NON-NEURONAL CHOLINERGIC MECHANISMS IN RED BLOOD CELL

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SUMMARY

A brief description will be done about the meaning of non-neuronal cholinergic system considering the identification of its components, the sites where they appear and the known physiological functions. The focus will be done on the red blood cell non-neuronal cholinergic responses to a stimulus.

GENERALITIES OF THE NON-NEURONAL CHOLINERGIC SYSTEM

Acetylcholine (ACh) was viewed as the "vagusstoff" after Loewi`s experiment with the frog heart. In 1936 Otto Loewi, with Henry Dale were recipients of the Nobel Prize in Physiology or Medicine, by their contribution for the acetylcholine discover as a neurotransmitter.

Outside of the central and peripheral nervous system there is evidence of the presence and of ACh that was firstly recognised in 1963 by Wittaker[1].

ACh is a phylogenetic old molecule present in prokaryotic and eukaryotic organisms. Recently Kawashima and collaborators have demonstrated the ubiquitous presence of ACh and the expression of its ACh-synthesizing enzyme among life forms without nervous system[2]. Acetylcholine participates in different cellular roles such as proliferation, differentiation, migration and immune functions, Figure 1[3-7].

ACh is considered as a universal cytotransmitter by Grando and colleagues[8,9], and is presented on epithelial, mesothelial, muscle, endothelial, and immune cells[8-10]. Endothelial cells and lymphocytes are able to synthesize and release ACh by the participation of choline acetyltransferase (ChAT) and vesicular acetylcholine transporter respectively (VAChT)[11-13]. Lymphocytes express most of the cholinergic elements and upon interaction with antigen presenting cells or endothelial cells, T cells produces increase amount synthesis and release of acetylcholine[14]. The autocrine ACh action depending on cell membrane receptor activates or not the lymphocytic cholinergic system enhancing in case of TCR/CD3, the expression of both ChAT and M5m ACh receptors[14].

At vascular wall the intrinsic intimae non-neuronal system is presented in the endothelium,

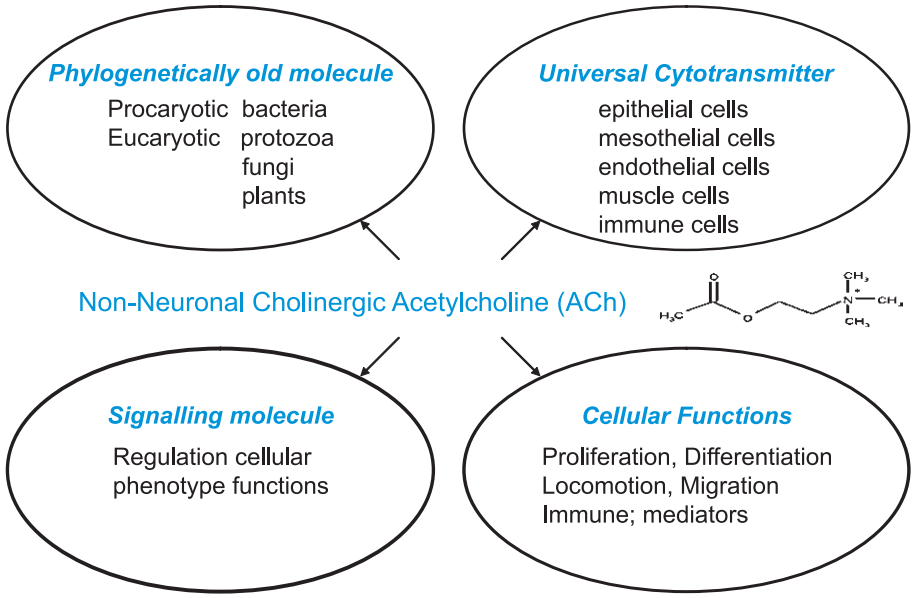

Fig. 1 – Schematic representation of the non-neuronal acetylcholine functions.

acting ACh on it by the auto-paracrine fashions[15]. Beyond the non-neuronal cholinergic components namely, ACh, ChAT and VAChT, above mentioned, the enzyme acetylcholinesterase (AChE) that conducted the hydrolysis of ACh, is also expressed[16].

