Amyloid peptide inhibits ATP release from human erythrocytes

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Abstract: The oxygen required to meet metabolic needs of all tissues is delivered by the erythrocyte, a small, flexible cell, which, in mammals, is devoid of a nucleus and mitochondria. Despite its simple appearance, this cell has an important role in its own distribution, enabling the delivery of oxygen to precisely meet localized metabolic need. When an erythrocyte enters in a hypoxic area, a signalling pathway is activated within the cell resulting in the release of ATP in amounts adequate to activate purinergic receptors on vascular endothelium, which trigger secretion of nitric oxide and other factors resulting in vasodilatation. Among other mechanisms, binding of deoxyhemoglobin to the cytoplasmic domain of the anion-exchange protein band 3 is probably involved in this pathway. The present study investigates the effect of amyloid β peptide exposure on this molecular mechanism. We report that deoxygenated human erythrocytes fail to release ATP following 24 h exposure to amyloid β peptide. Concurrently, amyloid β peptide induces caspase 3 activation. Preincubation of amyloid β peptide treated erythrocytes with a specific inhibitor of caspase 3 prevents amyloid-induced caspase 3 activation and restores the erythrocyte's ability to release ATP under deoxygenated conditions. Since the activity of red cell phosphofructokinase, a key step in glycolytic flux, is not modified within the red cell following amyloid peptide exposure, it is likely that ATP release reduction is not dependent on glycolytic flux alterations. It has also been suggested that the heterotrimeric G protein, Gi, and adenylyl cyclase are downstream critical components of the pathway responsible for ATP release. We show that cAMP synthesis and ATP release are not failed in amyloid-peptide-treated erythrocytes in response to incubation with mastoparan 7 or forskolin plus 3-isobutyl-1-methyl xanthine, agents that stimulate cAMP synthesis. In conclusion, these results indicate that amyloid β peptide inhibits ATP release from deoxygenated erythrocytes by activating red cell caspase 3, suggesting a pathophysiologic role for vascular amyloid peptide in Alzheimer's disease.

Key words: band 3, erythrocyte, caspase 3, amyloid β peptide, ATP, cAMP.

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Abbreviations: Abbreviations: Aβ, amyloid β; cdb3, cytoplasmic domain of band 3; Hb, hemoglobin; AD, Alzheimer's disease; Z-DEVD-FMK, benzyloxycarbonyl-Asp(OCH_3)-Glu-(OCH_3)-Val-Asp(OCH_3)-fluoromethylketone; IBMX, 3-isobutyl-1-methyl xanthine.

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Introduction

It has become increasingly clear that, in addition to functioning as an intracellular energy source, ATP can serve as an important extracellular signalling molecule (Gordon 1986). ATP activates P2 receptors on endothelium, platelets (Wang et al. 2003), and other blood cells (Di Virgilio et al. 2001), regulating several physiological responses including vascular tone (González-Alonso et al. 2002) platelet aggregation, and the release of endothelial factors. The matching of oxygen supply with demand requires a mechanism that increases blood flow in response to decreased tissue oxygen levels. The erythrocyte, by virtue of its ability to release ATP in response to exposure to reduced oxygen tension (Bergfeld and Forrester 1992; Dietrich et al. 2000; Ellsworth et al. 1995) or to mechanical deformation (Sprague et al. 1996), can participate in local control of vascular calibre (McCullough et al. 1997; Collins et al. 1998; Dietrich et al. 1996; Ellsworth 2000). Erythrocyte-derived ATP has been shown to stimulate endogenous endothelial NO synthesis in the circulation of the lung (Sprague et al. 2003) as well as in striated muscle (McCullough et al. 1997). Isolated rat cerebral arterioles have been shown to vasodilate under low extraluminal O_2 only in the presence of red blood cells (RBCs). This vascular response was accompanied by a concomitant increase of ATP in the vessel effluent, resulting in activation of a vasodilator mechanism operative in these vessels, possibly NO (Kelm et al. 1988). Similarly, ATP applied locally caused conducted vasodilation in isolated rat cerebral arterioles (Dietrich et al. 1996).

