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Palytoxin Induces Functional Changes of Anion Transport in Red Blood Cells: Metabolic Impact

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Abstract Palytoxin (PTX) is classified as one of the most powerful marine biotoxins (of high molecular weight and no protein origin) because it is able to interact strongly with important cellular structures influencing their function in different biological processes. This study of the effects of PTX on red blood cells (RBC) extends the knowledge about its toxicity, which concerns not only the well-known action on Na^+/K^+-ATPase but also band 3 protein (B3 or AE1), the role of which is essential for anion transport and for the structure, function, and metabolic integrity of the erythrocyte. The effects of PTX on RBC can be summarized as follows: it alters the anionic flux and seriously compromises not only CO_2 transport but also the metabolic modulation centered on the oxy–deoxy cycle of hemoglobin; it stabilizes the plasma membrane by preventing lipid peroxidation; and its effect does not lead to activation of caspases 3 and 8. From what is reported in steps 2 and 3, and on the basis of the results obtained on hemolysis, methemoglobin levels, and phosphatase activity, an increase of the reducing power of the erythrocytes (RBC) in the presence of PTX clearly emerges. The results have enabled us to outline some metabolic adaptations induced in the RBC by PTX.

Keywords Band 3 protein · Caspase 3 · Erythrocytes · Hemoglobin · Metabolism · Palytoxin

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PTX, one of the most powerful and lethal marine toxins (Rhodes et al. 2002; Rhodes and Munday 2004; Wiles et al. 1974), first isolated from the marine coelenterate *Palythoa toxica* (Moore and Scheuer 1971), is a nonprotein marine biotoxin of high molecular weight (Moore et al. 1982) (Fig. 1). The toxicity reported in humans appears to be due to the supply of marine organisms (clupeid fish), and due to the respiratory inhalation of marine aerosols generated by ocean waves (Ciminiello et al. 2006; Gallitelli et al. 2005; Onuma et al. 1999).

The main molecular target of PTX is the plasma membrane Na^+/K^+-ATPase (Artigas and Gadsby 2003; Hilgemann 2003), a sodium pump composed of two α and two β subunits (Beaugè and Glynn 1979; Glynn 1988). This protein pump is ubiquitous and regulates and guarantees the maintenance of sodium and potassium gradients across the cell membrane, a process that requires ATP (Artigas and Gadsby 2003, 2004; Läuger 1991).

Extremely low concentrations of PTX (1 pM), due to its binding to the α subunit of Na^+/K^+-ATPase, depolarizes

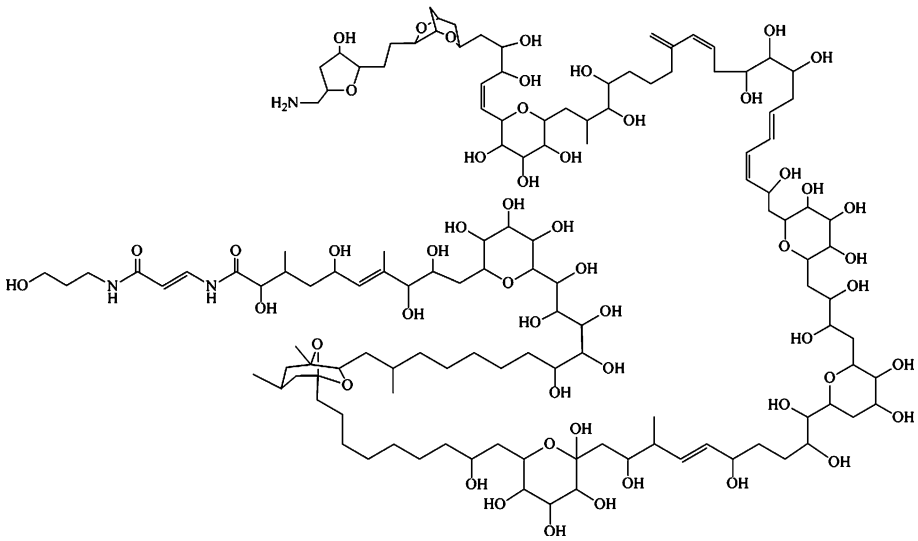

Fig. 1 Chemical structure of PTX according to Uemura et al. (1985)

the cell by causing a lower conductance and the appearance of relatively nonselective cation channels, which likely include at least part of the pump's ion translocation pathway (Artigas and Gadsby 2004; Habermann and Chhatwal 1982; Ikeda et al. 1988; Muramatsu et al. 1988; Tosteson et al. 1991). Altered ionic homeostasis, induced by PTX, influences other equilibria such as those of Ca^{2+} and H^{+} ions. At the cellular level, this results in a collapse of the mechanisms controlling the ion–water balance, triggering a variety of different cellular effects (Frelin and Van Renterghem 1995; Ikeda et al. 1988; Muramatsu et al. 1984; Sauviat 1989).

