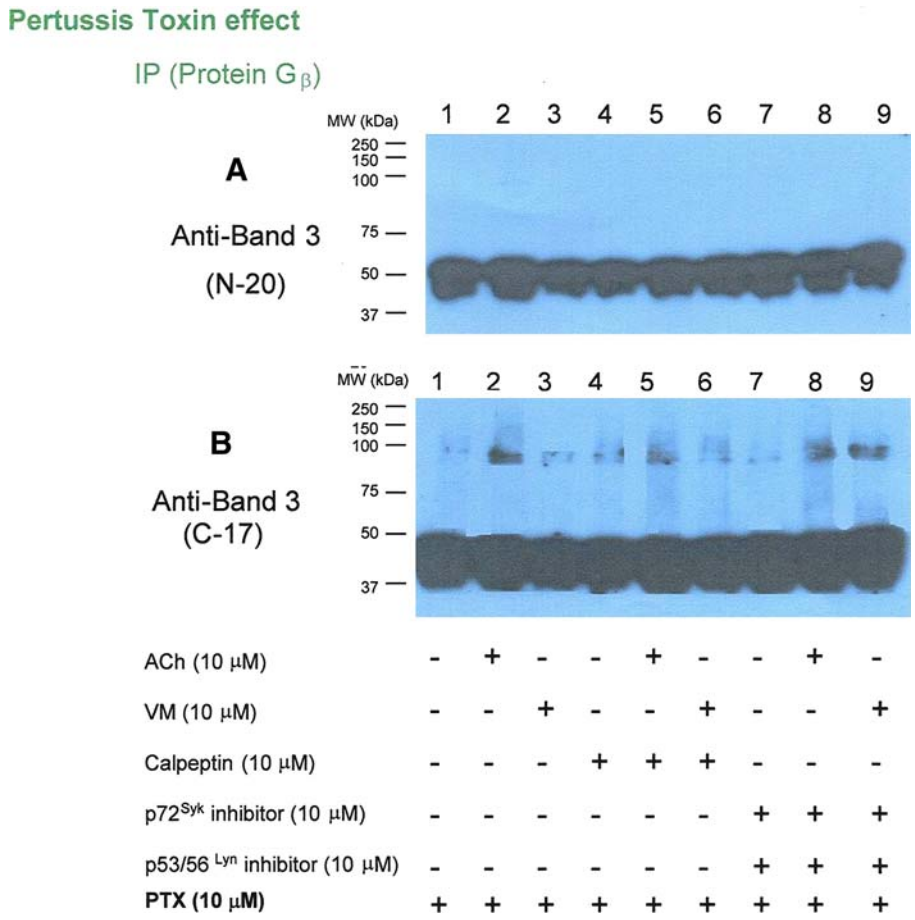

Fig. 5 Effect of pertussis toxin on immunoprecipitation of protein Gβ in erythrocyte membrane-soluble extracts previously incubated with control (lane 1), 10 μM ACh (lane 2), 10 μM VM (lane 3), 10 μM $p72^{syk}$ inhibitor (lane 4) with ACh (lane 5) or with VM (lane 6), and 10 μM $p53/56^{lyn}$ inhibitor (lane 7) with ACh (lane 8) or with VM (lane 9). The immunoprecipitate was electrophoresed and immunoblotted with anti-band 3 antibodies for the C-terminal and N-terminal

influence AChE enzyme activity as documented by us in the past (Santos et al. 2002).

In the present study, we verified that changes in band 3 protein phosphorylation/dephosphorylation status induce a statistically significant decrease in AChE enzyme activity. Nevertheless, values of enzyme activity obtained after induced maintenance of phosphorylated band 3 (calpeptin aliquots) are higher than those determined by the effectors of band 3 dephosphorylation (Syk + AMGT aliquots), in both the presence and the absence of ACh. Previously, a parallel dependence on the degree of band 3 phosphorylation has been described by us for the erythrocyte aggregation index (Saldanha et al. 2007).

Whereas band 3 extraction occurring under nondenaturing conditions (Triton X-100) is accompanied by AChE, as verified by us (Carvalho et al. 2004), there is no evidence of any kind of structural interactions between them. We have confirmed, using fluorescent probes, that the process of erythrocyte exovesiculation originates microvesicles enriched with AChE (Saldanha et al. 2002), while others have verified more stable tyrosine phosphorylation of band 3 in vesicles than in whole erythrocytes (Minetti et al. 2004).

