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Tubulin–Na^+,K^+-ATPase interaction: Involvement in enzymatic regulation and cellular function

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Abstract

A new function for tubulin was described by our laboratory: acetylated tubulin forms a complex with Na^+,K^+-ATPase (NKA) and inhibits its activity. This process was shown to be a regulatory factor of physiological importance in cultured cells, human erythrocytes, and several rat tissues. Formation of the acetylated tubulin–NKA complex is reversible. We demonstrated that in cultured cells, high concentrations of glucose induce translocation of acetylated tubulin from cytoplasm to plasma membrane with a consequent inhibition of NKA activity. This effect is reversed by adding glutamate, which is coctransported to the cell with Na^+. Another posttranslational modification of tubulin, detyrosinated tubulin, is also involved in the regulation of NKA activity: it enhances the NKA inhibition induced by acetylated tubulin. Manipulation of the content of these modifications of tubulin could work as a new strategy to maintain homeostasis of Na^+ and K^+, and to regulate a variety of functions in which NKA is involved, such as osmotic fragility and deformability of human erythrocytes. The results summarized in this review show that the interaction between tubulin and NKA plays an important role in cellular physiology, both in the regulation of Na^+/K^+ homeostasis and in the rheological properties of the cells, which is mechanically different from other roles reported up to now.

KEYWORDS
erythrocytes, membranes, Na^+, K^+-ATPase, Na^+/K^+ homeostasis, tubulin

1 | INTRODUCTION

Tubulin and Na^+,K^+-ATPase (NKA) are two highly conserved proteins in almost all eukaryotic cells. Tubulin is an important component of the cytoskeleton, while NKA regulates sodium and potassium homeostasis. These proteins fulfill different functions and have different locations in the cell: NKA is found in the plasma membrane and tubulin is a cytosolic protein. In spite of their disparity of function and intracellular distribution, we demonstrated that both proteins are able to interact in a direct manner (Zampar et al., 2009). Even though tubulin is a hydrophilic protein, interaction between NKA and tubulin allows that some tubulin molecules behave as hydrophobic entities, due to the prevailing hydrophobic nature of NKA. Formation of the NKA–tubulin complex inhibits NKA activity in a reversible fashion, so this process can be an important regulatory system for NKA activity with key implications in cell functions (Amaiden et al., 2011, 2012; Casale, Alonso, & Barra, 2001; Casale, Previtali, & Barra, 2003; Casale, Previtali, Serafino, Arce, & Barra, 2005).

Due to the broad distribution and importance of NKA, regulation of its enzymatic activity has been the focus of numerous studies (Al-Khalili et al., 2004; Arystarkhova, 2016; Bertorello, Aperia, Walaas, Nairn, & Greengard, 1991; Chibalin, Vasilets, Hennekes, Pralong, & Geering, 1992; Juel, 2014; Juel, Hostrup, & Bangsbo, 2015; Li & Anghans, 2015; Lingrel, 2010; Lubarski-Gotliv, 2016; Petrushanko

et al., 2012; Shah, Martin, Yan, Shapiro, & Liu, 2016; Yeh, Ling, English, & Cantley, 1983). Several regulatory systems of NKA activity are known, such as gene expression, posttranslational modifications of the enzyme and interaction with regulatory proteins. Each regulatory system has a specific molecular mechanism involved in the response to different situations to which cells are exposed. These NKA regulatory mechanisms not only affect enzymatic activity but also establish new functions for the enzyme in the cell. Regulation of NKA activity by association with tubulin was described for the first time in 1998 (Alonso, Nuñez-Fernandez, Beltramo, Casale, & Barra, 1998). In the following 5 years, it was demonstrated that the interaction between tubulin and NKA regulates NKA activity in different types of cell, and that this interaction has an important physiological role (Casale et al., 2001, 2003). These findings highlighted a new mechanism for the regulation of NKA activity: The reversible tubulin–NKA interaction, which was then extended to other P-ATPases such as PMCA and H^+-ATPase (Arce, Casale, & Barra, 2008; Campetelli et al., 2013; Campetelli, Previtali, Arce, Barra, & Casale, 2005; Monesterolo et al., 2008, 2012).

Tubulin is formed by two different polypeptides (α and β) and each of them is codified by several genes. In addition, different isoforms of tubulin are generated by posttranslational modifications (Janke, 2014). Tubulin–NKA interaction is dependent on these modifications. In fact, tubulin must be acetylated to form the complex with membrane NKA (Santander et al., 2006). Tubulin acetylation occurs on Lys^{40} of α-tubulin, and acetylated tubulin is the posttranslational modification most abundant in the membrane and the least frequent in the cytoplasm (Beltramo, Alonso, & Barra, 1992; Beltramo, Nuñez, Alonso, & Barra, 1994). A second system playing an important role in the regulation of NKA activity is another posttranslational modification, namely, "detyrosination/tyrosination" at the C-terminal end of α-tubulin.

Two important characteristics of the tubulin–NKA interaction are: (a) tubulin regulates NKA activity from inside of the cell, and (b) the formation of this complex is reversible (Casale et al., 2003, 2005). Besides maintaining Na^+ and K^+ homeostasis, the interaction of NKA with different proteins has other purposes. For example, it is known that NKA is involved in cellular movement when it interacts with dysadherin, a member of the FXYD family (Arystarkhova, 2016; Lubarski-Gotliv, 2016). In addition, NKA has an active participation in the function of skeletal muscle (Al-Khalili et al., 2004; Bertorello et al., 1991; Chibalin et al., 1992) and it regulates cellular growth by activation of key signals (Lingrel, 2010; Shah et al., 2016). There is no report on the participation of tubulin in the events mentioned, but tubulin–NKA interaction could be related with the anchorage of microtubules to the membrane, and with changes in the rheological properties of human erythrocytes (Amaiden et al., 2012; Nigra et al., 2016; Zampar et al., 2009). This would mean that tubulin–NKA interaction is an important factor to determine membrane properties of the cells, giving to this interaction a new role for tubulin and NKA distinguishable from those already known.