The action site of PTX was evidenced by means of ouabain and other cardiac glycosides, specific ligands of the sodium pump, which allowed the identification of the Na^{+}/K^{+}-ATPase as one of the main target of the toxin (Kim et al. 1991). In contrast, vanadate, a specific inhibitor of all type P ATPases (which the Na^{+}/K^{+}-ATPase belongs), inhibits the toxin action acting as an analog of phosphate (Kim et al. 1995; Tosteson et al. 1991). Vanadate forms a stable complex with the sodium pump on the cytoplasmic side of the red blood cells (RBC), stabilizing the dephosphorylated form (Cantley et al. 1978).

Moreover, PTX, appears to exert its toxicity by modulating the cytoskeleton properties, in particular by acting on actin filaments (dos Remedios et al. 2003). Studies on neuronal cells of human neuroblastoma BE (2)-M17, performed to evaluate the anticytoskeletal effects and the cationic flux changes induced by the marine toxins, showed that PTX triggers a series of cytotoxic events that range from the rapid depolarization and cytosolic Ca^{2+} increase to the cytoarchitecture reorganization (Louzao et al. 2006, 2007; Vilariño et al. 2008). More recent studies have suggested that Na^{+}/K^{+}-ATPase, as a result of its interaction with the toxin, may act as a signal transducer, activating some mechanisms closely associated with the cytoskeleton (Louzao et al. 2008; Xie and Askari 2002).

In RBC, the Na^{+}/K^{+}-ATPase, like the glucose transport protein, is either in contact with or closely linked to B3. All this would be consistent with a transport protein complex centered on B3 and responsible for the whole transport process, which overcomes the actual carriage of cations and anions being linked to energy production (Janoshazi and Solomon 1989).

B3 is the major transmembrane glycoprotein (number of copies of 1.2×10^{6}) of RBC. It mediates the electroneutral exchange of chloride and bicarbonate ions across the plasma membrane and plays an essential role in the transport mechanism of CO_2 (Passow 1986). From a structural point of view, the protein is characterized by two distinct functional domains: first, an N-terminal cytosolic domain (CDB3) (residues 1–360) that binds a variety of proteins, including hemoglobin (Hb) and some glycolytic enzymes (Zhang et al. 2000), and anchors the RBC membrane to the cytoskeleton through ankyrin and protein 4.2 (Lux and Palek 1995), and second, a C-terminal domain (residues 361–911) crossing the bilayer 12 folds, that mediates the anion transport through the membrane (Tanner 1997) and binds carbonic anhydrase II (Sterling et al. 2001; Vince and Reithmeier 2000).

The protein regulates anion exchange and erythrocyte metabolism by interacting competitively through its N-terminal end with some glycolytic enzymes and Hb. This allows an adequate and timely glucose 6-phosphate (G6P)

flux toward the pentose phosphate pathway (PPP) in the high-oxygenation state or to the glycolytic one (EMP) in the low-oxygenation state (Giardina et al. 1995; Lewis et al. 2009; Russo et al. 2008). The correct modulation of the G6P metabolic fluxes (centered on the deoxygenation–oxygenation cycle of hemoglobin) protects erythrocytes against oxidative stress and maintains their structural and functional integrity, guaranteeing an adequate life cycle. An increase of the oxidative level in RBC promotes caspase activation (caspase from procaspase), which in turn is followed by CDB3 cleavage and the consequent loss of the RBC metabolic regulation (oxygen dependent) (Mandal et al. 2003). All this triggers a vicious cycle that leads to caspase 3 activation and to the conclusion of the RBC life cycle (Ficarra et al. 2009; Galtieri et al. 2010; Tellone et al. 2008).

It should be recalled that the proteolytic detachment of CDB3 also prevents the release of ATP (Misiti et al. 2008) from RBC, lowering the paracrine action that this nucleotide exerts on the vascular epithelium, necessary for nitric oxide (NO) synthesis. In this work, we study the effects (direct or indirect) of PTX on the anion transport operated by B3 in RBC and highlight possible new cytotoxic aspects.