We are the first to perform the biochemical characterization of human umbilical vein endothelial cell membrane (HUVECs) protein bound form of the AChE. We have identified, with C-terminal anti-AChE, the expression of one molecular form membrane with 70kDa, (the molecular mass characteristic of the human monomeric form of AChE). When the N terminal anti-AChE was used two molecular forms with approximately 66kDa and 77kDa are expressed at membrane bound level[17]. The molecular form of 70kDa is also expressed at cytoplasm and nuclear compartments, where the latter also expressed an AChE isoform with approximately 55kDA[18]. We verified that the nuclear expression is not endothelial cell-specific but is also evidenced in non-neuronal and neuronal cells.

Studies of Borovikova[19] have showed the anti-inflammatory effect of ACh in the rat's systemic inflammatory response to endotoxin. The anti-inflammatory action attribute to ACh is associated with the extrinsic vascular cholinergic system, at the perivascular nerve fibres[20].

Tracey and co-workers have recently demonstrated the existence of the cholinergic anti-inflammatory pathway, which requires the action of the parasympathetic neuronal system[19,21]. More precisely, it relies on the activity of the sensorial vagus nerve that can sense inflammatory stimuli and further provide input to brain networks (nucleus tractus solitarius in the brainstem medulla oblongata) eliciting a motor anti-inflammatory response. This "wondering" nerve innervates various organs, such as the spleen and liver. At these organs the vagus nerve terminals release ACh that further triggers a decrease in the production of pro-inflammatory cytokines by resident macrophage or other cytokine-producing cells. The "cholinergic anti-inflammatory pathway contribute with the humoral anti-inflammatory mechanisms, comprising external signals and intracellular mediators, to limited the vascular inflammation. As a result there is restraining or counter-regulating cytokine release[22].

During an inflammatory state, blood leukocyte rolls, adheres and after transmigrates through the endothelial cells where then migrates to the injured tissues[23], where there is a tissue-pool of cytokines coming from the macrophages and/or resident monocytes. The steps developed in the leukocytes/endothelial cell interactions are influenced by the tissue-pool cytokines and also from those synthesised and secreted by activated endothelial cells.

Inhibitors of AChE modulate leukocyte activation[24]. We have observed that the intravenous administration of velnacrine maleate (an inhibitor of AChE) in Wistar rats previously submitted to the administration of LPS induced an increased leukocyte adhesion to mesenteric post-capillary venules[25].

The inhibition of AChE by velnacrine also modulates leukocyte-endothelial interaction in the rat cremaster network as showed by us[26]. We observed that there is an increased number of the rolling and adherent leukocytes to the endothelial wall of the post-capillary venules of Wistar rats`mesentery muscle perfuse with velnacrine. That number of leukocytes decreased when ACh was perfused after velnacrine.

Fujii and co-workers, have determined, at normal physiological conditions, the plasma and blood levels of ACh and have verified differences among species[27].

Depending on the degree of endothelium integrity the circulating ACh induce vasodilation or vasoconstriction according the amount of nitric oxide (NO) synthesised and released[28,29].

The NO released from endothelial cells and platelets is scavenged by erythrocyte and blood cell free hemoglobin[30]. Shapiro and co-workers[31] have done NO competion experiments, between plasma cell free hemoglobin and red blood cells (RBCs), under oxygenated and deoxygenated conditions at different hematocrits. They observed that external diffusion of NO to oxygenated erythrocytes is slower than to cell free hemoglobin. The hematocrit dependence at deoxygenated conditions disappears at contrast with oxygenated conditions where lower values of erythrocyte scavenger rate in relation to cell free hemoglobin was obtained at lower hematocrit. Recently, Shapiro and collaborators[32] attribute the above results to the increased erythrocyte membrane permeability verified at anoxic conditions.

Low tissue oxygen tension induced NO participation in the hypoxic vasodilation and several studies demonstrated the involvement of the hemoglobin structural allosteric transitions as oxygen sensor[33-34]. Among the heterotrophic effectors of oxygen binding hemoglobin, NO binds to the thiol group of cysteine β93 at high tissue oxygen tension. At low tissue oxygen tension there is a NO release from either S-nitrosothiol of the S-nitrosated hemoglobin or from the reduction of the anion nitrite to NO[35,36]. It knows that the T state of SNO-Hb promotes the transnitrosation by which NO groups are transferred to thiol acceptors biomolecules in RBCs[37]. One of these is the protein band 3[38], but the exact mechanism by which NO escape from erythrocyte membrane still remain uncertain[37,38].