We hypothesize that the degree of band 3 phosphorylation may explain our statistical significant results on enzyme AChE activity changes, as a consequence of a conformational modulation between the two proteins. Bordin has additionally demonstrated a functional relation between band 3 phosphorylation and crenation of red blood cells (Bordin et al. 1995; Chu and Low 2006).

In the present study we further confirmed that human erythrocytes have different types of G proteins, namely, $G\alpha_{i1}$, G_{β}, $G\alpha_{i1/\alpha_{i/2}}$, $G\alpha_{i3}$, $G\alpha_{i3}/G\alpha_{0}$, and $G_{\alpha S}$, as described in the literature (Iyengar et al. 1987; Olearczyk et al. 2004a, b; Escribá et al. 2003; Moss et al. 1983; Hsia et al. 1984). We verified that anti-$G\alpha_{i1/\alpha_{i/2}}$ protein is able to immunoprecipitate band 3, as shown in Fig. 4. The N-terminus of band 3 is visualized in all lanes, except for the control and ACh-treated aliquots, which show higher AChE activity (Fig. 4). The antibody against a peptide mapping within an internal region (110 to 160 amino acids) near the N-terminus of band 3 is unable to bind and therefore is not seen. Our data pointed out an association between protein $G\alpha_{i1/\alpha_{i/2}}$ and band 3 at either the N- or the C terminus, which is independent of band 3 phosphorylation status. The same was referred to above for AChE enzyme activity

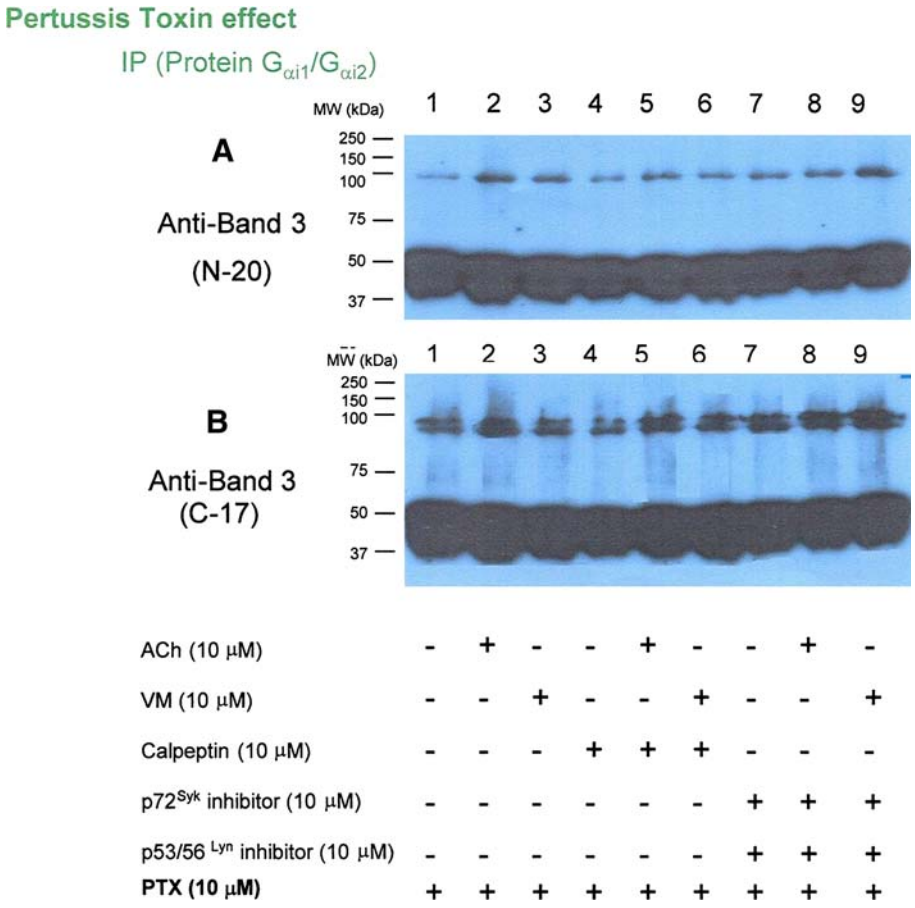

Fig. 6 Effect of pertussis toxin on immunoprecipitation of protein Gαi1/Gαi2 in erythrocyte membrane-soluble extracts previously incubated with control (lane 1), 10 μM ACh (lane 2), 10 μM VM (lane 3), 10 μM $p72^{syk}$ inhibitor (lane 4) with ACh (lane 5) or with VM (lane 6), and 10 μM $p53/56^{lyn}$ inhibitor (lane 7) with ACh (lane 8) or with VM (lane 9). The immunoprecipitate was electrophoresed and immunoblotted with anti-band 3 antibodies for the C-terminal and N-terminal