Materials and Methods

Materials

All reagents were purchased from Sigma-Aldrich (St. Louis, MO). Citrate fresh human blood was obtained from informed healthy donors who declared that they had abstained from all drug treatment for at least 1 week before sample collection, in accordance with the principles outlined in the Declaration of Helsinki.

Preparation of RBC

Citrate blood samples were washed three times with an isoosmotic NaCl solution. During washing, the white blood cells were discarded from the pellet. After washing, the RBC were resuspended (hematocrit 3%) in the incubation buffer (35 mM Na_2SO_4, 90 mM NaCl, 25 mM HEPES [*N*-(2-hydroxyethyl)-piperazine-N^1-2-ethanesulfonic acid], 1.5 mM $MgCl_2$), adjusted to pH 7.4 and 310 ± 20 mOsmol/kg, measured by an Osmostat OM-6020 apparatus (Daiichikagakuco, Kyoto, Japan).

Methemoglobin (met-Hb) levels and the degree of hemolysis were determined at the end of the incubation time as follows: hemolysis was determined by spectrophotometrically measuring Hb concentrations in the supernatants obtained from centrifugation at $2500 \times g$ for 5 min at 4°C; met-Hb levels were determined spectrophotometrically on lysed cells (Zijlstra et al. 1991).

Kinetic Measurements

Cells were incubated in the above incubation buffer at 25°C in the absence or presence of 1.0 pM of PTX, 1.0 mM of orthovanadate (OV), or 0.1 mM of ouabain under different experimental conditions. At several time intervals, 10 mM of the stopping medium SITS (4-acetamido-4′-isothiocyanostilbene-2,2′-disulfonic acid) was added to each test tube containing the RBC suspension. Cells were separated from the incubation medium by centrifugation (J2-HS Centrifuge, Beckman, Palo Alto, CA) and washed three times at 4°C with a sulfate-free medium to remove the sulfate trapped on the outside. After the final washing, the packed cells were lysed with perchloric acid (4%) and distilled water. Lysates were centrifuged for 10 min at $4000 \times g$ (4°C), and membranes were separated from the supernatant. Sulfate ions were precipitated from the supernatant by adding a glycerol/distilled water mixture (1:1, v/v), 4 M NaCl and 1 M HCl, 1.23 M $BaCl_2 \cdot 2H_2O$, to obtain a homogeneous barium sulfate precipitate. The absorbance of this suspension was measured at 350–425 nm.

The sulfate concentration was determined using a calibrated standard curve, obtained by measuring the absorbance of suspensions with known amounts of sulfate (Romano et al. 1998). The experimental data on sulfate concentration as a function of the incubation time were analyzed by best-fit procedures by the equation $c(t) = c_\infty (1 - e^{-kt})$, where $c(t)$ is sulfate concentration at time t, c_∞ the intracellular sulfate concentration at equilibrium, and k the rate constant of sulfate influx.

Determination of Phosphatase PTP-1B Activity

Phosphatase phosphotyrosine phosphatase (PTP)-1B activity was determined using *p*-nitrophenyl phosphate (*p*-NPP) as substrate. Briefly, membranes were suspended in 25 mM HEPES buffer of pH 7.3, containing 0.1 mM phenylmethanesulfonylfluoride, 20 mM $MgCl_2$ and 15 mM *p*-NPP, and incubated at 37°C for 30 min. After centrifugation, the release of *p*-nitrophenol was measured in the supernatant at 410 nm (Maccaglia et al. 2003).

Caspase 3 and 8 Assays

Citrate blood samples were washed three times with an isoosmotic NaCl solution. The white blood cells were discarded from the pellet during washing. After washing, the RBC were resuspended (hematocrit 3%) in the incubation buffer (35 mM Na_2SO_4, 90 mM NaCl, 25 mM HEPES, 1.5 mM $MgCl_2$), adjusted to pH 7.4, and incubated for 2 h

at 37°C in the absence or presence of 1.0 pM of PTX, 1.0 mM of OV, or 1.0 pM PTX plus 1.0 mM OV. After treatment, erythrocytes were collected by centrifugation at 3000 rpm for 5 min, resuspended in HEPES buffer (100 mM HEPES pH 7.5, 20% glycerol, 5 mM DTT and 0.5 mM ethylenediaminetetraacetic acid [EDTA]), and lysed by sonication. The cell lysates were clarified by centrifugation at 15,000 rpm for 10 min. The supernatant was passed through a Microcon YM 30 filter device (nominal molecular weight limit 30,000). The cell lysates (100 µl) were incubated in a final volume of 600 µl at 37°C for 1 h with enzyme-specific colorimetric substrates (Ac-DEVD-pNA or Ac-IEPD-pNA 100 µM in HEPES buffer for the determination of caspase 3 or 8 activity, respectively). Samples were then analyzed with a spectrophotometer after pNA release at 405 nm and expressed as the *n*-fold value of the untreated sample.