NON-NEURONAL CHOLINERGIC MECHANISMS IN RED BLOOD CELL

Red blood cells (RBCs) express at external membrane surface the enzyme AChE that has the particularity to be inhibited by its substrate ACh at high concentrations. Different AChE enzyme complex forms may be presented namely, active and less active ones according the amount of ACh existent.

As also referred the erythrocytes controls diffusion of small gas molecules such as NO, and inside the erythrocyte nitric oxide, can react with deoxyhemoglobin heme to form nitrosylhemoglobin or with haemoglobin thiols to form S-nitrosohaemoglobin[36,37]. For these reasons, S-nitrosohaemoglobin has been considered a reservoir of nitric oxide and as mentioned above ACh is an endog-

enous compound with vasoactive properties, present in blood circulation. We hypothesised that the non-neuronal cholinergic system participates in erythrocyte NO mobilization and translocation. So, we have questioned whether ACh induces changes in RBCs NO mobilization. In order to answer, human erythrocyte suspensions, in presence of ACh, were loading with the permeable non fluorescent probe diamino fluoresceine-2 diacetate (DAF-2Da). After we quantified by spectrofluorometry analysis the appearance of intra erythrocyte fluorescence intensity of triazolofluorescein (DAF-2T) which results from the reaction between NO and the 4, 5- diaminofluorescein, we concluded that ACh, in a concentration dependent way, is able to induce NO mobilization inside the erythrocyte[39]. Based on these results and on the vasoactive role of ACh at endothelium wall we have hypothesised that ACh induce changes in erythrocyte deformability as well as in the levels of NO metabolites namely nitrites (NO_2^-) and nitrates (NO_3^-). We have verified that in presence of ACh there is an increased of the erythrocyte deformability, of the NO_2^-, NO_3^- levels and the oxygen hemoglobin affinity and a decreased of erythrocyte aggregation, Figure 2[40,41].

The lower erythrocyte deformability expressed by hypertensive, hypercholesterolemic and kidney transplant patients was associated with a higher nitric oxide efflux under ACh stimulation as we verified by studies conducted *in vitro*[42]. This may be a compensate erythrocyte ability that allows in vivo at microcirculatory network NO and oxygen donation otherwise compromised by the erythrocyte deformability deficiency.

Human erythrocyte deformability and the oxygen hemoglobin affinity increase, without changes in membrane lipid fluidity and peroxidation, when erythrocytes were exposed to NO 10-7M. However when erythrocytes were exposed at NO 10-5M an increase in membrane lipid fluidity and peroxidation values was verified, while at NO 10-3M the methemoglobin levels increase with decreased of erythrocyte deformability[43]. The methemoglobin formation may result from the reaction between NO and oxyhemoglobin catalysed by the hemoglobin reductase enzyme where nitrates are also produced[44].

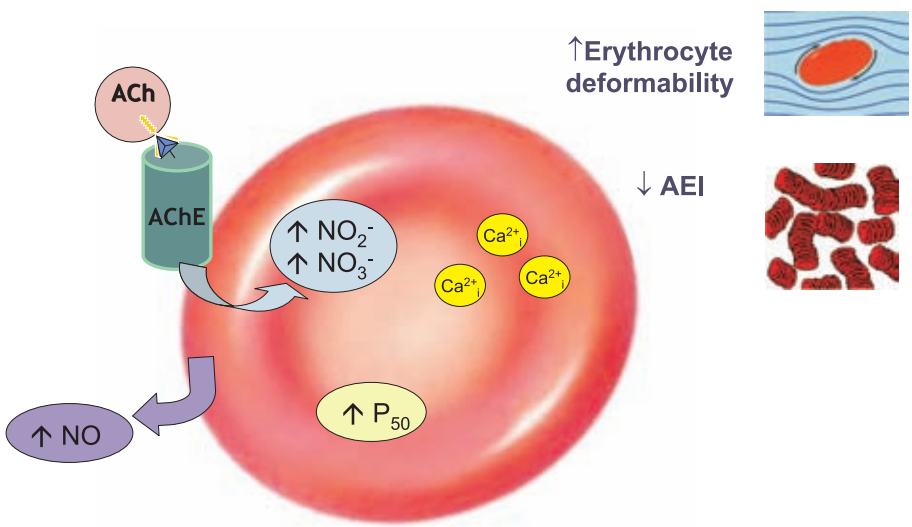

Fig. 2 – Schematic representation of the erythrocyte responses to the action of the non-neuronal acetylcholine obtained from *in vitro* studies.