The signal transduction pathway that relates both pharmacological and physiological stimuli to ATP release from erythrocytes has been described. Components of this pathway include the heterotrimeric G proteins G_s (Olearczyk et al. 2001), G_i (Olearczyk et al. 2004), adenylyl cyclase (AC), cyclic AMP (cAMP) dependent protein kinase A (Sprague et al. 1998), and the cystic fibrosis trans-membrane conductance regulator or pannexin as the final conduit for release (Liang et al. 2004). In human erythrocytes, pharmacological activation of G_i results in stimulation of AC activity, leading to increased cAMP synthesis and release of ATP (Olearczyk et al. 2004), a stimulus for endothelial NO synthesis. It has been hypothesized that the first signal pathway responsible for ATP release from deoxygenated erythrocytes involves an interaction between deoxyhemoglobin and the cytoplasmic domain of the anion-exchange protein band 3, i.e., cdb3 (Jagger et al. 2001). Recently, we have shown that amyloid β peptide induces caspase 3 activation in human erythrocytes under normoxic conditions (Clementi et al. 2007) and it has been shown that this event leads to cdb3 cleavage (Mandal et al. 2003). Previous studies showed that important parameters indicative of RBC function and integrity might be negatively affected in RBCs in AD patients (Kay and Goodman 1994; Engstrom et al. 1995; Clementi et al. 2004; Delibas et al. 2002; Jayakumar et al. 2003; Mohanty et al. 2008). Here, we investigate the hypothesis that amyloid peptide alters the signal transduction pathway relating to ATP release from erythrocytes in response to reduced oxygen tension.

Materials and methods

Chemicals

Aβ(1–42) peptide was obtained from Peptide Specialty Laboratories GmbH (Heidelberg, Germany). Analysis of the peptide by reverse-phase, high-performance liquid chromatography (HPLC) and mass spectrometry revealed a purity >98%. All other reagents were of the highest grade and obtained from Sigma-Aldrich Chemical Co. (St. Louis, Missouri).

Buffer A: (25 mmol/L HEPES, 1 mmol/L NaH_2PO_4, 110 mmol/L NaCl, 5 mmol/L KCl, and 2 mmol/L $MgCl_2$) at pH 7.4 and 290 ± 5 mOsm·Kg^{-1}, measured by an Osmostat OM-6020 apparatus (Daiichikagakuco Ltd., Kyoto, Japan). Buffer B: (25 mmol/L HEPES, 1 mmol/L NaH_2PO_4, 110 mmol/L NaCl, 5 mmol/L KCl, and 2 mmol/L $MgCl_2$) at pH 7.3 and 290 ± 5 mOsm·Kg^{-1}, measured by an Osmostat OM-6020 apparatus (Daiichikagakuco Ltd.). The control experiments were performed by replacing Aβ peptide with a solution of an equal volume of HEPES buffer (untreated red blood cells).

Preparation of red blood cells

Heparinized fresh human blood was obtained from informed healthy donors in accordance with the principles outlined in the Declaration of Helsinki and immediately processed. Plasma separation was obtained by centrifuging at 2500*g* for 5 min. Human erythrocytes were isolated by density gradient centrifugation by Ficoll. After a separation procedure, the packed cells were gently resuspended with the washing HEPES buffer, divided in 2 samples, and suspended in buffer A or B (50%, *v*/*v*). Control cells (normoxic, buffer A) were prepared by equilibration under air at 37 °C. Samples with low levels of oxygenated hemoglobin (deoxygenated erythrocytes, buffer B) were prepared by submitting samples to cycles of in vacuum deoxygenation and nitrogen (ultrapurum) saturation (at a pressure of 760 Torr (1 Torr = 133.3 Pa). Two minutes after the deoxygenation process ended, cells and a 200-μL sample of the buffer-containing cells were removed for analysis of cAMP and ATP concentration. Buffer B was 0.1 pH unit lower than buffer A, to compensate for the Haldane effect occurring during the deoxygenation step. Saturation of hemoglobin with oxygen was determined by spectrophotometric measurements (Cary

3E, Varian, Palo Alto, California) utilizing the millimolar absorptivities reported by Zijlstra et al. (1991). In experiments performed under low PO_2 conditions, the measured percentage of deoxygenated hemoglobin was 60% ± 0.32%. The amyloid β peptide concentration was fixed to 2 and 10 μmol/L. To exclude the possibility that erythrocyte lysis contributed to the measurement of ATP release, after measuring ATP in the erythrocyte suspension, erythrocytes were sedimented by centrifugation at 500*g* for 10 min. The presence of hemoglobin in the supernatant was determined by light absorption at 405 nm (Cary 3E, Varian).