Lipid Peroxidation Assay

Erythrocyte isolated as above were incubated for 2, 6, and 24 h in the absence or presence of 1.0 pM of PTX, 1.0 mM of OV, 1.0 pM of PTX plus 1.0 mM of OV, or 3.0 mM of t-butyl hydroperoxide (t-BOOH). After incubation, the samples were washed three times with 10 volumes of 0.9 % NaCl and centrifuged at 2500 rpm for 5 min. During the last washing, the packed cells were resuspended in 30 volume with ice-cold hypotonic medium containing 5 mM Tris and 5 mM KCl to yield hemolysate, then centrifuged for 10 min at 12,000 rpm. This operation was repeated three times. Last, the hemolysate were resuspended in 0.9% of NaCl and used for lipid peroxidation assay by the thiobarbituric acid reactive substances (TBARS) methods (Yagi and Rastogi 1979).

Statistical Analysis

Data are presented as mean ± standard deviation (SD). The data were analyzed by one-way analysis of variance. The significance of the differences in relation to the respective controls for each experimental test condition was calculated by Student's *t*-test for each paired experiment. A P value of <0.05 was regarded as indicating a significant difference.

Results

PTX remarkably influences B3 anion flux (Fig. 2). PTX induces a decrease of ~50% of the rate constant (RC) value that goes from 0.012 to 0.0064 min^{-1}, respectively, for the erythrocytes incubated in the absence and presence of 1 pM of the marine toxin.

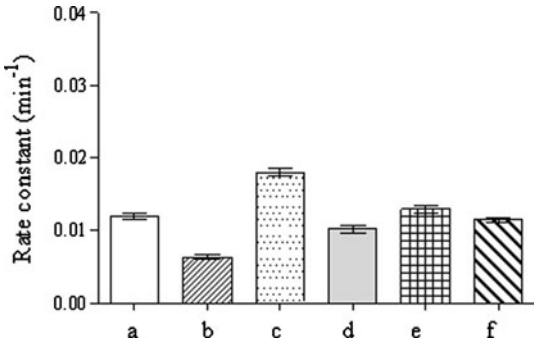

Fig. 2 Rates of sulfate transport in normal human RBC, incubated in the absence (*a*) or presence of 1.0 pM of PTX (*b*), 1.0 mM of OV (*c*), 1.0 pM of PTX plus 1.0 mM of OV (*d*), 0.1 mM of ouabain (*e*), or 1.0 pM of PTX plus 0.1 mM of ouabain (*f*). Values are the means ± SD of at least three different experiments

Because of its high molecular weight (2680 Da) and chemical composition, PTX is not able to cross the erythrocyte membrane. Hence, modulation of B3 is probably due to structural modifications that start from the outside of the membrane. A possible explanation for PTX effects on anion antiport could be its direct interaction with the extracellular side of B3. Another hypothesis for a PTX indirect action could be related to its high affinity for Na^+/K^+-ATPase. In this case, this protein could act as membrane receptor, able to transduce a signal that negatively affects the anion flux and B3 functionality. To prove that what we measured was only the anion flux through B3 channel, and not a kind of nonspecific ion pore generated by PTX, we tested the anion kinetics in RBC pretreated with 2 mM SITS, a known inhibitor of B3 functionality (Lepke et al. 1976). The results indicated that under these conditions, no anion exchange could be observed in the presence of PTX, demonstrating that the changes observed in the above experiments on B3 activity are an effective response to the toxin influence (data not shown).

We also carried out anion flux experiments in the presence of 1.0 mM of OV, a well-known antagonist of PTX with respect to Na^+/K^+-ATPase (Kim et al. 1995; Tosteson et al. 1991). The results showed an increase in the RC values from 0.0064 to 0.0102 min^{-1}, respectively, for RBC incubated in the presence of 1.0 pM of PTX or 1.0 pM of PTX plus 1.0 mM of OV. The values highlighted a clear recovery of B3 functionality, with a decrease in the percentage of inhibition from ~50 to 15%. This provides an evidence for an indirect PTX influence on B3, noticeably inhibited by OV. This latter, in fact, should counteract the PTX influence by acting, at intracellular level, on one hand with CDB3 and on the other hand with the Na^+/K^+-ATPase.