If auto-oxidation of haemoglobin does occurred the superoxide anion will be produced which generates peroxynitrite after reaction with NO[45]. The decomposition of peroxynitrite molecules leads to nitrite and nitrate[46-48], and the reaction between peroxynitrite and haemoglobin generates SNOHb, which could decompose to nitrosothiol and nitrate[46-49].

Another origin for nitric oxide metabolites appearance in erythrocyte suspensions during incubation with or without ACh may resulted from the nitrosothiols decomposition (e.g. S-nitrosoglutathione) as described by Jia et al[50].

Glutathione is an abundant molecule inside erythrocytes and has a thiol group that can react with nitric oxide or other molecules to form nitrosothiols such as S-nitrosoglutathione[51]. This nitric oxide reserve attributed to glutathione could be affected by the inactivation of glutathione reductase induced by oxidative stress[52]. The thiol/disulfide reagents like as oxidised and reduced gluthathione (which is present at high level inside RBCs), has a suitable redox potencial what made it useful for regeneration proteins. For instance dithiothreitol (DTT) is a thiol reducing agent enable for regenerating disulfide-contains proteins and establish interchangeable thiol-disulfide reaction with glutathione[53]. We have hypothesised that the manipulation of erythrocyte thiol status will be able to change the NO mobilization that occurs at absence or presence of AChE effectors. We have conducted in *in vitro* studies upon redox status modulation using DTT. The following results here present are accepted for publication. We verified that NO is strongly mobilized inside RBCs but much less released to the extracellular compartment under DTT influence that when compared with the effect of acetylcholine--AChE or velnacrine-AChE complexes. Higher levels of intracellular NO are responsible for the enhanced metabolites production, explaining the greater mobilization via GSNO, although the same didn't occur with peroxynitrite concentration. DTT is an activator of the glutathione reductase enzyme activity[54] which allowing S-nitrosoglutathione renovation may be one explanation for the observed NO mobilization. Conversion of metHb into oxyHb was slightly triggered by DTT, which may be associated with a thiol-dependent activity of metHb--reductase as has been described[55].

Acetylcholine significantly prompted DTT--induced nitric oxide mobilization, since higher levels of NO and its metabolites were determined respectively in the extracellular and inner compartments. Regarding the action of velnacrine in presence of DTT, only nitrite concentration increased, while nitrate and NO values were lower. ACh or VM do not significantly modify the DTT influence on peroxynitrite levels. However, velnacrine plus DTT showed a higher increase on GSNO concentration.

An interesting finding was that reduced glutathione (GSH) concentration is not modified when DTT or AChE effectors are present, denoting that the RBCs antioxidant mechanisms are conserved. Overall peroxynitrite production was due to erythrocyte-velnacrine stimulation, while acetylcholine scarcely altered its basal levels. Reduced environment states, by turn, favoured the effects of VM but went against those of ACh in the way of higher formation of peroxynitrite.

We verified for the first time, as far as we are concerned, that the presence of increasing amounts of, DTT, do not significantly modify the red blood cell elongation index, aggregation index and membrane lipid fluidity, when incubated in blood samples of healthy subjects. However the erythrocyte deformability assessment showed a significant decrease ($p<0.05$), at low shear stress, when the AChE inhibitor, velnacrine, is present with each of the following concentrations DTT 10-6M, 10-5M 5x 10-5M in blood samples aliquots. The active ACh--AChE complex does not exert major influence on erythrocyte deformability property. Both acetylcholine and velnacrine diminish significantly the erythrocyte aggregation index, in stasis during 5 and 10 seconds, for all DTT 10-6M, 10-5M 5x 10-5M concentrations in relation to the values obtained in DTT blood samples aliquots, (CHM in

publication). These results seems, as previous documented for ACh, that the erythrocyte hemorheological profile may be dependent on its elements of the non-neuronal cholinergic system[40,42], to be in accordance with preview more general idea postulated by Paulischke and cow-workers that external and integral membrane proteins[56] influence the erythrocyte hemorheological properties.