Measurement of ATP

ATP was measured by the luciferin–luciferase technique (Bergfeld and Forrester 1992; Dietrich et al. 2000), which uses the ATP concentration dependence on light and is generated by the reaction of ATP with firefly tail extract. Sensitivity was augmented by the addition of synthetic D-luciferin to the crude firefly tail extract. A 200-μL sample of the RBC suspension was injected into a cuvette containing 100 μL of crude firefly tail extract (10 mg/mL distilled water, FLE 250; Sigma-Aldrich) and 100 μL of a solution of synthetic D-luciferin (50 mg/100 mL distilled water; Sigma-Aldrich). The light emitted was detected using a luminometer (Autolumat LB 953, EG & G, Turku, Finland). A standard curve was obtained on the day of each experiment.

Measurement of cAMP

Erythrocytes were added to 4 mL of ice-cold absolute ethanol containing HCl (1 mmol/L), and the mixtures were centrifuged at 14 000*g* for 10 min at 4 °C. The supernatants were removed and stored overnight at –20 °C to precipitate the remaining proteins. Samples were then centrifuged a second time at 3700*g* for 10 min at 4 °C. The supernatant was removed and dried under vacuum centrifugation. Concentrations of cAMP were then determined with a cAMP Biotrak enzyme immunoassay system (Amersham Biosciences).

Incubation of RBCs with agents that alter cAMP concentration or activity

Amyloid treated and untreated erythrocytes were incubated for 2 min either with mastoparan 7 (activator of G_i, 10 μmol/L; Sprague et al. 2005) or forskolin (10 μmol/L dissolved in ethanol, final ethanol concentration 0.2%) to stimulate adenylyl cyclase plus 3-isobutyl-1-methyl xanthine (IBMX) (100 μmol/L) to prevent cAMP degradation or their respective vehicles. Amounts of ATP released in response to mastoparan 7, forskolin, and IBMX, as well as the effects of these agents on cAMP concentration, were determined.

Analysis of caspase 3 activity

Caspase activity was measured by using a Sigma-Aldrich assay kit following the manufacturer's instructions. DEVD-pNA was used as a colorimetric substrate. Human deoxygenated erythrocytes, after treatment with amyloid peptide (2 and 10 μmol/L for 24 h at 37 °C), were pelleted by centrifugation. The pellets were washed with PBS and lysed in 50 mL of chilled cell lysis buffer and left on ice for 10 min. Lysate was centrifugated at 10 000*g* for 1 min at 4 °C, and the supernatant was used for the caspase 3 assay. The protein concentration was confirmed by bicinchoninic acid (BCA) assay. The protease activity was determined after 25 min at 37 °C by spectrophotometric detection at 405 nm of the chromophore *p*-nitroanilide (pNA) after its cleavage by caspase 3 from the labelled caspase 3-specific substrate (DEVD-pNA). Before performing spectrophotometric determinations, erythrocyte lysates were filtered (cut-off 50 000), to eliminate interferences by hemoglobin spectra. Additional control assays with the presence of specific caspase 3 inhibitor (Z-DQMD-FMK) and in the absence of recombinant human caspase were performed for measuring the nonspecific hydrolysis of the substrate (data not shown (SDS–PAGE)). Erythrocytes were osmotically lysed in hypotonic solution and the lysate was incubated overnight with rabbit anti-caspase 3 (Santa Cruz, Biotechnology, Santa Cruz, California) antibody at 4 °C. Protein A/G agarose (Santa Cruz, Biotechnology) was added and incubated for 1 h at room temperature and the immunoprecipitate was washed, denatured, and separated by SDS–PAGE. Before starting SDS–PAGE, the amount of protein in the samples derived from treated and untreated cells was determined to load the same amount of protein (Lowry et al. 1951). After SDS–PAGE, proteins were electrophoretically transferred to a nitrocellulose membrane and probed with anti-caspase 3 antibody, followed by alkaline phosphatase linked goat anti-rabbit secondary antibody (Santa Cruz, Biotechnology), and finally visualized using Bio-Rad color developing reagent (Bio-Rad Laboratories Inc.,Hercules, California). Images of nitrocellulose membranes were acquired (Bio-Rad Gel Doc 2000) and scanned (Bio-Rad GS800) using Bio-Rad Quantity One software.

Phosphofructokinase activity

Phosphofructokinase (PFK) enzymatic activity was determined by the method described previously (Beutler 1975). Determinations were performed on hemolysates obtained from RBCs after treatment with amyloid peptide and from untreated and control RBCs. PFK experimental data were analysed with the Hill equation and 3 kinetic parameters, V_{max}, S_{50}, and n_{50}, were determined by the least-squares curve fitting method.