PTX binds reversibly to the Na^+/K^+ pump and inhibits the Na^+/K^+-activated ATPase, converting the pump into a nonspecific cation channel (Habermann 1989; Tosteson

Table 1 Increase of hemolysis (a) or met-Hb (b) percentage with respect to control in normal human RBC

Compound	Incubation time (h)[a]					
	1/2		2		6	
	a	b	a	b	a	b
PTX (1.0 pM)	0	8.0 ± 2.2	0	8.7 ± 1.3	3 ± 0.5	0
OV (1.0 mM)	0	15.1 ± 3.2	30 ± 4.5	17.3 ± 3.4	62 ± 4.2	0
PTX (1.0 pM) + OV (1.0 mM)	0	8.2 ± 1.7	12 ± 2.3	8.4 ± 1.6	45 ± 3.7	0

[a] Values are the means ± SD of at least three different experiments

et al. 1991, 1995). This generates a perturbation of cellular physiological conditions that could destabilize the normal anion exchange. To support the idea of an indirect effect of PTX on B3 functionality, we analyzed the anion flux in the presence of ouabain, which is known to block the effects of PTX on Na^+/K^+-ATPase (Ozaki et al. 1985). The results reveal that the influence of PTX on B3 activity was reversed upon addition of ouabain to the medium, restoring RC values from 0.0064 min^{-1} (RBC incubated in presence of 1.0 pM of PTX) to 0.0114 min^{-1} (RBC incubated in presence of 1.0 pM of PTX plus 0.1 mM of ouabain) (Fig. 2). In other words, the presence of ouabain antagonizes the effect of PTX on anion flux, suggesting that when PTX is not able to bind Na/K pump, its influence on B3 almost completely disappears.

To assess the PTX influence on RBC hemolysis, we determined the change in Hb concentration in RBC incubated in the presence of PTX, OV, and PTX plus OV. As shown in Table 1, PTX induced a weak influence in the RBC hemolysis process, in contrast to OV. The absence of hemolysis within the first 6 h of incubation time with PTX and the remarkable inhibition of the same percentage in RBC incubated in the presence of PTX plus OV strongly support the ability of the toxin to induce a given degree of membrane stability through important cellular changes.

Our results are confirmed by the met-Hb levels observed after RBC incubation in the presence of PTX, OV, and PTX plus OV; the values depicted in Table 1 show only a slight increase in the values of met-Hb within 2 h of incubation time. Nevertheless, it is interesting to note that the met-Hb percentage in the presence of OV was promptly reduced when RBC were incubated in the presence of OV and PTX. The analysis of caspase 3 and 8 activity is in agreement with this observation. In fact, the results of the experiments illustrated in Fig. 3 show that the presence of PTX did not cause significant variation in both caspase 3 and 8 activity in comparison with the control after 2 and 6 h of incubation time. Completely different was the effect of OV, which led to an increase of caspase 3 activity. This effect is counteracted by the presence of PTX.

In this respect, Hughes et al. (1997) reported a link between potassium leak from the intracellular compartment and activation of caspases, which are normally kept inactive at physiological ion concentrations.

The presence of a different redox balance is also supported by the paradoxical lack of a series of effects due to