Based in all the above mentioned reactions we have imagined a hypothesis to explain the signal transduction mechanism that may be associated with nitrite, nitrate production, and NO mobilization in presence of AChE substrate or inhibitor, in, *in vitro,* erythrocyte suspensions, Figure 3. Erythrocyte membrane protein band 3 binds some glycolytic enzymes and haemoglobin[57-59] and is the most abundant protein expressed in human red blood cell membranes. Protein band 3, known as a spanning erythrocyte membrane protein, could be phosphorylated in a tyrosine residue by protein tyrosine kinase (PTK) and then dephosphorylated by the protein tyrosine phosphatase (PTP)[60]. Besides the enrichment of shed vesicles in AChE at variance with poor band 3 protein molecules[61], we can hypothesize that changes in band 3 protein conformations could occur when ACh or velnacrine binds to AChE. In our idea either the enzyme–substrate or the enzyme–inhibitor complex induces conformational changes in an erythrocyte G protein[62], that in turn may activates the PTK enzyme responsible for band 3 phosphorylation with sorting of glycolytic enzyme and consequently increasing the glycolytic pathway rate. The NADH produced may participate in the methahemoglobin conversion to haemoglobin by haemoglobin reductase action[63].

The S-nitrosohaemoglobin (SNOHb) molecules binding to the phosphorylated band 3 protein, that has exposed SH group, allows the trans-nitrosylation reaction with the band 3. The NO·molecules after be transferred are released from the erythrocytes[64].

In summary, we hypothesis a possible acetylcholinesterase role in the signal transduction mechanism in response to the action of acetylcholine that originate NO mobilization and nitrite and nitrate changes concentrations, in human erythrocyte suspensions. The trans-nitrosylation process coupled between band 3 proteins and SNOHb could be associated with an unknown mechanism mediated by the AChE-ACh complex, with the participation of PTK and PTP which may be dependent on G protein (G Prot)[41].

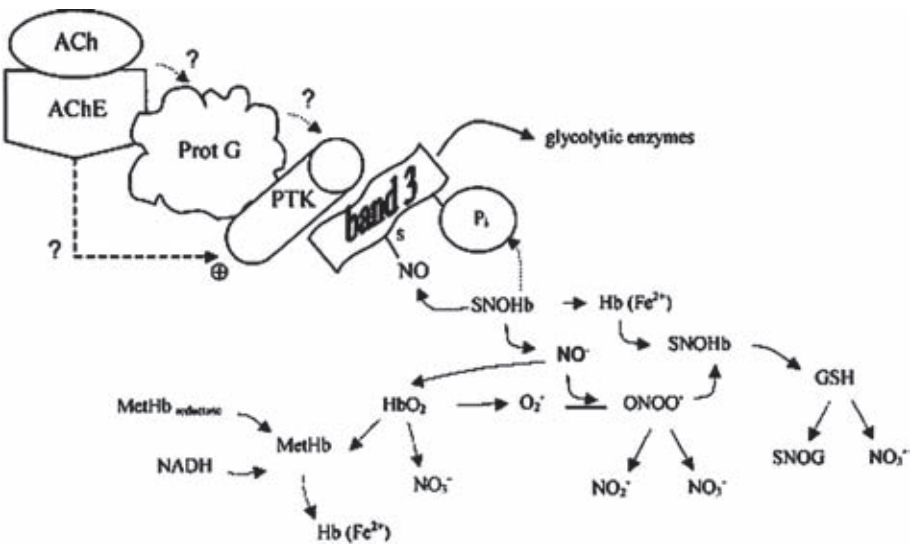

Fig. 3 – Schematic representation of the signal transduction mechanism proposed for the erythrocyte non-neuronal cholinergic system[41].

In order to verified our hypothesis we start by studying the influence of the band 3 protein phosphorylation degree in the RBCs hemorhreological properties. After we have studied the influence of the AChE substrate and inhibitor on the band 3 phosphorylation degrees, and we have identified the G protein associated with these band 3 states of phosphorylation in absence and presence of AChE effectors.

When we modulate protein band 3 phosphorylation states with PTK $p53/56^{lyn}$ inhibitor (Aminogenistein or AMGT) and PTP inhibitor (Calpeptin), the erythrocyte elongation index (EEI), or deformability, was not affected[65]. At variance erythrocyte aggregation increased when band 3 protein is phosphorylated and decreased when at dephosphorylated state. However both manipulated states induced lower erythrocyte aggregation values than blood samples aliquots control[65].