Statistical analysis

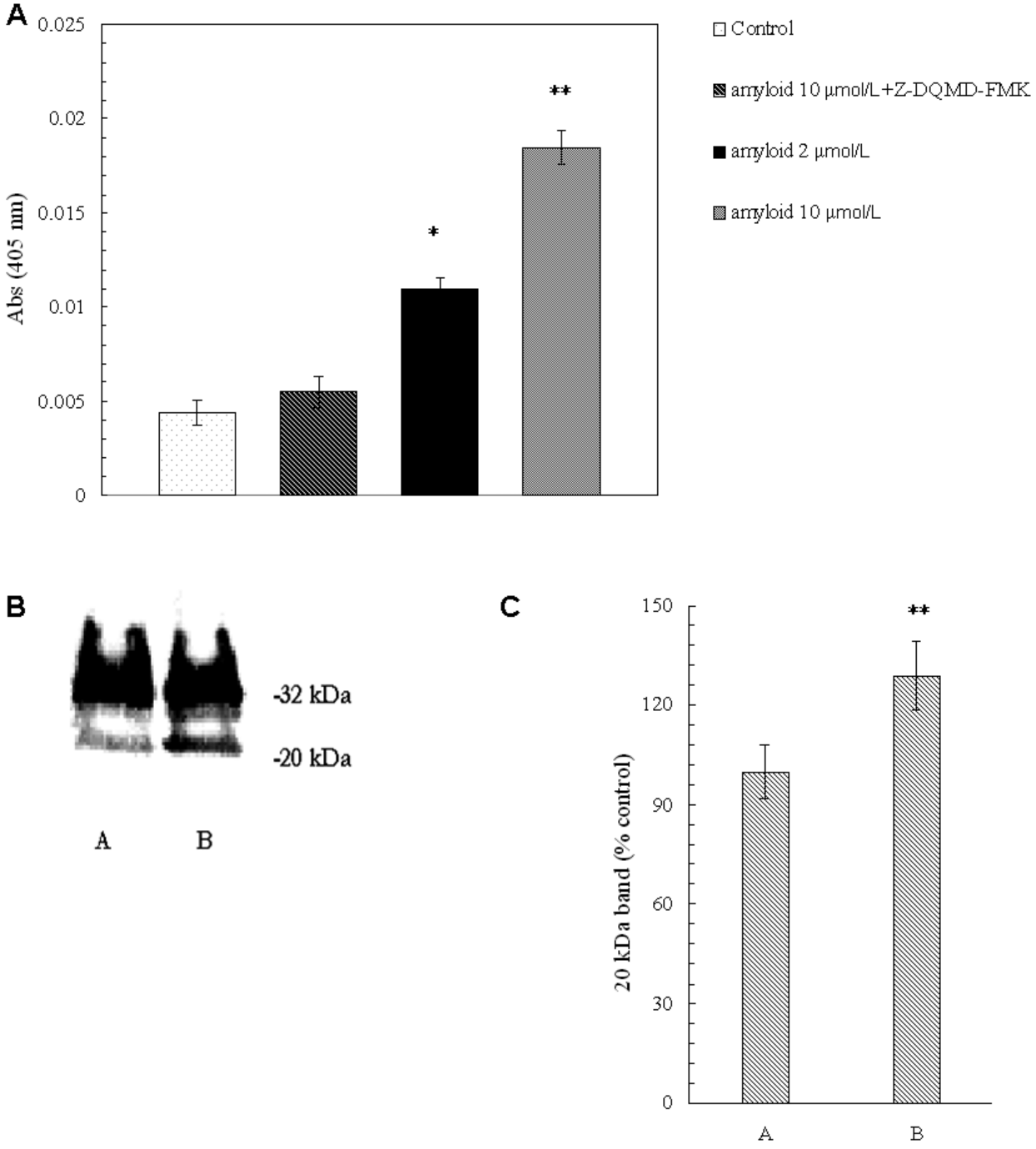
The data were analyzed by one-way ANOVA, followed by a post hoc Newman–Keul test for multiple comparisons among group means, and differences were considered statistically significant if $p < 0.05$. All results are presented as the mean ± SE of at least 3 different experiments performed in triplicate, unless otherwise specified.