values, which are also independent of the phosphotyrosines (except for control and ACh-treated). Concerning G_β protein, an association occurs with band 3 at (i) the C-terminus, independently of any AChE activity or band 3 phosphorylation, and (ii) the N-terminus when band 3 is mostly phosphorylated (calpeptin) or mostly dephosphorylated (PTK inibitors), in the presence of velnacrine only. These two erythrocyte suspensions have in common the absence of methemoglobin and a higher oxygen content (98.6%), while the remaining contain oxyhemoglobin concentrations ranging from 11.3% to 32%. Low and Chu have demonstrated that deoxygenation can shift the glycolytic enzymes from band 3 to the cytosol, thereby triggering changes in erythrocyte membrane mobility (Corbett et al. 1994; Chu and Low 2006). Furthermore, the presence of pertussis toxin showed similar lane profiles for the band 3 C-terminus. No band 3 traces were immunodetected at the near N-terminus with pertussis toxin (Fig. 5). Additionally, it seems that there is no association between band 3 and G_β protein at the N-terminus for any aliquots with pertussis toxin. Our results with pertussis toxin suggest a potential association between band 3 and $G\alpha_{i1/\alpha i/2}$ protein occurring independently of AChE enzyme activity and degree of band 3 phosphorylation.

Given that pertussis toxin is able to rybosilate Gi proteins and consequently inactivate them but maintain the subunits together (Moss et al. 1983; Hsia et al. 1984), we hypothesized that there is no linkage between Gi protein and band 3 in the near-N-terminus domain, but near the C-terminus. By this rationale, we may speculate that without pertussis toxin, Gi protein seems to remain at an unknown level of dissociated and/or associated subunits. If so, we may suppose that (i) Gi protein ($G\alpha_{i1/\alpha i/2}$ G_β) is associated with the C-terminus independent of the degree of band 3 phosphorylation and AChE activity; (ii) $G\alpha_{i1/\alpha i/2}$ is associated with the N-terminus at lower AChE activity; and (iii) G_β protein is associated with the N-terminus when band 3 is phosphorylated and dephosphorylated assembled with velnacrine. From our results, we conclude that the erythrocyte Gi protein is not dissociated, and binding at the C-terminus under physiological conditions follows this dissociation when AChE binds velnacrine and band 3 is mostly dephosphorylated. When AChE is less active (in the presence of velnacrine), the Gi protein subunits also bind to the band 3 N-terminus. Furthermore, there is a study supporting the idea that an erythrocyte Gi protein is involved in the ATP efflux, by an unknown mechanism, which occurs when erythrocytes are submitted to mechanical deformability (Olearczyk et al. 2004a, b).

It is important to recall that glycolytic enzymes bind to the band 3 protein N-terminus (dependent on the degree of band 3 phosphorylation and hemoglobin oxygenation) as well as to 2,3-biphosphoglycerate, PTK, PTP, ankyrin, and deoxygenated hemoglobin (Campanella et al. 2005; Bordin et al. 2002, 2005; Anong et al. 2006). Recently, it has been reported that band 3 is a substrate for erythrocyte transglutaminase yielding a cross-link at the N-terminus near the binding site for band 4.2 protein (Gutierrez and Sung 2007). Part of the molecular binding reported in the N cytoplasmic domain of band 3 (cd3) originates covalent ligations, which may induce conformational modifications most likely triggering several anchoring sites recognized by Gi protein. However, further studies must be performed in order to clarify this hypothesis.

We thus conclude that an erythrocyte G protein (with subunits $G\alpha_{i1/2}$ and G_{β}) is associated with band 3 protein. AChE enzyme activity is dependent on protein band 3 phosphorylation status and may be involved in a conformational linkage between G protein and band 3. Therefore, it would be interesting to verify whether, in transgenic rats overexpressing AChE, it is possible to reduce its activity using AChE and PTK inhibitors.

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