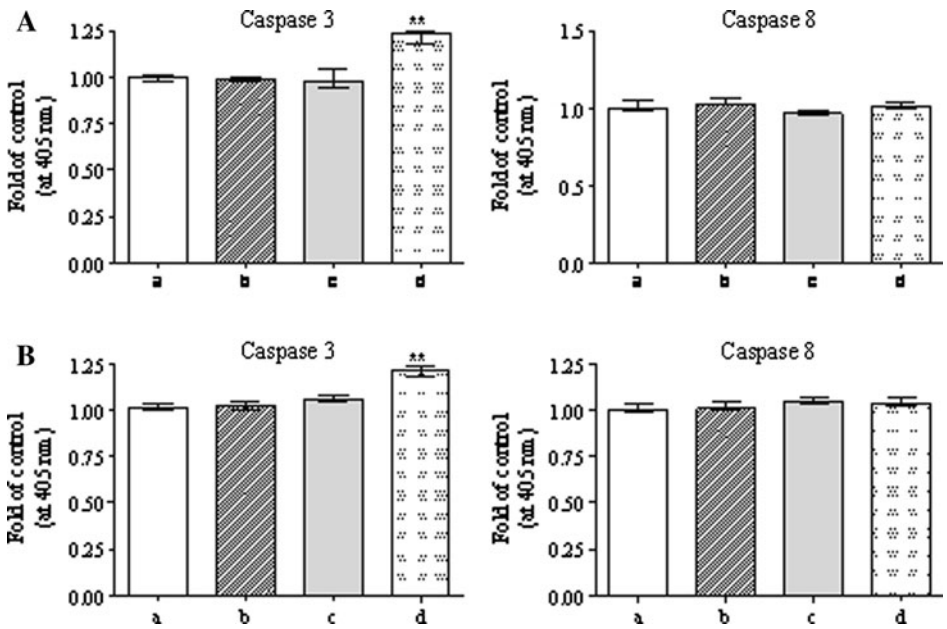

Fig. 3 Normal human RBC incubated 2 h (**A**) or 6 h (**B**) in the absence (*a*) or presence of 1.0 pM of PTX (*b*), 1.0 pM of PTX plus 1.0 mM of OV (*c*), and 1.0 mM of OV (*d*) and analyzed for caspase 3 and 8 activity. **Significant differences compared to control at $P < 0.05$

the inhibition of the phosphatase activity induced by PTX (Fig. 4). Inhibition of the enzyme is probably due to detachment of the phosphotyrosine phosphatase from B3, caused by the increased concentration of Ca^{2+} in cells exposed to PTX (Satoh et al. 2003).

This dissociation strongly influences the delicate balance of phosphorylation–dephosphorylation cycle in RBC (Zipser et al. 2002), promoting the tyrosine phosphorylation of CDB3 and the consequent release of glycolytic enzymes. This particular context would drive the erythrocyte to a preferential use of G6P in the EMP pathway instead of PPP, triggering a cascade of signals that would induce activation of caspase 3, as shown in our previous studies (Barreca et al. 2009; Ficarra et al. 2009; Galtieri et al. 2010; Tellone et al. 2008). The lack of these events, highlighted by our experiments, could be correlated to the increase of ATP concentration due to the PTX inhibition of ATP hydrolysis by Na^+/K^+-ATPase. The nucleotide is a strong negative modulator of EMP pathway, and its increase shifts the flux of G6P toward PPP, resulting in an enhancement of NADPH production. This could well counteract the oxidative stress and therefore oxidation of the heme iron of Hb.

The existence of a higher reductive power in the presence of PTX was also highlighted by a lipid peroxidation assay of the RBC membrane. Figure 5 shows lipid peroxidation values in the sample incubated for 2, 6, and 24 h in the presence of 1.0 pM of PTX, 1.0 mM of OV, and both compounds (1.0 pM of PTX plus 1.0 mM of OV). The results highlight that the value obtained in the presence of PTX was almost completely superimposable to the one obtained for the control, also after 24 h of incubation. OV, on the other hand, induced a slight increase in the peroxidation level, which became evident after 24 h of incubation—although it was clearly inferior with respect to a strong oxidant like t-BOOH.

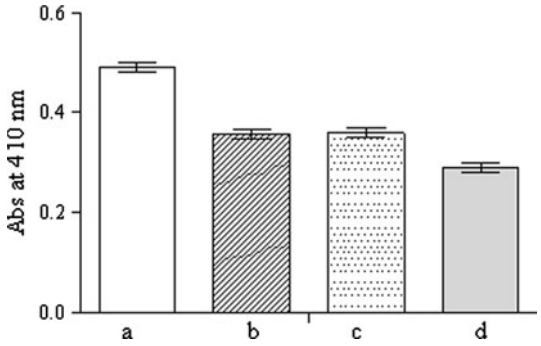

Fig. 4 Phosphatase activity in normal human RBC, incubated in absence (*a*) or presence of 1.0 pM of PTX (*b*), 1.0 mM of OV (*c*), or 1.0 pM of PTX plus 1.0 mM of OV (*d*). Values are the means ± SD of at least three different experiments

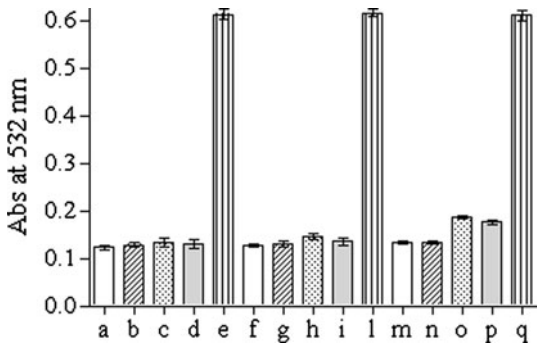

Fig. 5 Lipid peroxidation assay of RBC membrane. The RBC were incubated in absence (*a*, *f*, *m*) or in the presence of 1.0 pM of PTX (*b*, *g*, *n*), 1.0 mM of OV (*c*, *h*, *o*), 1.0 pM of PTX plus 1.0 mM of OV (*d*, *i*, *p*) or 3.0 mM of t-BOOH (*e*, *l*, *q*) for 2 h (*a*, *b*, *c*, *d*, *e*), 4 h (*f*, *g*, *h*, *i*, *l*), and 24 h (*m*, *n*, *o*, *p*, *q*) and assay for the determination of lipid peroxidation level