Results

Amyloid peptide activates caspase 3 in deoxygenated erythrocytes

As shown in Fig. 1A, following amyloid β peptide treatment caspase 3 activity increases. Pretreatment of amyloid-exposed RBCs with a specific caspase 3 inhibitor, i.e., Z-DQMD-FMK, inhibits amyloid-mediated caspase 3 activation, ruling out the presence of unspecified proteolytic activities, i.e., calpains. Western blot analysis (Fig. 1B) indicates

Fig. 1. (A) Caspase 3 activation in deoxygenated erythrocytes. Activity of caspase 3 was determined in cell lysates obtained by erythrocytes at the end of treatment time with amyloid peptide ($N = 5$). **, $p < 0.01$ and *, $p < 0.001$ compared with control. (B) Caspase 3 activation in deoxygenated erythrocytes. Lysates were immunoprecipitated with anti-caspase 3 antibody and analysed for caspase 3 by Western blotting. Untreated cells, lane A; cells treated with 10 µmol/L amyloid peptide for 24 h, lane B; 20 and 32 kDa bands represent, respectively, the activated and inactivated form of caspase 3. (C) Quantification of the intensities of the band at 20 kDa determined by densitometric scanning of nitrocellulose membrane. ($N = 4$). **, $p < 0.001$ with respect to control.



the presence of the active form of caspase 3 in intact erythrocytes after treatment with 10 µmol/L amyloid for 24 h. The appearance of the active form of caspase 3 is represented by the band at 20 kDa (Fig. 1C). WB and caspase 3 activity obtained in the presence of 10 µmol/L of amyloid peptide confirms that previously shown under normoxic conditions (Clementi et al. 2007).

Effect of amyloid peptide on ATP release and on accumulation of cAMP

To test whether amyloid-mediated activation of caspase 3 may alter the mechanism responsible for ATP release from deoxygenated erythrocytes, we evaluated ATP release levels in amyloid-treated and untreated erythrocytes. (Olearczyk et al. 2004) reported that activation of G_i resulted in the stimu-

Fig. 2. Effect of amyloid treatment on ATP release from deoxygenated and control erythrocytes. Erythrocytes were treated with 2 μmol/L amyloid peptide at 37 °C for 24 h ($N = 4$). **, $p < 0.01$ compared with deoxygenated cells. Red blood cell, RBC.

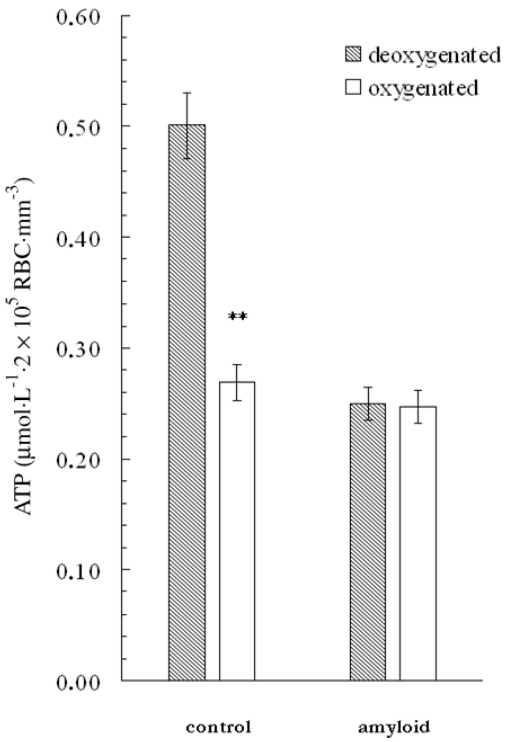
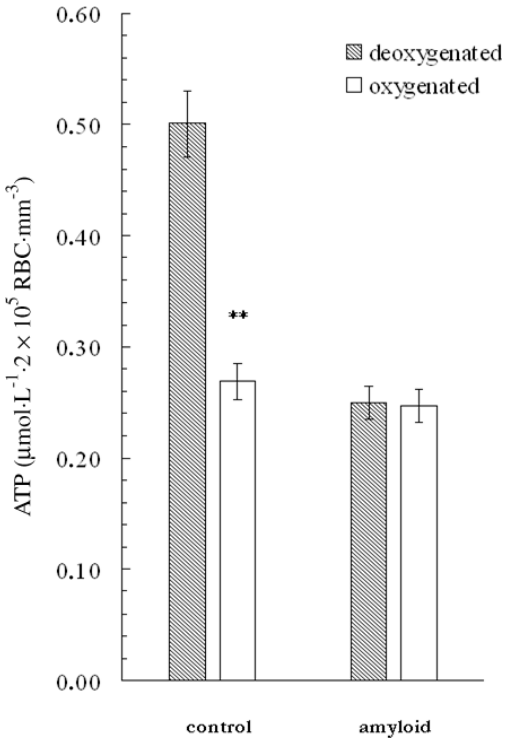