Concerning the AChE effectors ability to modify erythrocyte NO translocation we recall what is mentioned above, that manipulated red blood cells suspensions in the presence of ACh, shows increase of NO levels translocation. However the addition of $p72^{syk}$ inhibitor, (band 3 protein at partially phosphorylated state), to the RBCs suspensions, reveal a decrease of NO concentrations besides the presence of ACh. When in presence of calpeptin and ACh, band 3 being totally phosphorylated, we observed an increase of NO levels. The erythrocyte NO mobilization and its efflux are dependent of the AChE-ACh enzyme active complex and also from the protein band 3 phosphorylation degree, (in publication).

The addition of PTK_i, (inducing a band 3 protein partially phosphorylated state), showed higher NO_x levels in the presence of the velnacrine--AChE less active enzyme complex. In the presence of calpeptin, (protein tyrosine phosphatase inhibitor), band 3 being totally phosphorylated, we obtained the opposite NO mobilization.

Triton X-100 erythrocyte membrane soluble extracts revealed that the level of phosphorylated band 3 obtained in control samples was influenced by the presence of acetylcholine, velnacrine, calpeptin, and PTK_i. ACh maintains the level of band 3 phosphorylation induced by calpeptin at variance with the antagonism action caused by the less active enzyme AChE –velnacrine form. Both active and less active enzyme AChE complex do not abolished the dephosphorylation state of band 3 induced by the protein tyrosine kinase inhibitor.

These results suggested the key-role of the AChE-effectors enzyme complexes in the band 3 phosphorylation/dephosphorylation reactions PTK and PTP dependent in association with erythrocyte NO metabolism.

Recalling our hypothesis for the signal transduction mechanism the involvement of a G protein needs to be confirmed as well as its type.

From the literature it was described that the heterotrimeric G protein Gi, participates in the ATP release from erythrocytes[66] that the expression of G_{i2} is reduced in the erythrocyte membranes of humans with type 2 diabetes[67]. It was also verified a decreased of erythrocyte G proteins (Gαi, Gαo and Gβ) in hypertensive subjects in relation to healthy persons[68].

Our purpose was to identify the G protein type that could be linked to the band 3 protein phosphorylation degree states and to know whether each G protein sub-units (α,β,γ) is related to the active or less active enzyme AChE complex forms. For this propose we made *Western blotting* analysis using primary antibodies to different protein G sub-units such as 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 $G\alpha_{q/11}$. We could identify on erythrocytes membrane soluble extracts possible linkage between protein $G\alpha_{i1/2}$ and/or protein G_{β} with protein band 3. The results were then confirmed by immunoprecipitation of this two protein G sub-units with following analysis by Western blot using antibodies against protein band 3 (C-terminal) and band 3 (N-terminal).

From all the blood samples aliquots studied we verified that G protein sub-units $G\alpha_{i1/2}$ and G_{β} is linked with band 3 C-terminal site. Moreover when erythrocyte AChE was stimulated with acetylcholine and when is present with PTK inhibitors there was

an increase of the expression of the linkage between $G\alpha_{i1/2}$ – Band 3 (C- and N-terminal) and G_{β} – Band 3 (C –terminal). These two conformational states of G protein sub-units seem to be related with the phosphorylation band 3 protein states.

Our results of this work allow us to identify the linkage between protein Gαi1/Gαi2 and/or protein Gβ and protein band 3 on erythrocytes membrane. At band 3 C-terminal end both protein Gαi1/Gαi2 and protein Gβ are bonded. We observed that when the erythrocyte active AChE/ACh enzyme complex is formed in absence or presence of PTK inhibitors, there is an increase of the linkage Gαi1/Gαi2 – band 3 protein at C-terminal domain.

In summary, our data allows us to confirm that G_i protein is a necessary component of the signal transduction pathway that seems linked to the band 3 protein phosphorylation degree states and related to the activation or inhibition of acetylcholinesterase complex. We can conclude that our proposed signal transduction mechanism based on NNCS participates on erythrocyte NO translocation and contribute to understand some intracellular erythrocyte-dependent events. Some insights may contribute to better understand the erythrocyte functions at microcirculation level at physiological and pathological conditions

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