Discussion

The results of these experiments performed on human RBC exposed to PTX show a strong decrease of anion transport. These results make it easier to understand the toxicity of this molecule, which exerts its harmful effects not only on Na^+/K^+-ATPase, but also on other essential cellular structures like B3 (Fig. 6). What emerges is the action of PTX on B3, which indicates an indirect action of the toxin, probably linked to a signal transduction generated by the formation of a PTX–Na^+/K^+-ATPase complex. Within RBC, binding of PTX to Na^+/K^+-ATPase causes an excess of Na^+ input that is initially offset by the cell with a release of K^+. Moreover, the loss of the ions' cellular balance leads to a given degree of depolarization of the plasma membrane, which facilitates the entry of Cl^- ion, followed by swelling and failure of the cell membrane (Lang et al. 1998). In this context, the strong inhibition of the anion flux in the presence of PTX could be related to the significant imbalance between the Cl^- and HCO_3^- concentrations. The kinetic slowdown observed could also be due to the significant change of pH induced by PTX, characterized by an acidification of the RBC cytoplasm (Tosteson et al. 1991). Jin et al. (2003) indicated that His 834, located at the cytosolic surface of human B3, is an essential residue for anion exchange; the acidification would thus lead to the formation of hydrogen bonds, which would be followed by a slow anion intramembranous flux (as observed by Muller-Berger et al. 1995 in the mouse B3). Furthermore, the high cytoplasmic concentration of H^+ stabilizes the conformational T state of Hb, which is known to bind the CDB3 with a higher affinity than oxygenated Hb (R state). The formation of this complex would create a kind of steric hindrance at the level of the intramembranous channel, which could further hinder the flux, as we observed in our

previous kinetic studies that analyzed the move from a high-oxygenation state to a low one (Galtieri et al. 2002). Furthermore, we have to consider the notion that in RBC, the kinetics of anion transport under these conditions (low pH) would be further impaired by a decreased availability of HCO_3^- because of the shift to the left of the following reaction: $CO_2 + H_2O \rightleftarrows H_2CO_3 \rightleftarrows H^+ + HCO_3^-$.

The CO_2 further shifts the balance toward the T state of Hb, triggering a harmful and vicious circle. The hypothesis of an indirect action of PTX on the functionality of B3 is strongly supported by the anion kinetics obtained in the presence of PTX plus OV, and PTX plus ouabain. OV and ouabain, acting with different molecular mechanisms as antagonists of PTX on Na^+/K^+-ATPase (Tosteson et al. 1991), would not allow the toxin to alter the cationic flux, thereby also inhibiting the anionic exchange. Furthermore, the different molecular mechanisms based on the effects of ouabain and OV on B3 (also reflected by the different anionic RC values observed in the presence, respectively, of PTX plus OV, and PTX plus ouabain) confirm that the interaction between PTX and the membrane protein is promoted by ATP (Chhatwal et al. 1983; Habermann and Chhatwal 1982). Therefore, RBC in the two conditions (ouabain plus PTX, and OV plus PTX) appear to be characterized by different metabolic conditions that are due to the different use of G6P, which may lead to a significant difference in the availability of ATP. In contrast, the absence of caspase activation and hemolysis and the reduced percentage of met-Hb in the presence of PTX reveal an unexpected erythrocyte stability. These results are further supported by the low degree of hemolysis observed in the cell incubated with PTX plus OV. The PTX effects observed in RBC may therefore be summarized as follows: (1) it significantly slows the anion transport through B3 seriously altering the metabolic modulation linked to the oxygenation-deoxygenation cycle of Hb; (2) it stabilizes the RBC membrane by preventing lipid peroxidation; and (3) it does not lead to activation of caspases 3 and 8.