Fig. 3. Effect of amyloid treatment on cAMP accumulation in deoxygenated and control erythrocytes. Erythrocytes were treated with 2 μmol/L amyloid peptide at 37 °C for 24 h ($N = 4$). **, $p < 0.01$ compared with deoxygenated cells.



lation of AC activity, leading to increased cAMP synthesis and release of ATP; therefore, cAMP levels were also determined. As shown in Fig. 2, ATP release values from erythrocytes were significantly greater when the cells were exposed to solutions with low PO_2 than when the cells were exposed to solutions with normoxic conditions. When erythrocytes were treated with amyloid β peptide for 24 h, ATP release values from erythrocytes under low PO_2 were not significantly different with respect to the cells exposed to solutions with normoxic conditions. As shown in Fig. 3, cAMP accumulation levels in erythrocytes were significantly greater when the cells were exposed to solutions with low PO_2 than when the cells were exposed to solutions with normoxic conditions. When erythrocytes were treated with amyloid β peptide for 24 h, cAMP accumulation levels did not shown any significant difference from cells exposed to normoxic conditions. To clarify components of the signal transduction pathway responsible for amyloid-mediated effects on ATP release from erythrocytes under hypoxic conditions, further experiments were performed with mastoparan 7 and forskolin plus IBMX, agents able to stimulate cAMP synthesis and ATP release by activating Gi and cAMP synthesis, respectively. As shown in Figs. 4 and 5, ATP release values and cAMP levels were not different between erythrocytes incubated with mastoparan 7 or with forskolin plus IBMX in the presence and absence of amyloid peptide. These results rule out the involvement of G_i and AC in amyloid-mediated effects. To test whether amyloid-mediated activation of caspase 3 is a biochemical event linked with inhibition of ATP release from deoxygenated erythrocytes, further experiments were performed with a caspase 3-specific inhibitor, Z-DQMD-FMK. As shown in Figs. 4 and 5, pretreatment of erythrocytes with a caspase inhibitor abolishes the different ATP release values and cAMP levels shown by amyloid-treated erythrocytes and control cells under hypoxic conditions. Maximum effect was achieved when cells were treated with 100 μmol/L of Z-DQMD-FMK for 2 h prior to amyloid peptide exposure. This finding supports the hypothesis that caspase 3 mediated-band 3 cleavage could represent the key event responsible for inhibition of ATP release from erythrocytes following amyloid β exposure.

Effect of amyloid peptide on PFK activity

To test the possibility that ATP release inhibition following amyloid peptide exposure might be due to a reduced production of ATP by glycolitic flux, we performed an analysis of the activity of phosphofructokinase (PFK), the key regulatory step in glycolysis. We found that PFK activity analysed on the basis of the 3 parameters, V_{max}, S_{50}, and n_{50}, did not show significant differences between controls and amyloid-treated cells. The V_{max} value was 89.53 ± 4.18 mmol·L^{-1}·min·gHb^{-1} for control cells and 83.57 ± 3.24 mmol·L^{-1}·min·gHb^{-1} for treated cells , the n_{50} value was 1.29 ± 0.29 mmol/L for control cells and 1.29 ± 0.19 mmol/L for treated cells, and the S_{50} value was 0.240 ± 0.126 for control cells and 0.340 ± 0.123 for treated cells.

Hemolysis degree

An obvious potential source of extracellular ATP is spontaneous lysis of the erythrocytes. To determine cell lysis

Fig. 4. Effect of mastoparan 7 (10 μmol/L), forskolin (10 μmol/L) plus 3-isobutyl-1-methyl xanthine (IBMX, 100 μmol/L), and caspase 3 inhibitor, Z-DQMD-FMK (100 μmol/L), on ATP release from deoxygenated erythrocytes following amyloid exposure (2 μmol/L). Maximal ATP release in response to mastoparan 7 administration occurred 5 min after exposure for treated and untreated cells ($N = 5$). *, $p < 0.01$ compared with control cells;**, $p < 0.01$ compared with amyloid-treated cells.

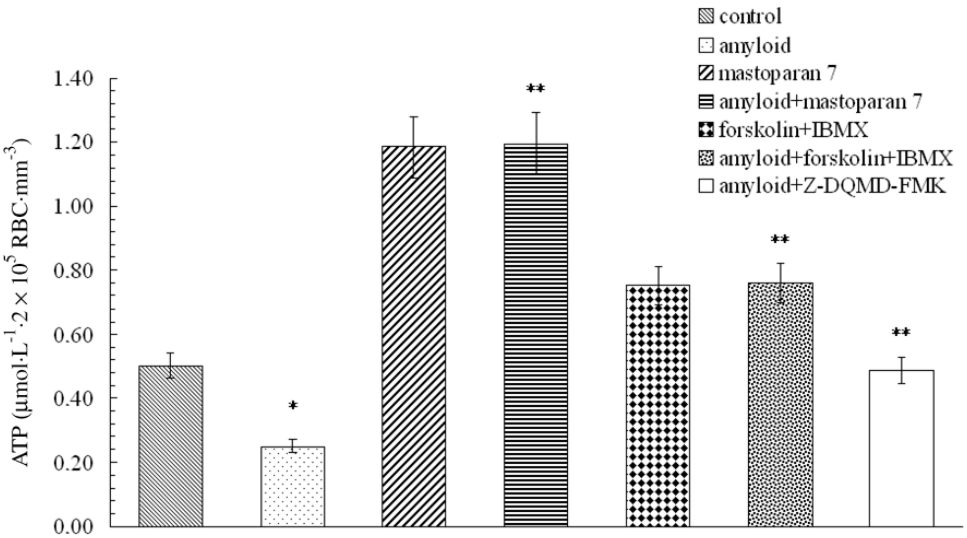
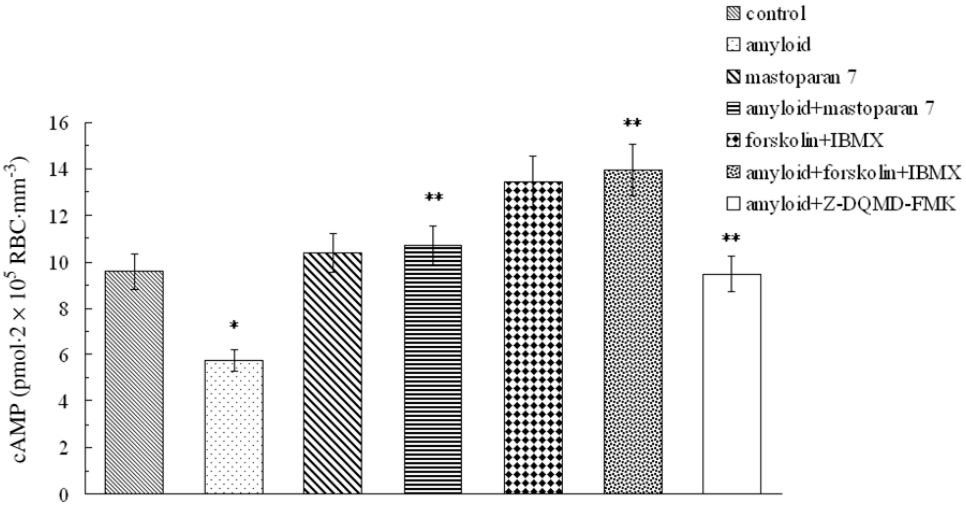


Fig. 5. Effect of mastoparan 7 (10 μmol/L), forskolin (10 μmol/L) plus 3-isobutyl-1-methyl xanthine (IBMX, 100 μmol/L), and caspase 3 inhibitor, Z-DQMD-FMK (100 μmol/L), on cAMP accumulation in erythrocytes following amyloid β exposure (2 μmol/L) ($N = 5$). *, $p < 0.01$ compared with deoxygenated cells; **, $p < 0.01$ compared with amyloid-treated cells.



after experiments, erythrocyte suspensions were analysed to evaluate haemoglobin concentration in the supernatant. The percentage of hemolysis was always <3%.