Characteristics (2) and (3) support the idea that RBC exposed to PTX are characterized by a higher reductive power due to a high availability of NADPH and the full efficiency of the system that produces it. This is a paradoxical situation because it is in contrast to the strong alteration of the intracellular ionic balance and in contrast to the increase of the G6P directed through the EMP pathway due to stabilization of the T state of Hb induced by acid pH values. Moreover, we also have to consider the inhibitory phosphatase activity of the toxin, which should induce further oxidative stress. The existence of a higher reductive power of the RBC is further supported by the absence of membrane lipid peroxidation and by the low values of met-Hb observed in the presence of PTX. Despite the devastating cytotoxic action that is expressed, PTX seems to promote some metabolic adaptations that compensate and mitigate its cellular "malignancy." In this context, because the interaction of PTX with the Na^+/K^+-ATPase inhibits ATP hydrolysis activity of the pump (Tosteson et al. 1991), we hypothesize that the increase in concentration of the triphosphate nucleotide is reflected on the functionality of EMP. In this regard, Tosteson et al. (1991) noted in RBC an inhibition of lactate production in

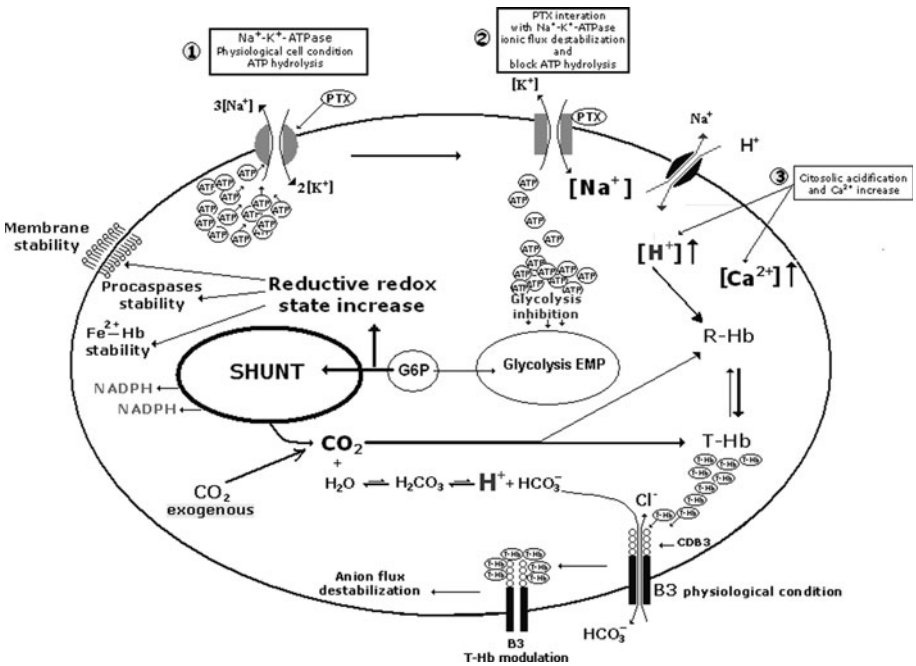

Fig. 6 PTX effect on RBC. Normal cellular conditions are perturbed by the interaction of PTX with the Na^+/K^+-ATPase on RBC membrane. Conversion of the pump into a nonselective ion channel and inhibition of ATP hydrolysis activity result in extensive perturbations of intracellular RBC metabolism. See text for details

the presence of PTX. The consequent shift of G6P toward the PPP leads the erythrocyte to a continuous supply of NADPH, allowing it to counteract the harmful oxidative stress, caspase 3 activation, membrane derangement, and premature hemolysis. On the other hand, it is necessary to emphasize that this metabolic state, made possible by the stability of procaspase, which guarantees the structural integrity of CDB3, aggravates the negative consequences of RBC exposed to the toxin because the cell is further flooded with CO_2, produced endogenously by the PPP, in a condition in which the function of band 3 is already seriously impaired. It should be noted that the procaspase and the membrane stability and the low levels of met-Hb observed in the presence of high availability of CO_2 are an indication of an effective control mechanism of the reactive oxygen species (Romero et al. 2006).

This could hasten the functional and metabolic recovery of the cell, allowing nonharmful and physiological elimination from the blood circulation, as probably occurred in a case of slight intoxication near Genoa in summer 2005 (Ciminiello et al. 2006). The results discussed here could support the notion that low amounts of PTX exert mild cellular damage, which could be counteracted by cell survival mechanisms (Bellocci et al. 2011).

Our findings open a window on the different biological effects of this toxin and broaden our understanding of the various mechanisms that are based on the action of this intriguing and dangerous marine toxin.

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