Discussion

It has been shown that erythrocytes participate in the regulation of vascular resistance in the lung (Dietrich et al. 2000; Sprague et al. 2003), striated muscle (McCullough et al. 1997), and isolated cerebral arterioles (Dietrich et al. 2000), releasing ATP in response to physiological stimuli such as reduced oxygen tension and mechanical deformation (Sprague et al. 2007). The initiating signal involved in the release of ATP from erythrocytes in response to reduced oxygen tension involves an interaction between deoxyhemoglobin and the cytoplasmic domain of cdb3. Following this event, it has been hypothesized that cytoskeleton conformational changes subsequent to deoxyhemoglobin binding to cdb3 cause stress on the membrane components, thus activating the downstream pathway relating to ATP release (Jagger et al. 2001). Consistent with this hypothesis, reorganization of the cytoskeleton and signalling pathway activation–deactivation are strongly linked with oxygenation–deoxygenation transitions of haemoglobin (Barvitenko et al. 2005; De Rosa et al. 2007; Chu et al. 2008).

We found that in human erythrocytes incubated in the presence of amyloid peptide, in association with caspase 3 activation, cAMP accumulation and ATP release from erythrocytes are reduced in response to incubation of these cells under low PO_2 conditions. Recently, Mandal et al. (2003) reported that in human erythrocytes, caspase 3 cleaves the peptide chain of the N-terminal cytoplasmic domain of band 3 protein at Asp45 and Asp205, and thereby the cdb3

interactions with several glycolytic enzymes, carbonic anhydrase II, and deoxyhemoglobin are lost. In the present study, erythrocyte pro-caspase 3 undergoes cleavage from the 32 kDa zymogen to the 20 kDa active form in amyloid-treated deoxygenated erythrocytes. Caspase 3 activation is confirmed by measuring the ability of lysates derived from amyloid-treated erythrocytes to cleave caspase 3 substrate, i.e., Ac-DEVD-pNA. It has been reported that 1–54 and 202–211 residues represent the most flexible regions in cdb3 (Zhang et al. 2000) and, consequently, the more accessible ones for caspase-mediated proteolysis. This finding is in line with previous reports that demonstrated an increase in breakdown of erythrocyte band 3 in AD subjects (Bosman et al. 1991; Kay and Goodman 1994).

Here we report that inhibition of ATP release from deoxygenated erythrocytes was abolished when these cells were incubated with the caspase 3 inhibitor Z-DQMD-FMK prior to treatment with amyloid peptides. On this basis, we suggest that caspase 3-mediated cleavage of cdb3 (Mandal et al. 2003), abrogating cdb3 binding sites for deoxy-Hb, interrupts the signal transduction pathway, which relates hemoglobin conformational transition (R→T) to heterotrimeric G protein G_i activation, ultimately resulting in ATP release inhibition. This finding supports previous papers regarding the importance of deoxyHb–band3 interaction in the regulation of key elements of erythrocyte function (Barvitenko et al. 2005; DeRosa et al. 2007; Chu et al. 2008).

The absence of any significant effect of amyloid peptide on the activity of phosphofructokinase (PFK) suggests that this enzyme, a key control step in the glycolytic pathway, is not a target of amyloid peptide action within the erythrocyte, corroborating that the inhibition of ATP release induced by amyloid peptide is not connected to an impairment of the glycolytic flux.

When heterotrimeric G proteins are activated, the subunit dissociates from the β complex. The subunit and the β complex can then regulate, either individually or synergistically, the catalytic activity of AC (Tang and Gilman 1991). Here we report that amyloid-treated and untreated erythrocytes generate comparable amounts of intracellular cAMP and ATP release following incubation with mastoparan 7 and forskolin–IBMX, agents that activate G_i and AC activities, respectively, suggesting that the failure of low PO_2 to stimulate ATP release in amyloid-treated erythrocytes is not explained by a simple decrease in the activity of subunit G_i in heterotrimeric G proteins and AC.

It should also be noted that mechanical deformation is known to stimulate ATP release from erythrocytes (Sprague et al. 1996). Thus, it is possible to hypothesize that amyloid peptide may also inhibit this mechanism of ATP release. Further studies are required to confirm such a speculation.

Although it remains uncertain whether micromolar concentrations are able to mimic the in vivo effects of amyloid peptides, our finding implies that ATP release from AD erythrocytes may be impaired, particularly in brain microvasculature regions after ischemia or where deposition of amyloid peptide may cause a narrowing of the lumen of cerebrovessels, inducing permanent hypoxic conditions. This event should lead to a reduced stimulus for endogenous NO synthesis in the microvasculature, which contributes to the increased severity of vascular complications in AD patients.

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