In this review, the results reported about tubulin–NKA interaction are collected, summarized and organized in a way that allows us to explain the molecular mechanism of the interaction and its influence on cellular function. The regulation mechanism of NKA by tubulin has potential physiological implications in different cells and can provide a new perspective to explain the function of different cellular processes in which both tubulin and NKA are involved.

2 | ASSOCIATION OF TUBULIN TO THE MEMBRANE: TUBULIN–NKA INTERACTION

NKA is an integral membrane protein essential to homeostasis of electrolytes and body fluids (Jorgensen, Håkansson, & Karlish, 2003; Skou & Esmann, 1992). NKA is a heterodimer of an α- and a β-subunit in a 1:1 stoichiometry. The α-subunit, of 110 kDa, has 10 transmembrane domains, and the N- and C-terminal ends are located in the cytosolic side of the membrane (Geering, Meyer, Paccolat, Kraehenbühl, & Rossier, 1985). The β-subunit, smaller than the α-subunit (55 kDa), has only one transmembrane domain, is highly glycosylated, supports stability of the α-subunit and assists in the transport of the enzyme from the reticulum to the membrane (Geering et al., 1985). Different isoforms of both NKA subunits are found in different tissues. NKA is a member of the ATPase family named P-ATPase, due to the fact that members are characterized by the formation of a phosphorylated intermediate form during their catalytic cycle (Lutsenko & Kaplan, 1995).

On the other hand, tubulin is a hydrophilic protein and thus is mainly found in the cytosol. However, a fraction of tubulin can acquire hydrophobic character by its association to the membrane (Nuñez Fernandez, Beltramo, Alonso, & Barra, 1997). This association of tubulin with membranes can involve its interaction with different membrane components such as certain members of the P-ATPase family (Arce et al., 2008), connexin 43 (Ambrosi et al., 2016), the α-subunit of Gs protein (Sarma et al., 2015), and so on. In this way, tubulin becomes a peripheral membrane protein, not inserted in the lipid bilayer. One of these interactions is the association of tubulin with NKA (Alonso et al., 1998). Formation of the tubulin–NKA complex was described by the first time through the use of isolated membranes and purified tubulin from rat brains (Alonso et al., 1998). In this in vitro system, it was found that the hydrophobic character of tubulin is due to its interaction with a protein present in the brain membrane: sequencing of the protein showed that it was the α-subunit of NKA. It was later described that NKA and tubulin are both partners in the same complex and that this complex is present in a variety of cell types, including human erythrocytes (Amaiden et al., 2011).

In vivo association of tubulin to the membrane is reversible (Casale et al., 2003, 2005) and sensitive to several effectors (Table 1). Since this association depends on the interaction between tubulin and NKA, the formation of the tubulin–NKA complex and, consequently, the inhibition of NKA activity will also be sensitive to the same effectors (Table 1). Treatment of cultured cells with high glucose concentration (higher than 25 mM) induces association of tubulin to cell membrane (Casale et al., 2003, 2005; Rivelli et al.,

TABLE 1 Effectors that regulate the association of tubulin to the membrane in different cells or tissues

Effector	Cell or tissue	Tubulin–NKA complex	NKA activity	Membrane tubulin	References
Glucose	Astrocytes, CHO, HEP-2, 3T3 NIH cells	ND	Inhibition	Increase	Casale et al. (2003, 2005)
	Human erythrocytes	Increase	Inhibition	Increase	Amaiden et al. (2012), Rivelli et al. (2012)
	Erythrocytes, brain and liver of rat	ND	Inhibition	Increase	Rivelli et al. (2012)
	Heart and skeletal muscle of rat	ND	No variation	No variation	Rivelli et al. (2012)
	COS cells	Increase	Inhibition	Increase	Rivelli et al. (2012)
Glucose/ quercetin	COS cells	Decrease	Activation	Decrease	Rivelli et al. (2012)
Glutamate/Na^+	Astrocytes, CHO, HEP-2, 3T3 NIH cells	ND	Activation	Decrease	Casale et al. (2003, 2005)
DAG	Human erythrocytes	ND	ND	Increase	Monesterolo et al. (2012)
Nocodazole	Human erythrocytes and COS cells	ND	Activation	Decrease	Amaiden et al. (2012), Casale et al. (2005)
Taxol	Human erythrocytes and COS cells	ND	Inhibition	Increase	Amaiden et al. (2012), Casale et al. (2005)

Note. ND: nondetermined; NKA: Na^+,K^+-ATPase; DAG: diacylglycerol.

2012). This increment in the level of membrane tubulin can be rapidly reverted (in <15 min) by removing glucose and treating cells with glutamate and Na^+ (Casale et al., 2003, 2005). Similar results can be observed in several rat tissues (Rivelli et al., 2012). Intraperitoneal administration of glucose to rats causes an increment in the tubulin associated to the membrane of the erythrocytes, brain and liver, this increment is temporary because tubulin is dissociated when glucose is metabolized. Besides glucose and glutamate, other drugs are able to induce association/dissociation of tubulin with the cell membrane. In human erythrocytes, it was found that drugs that promote assembly or disassembly of microtubules, such as taxol and nocodazole, also induce association or dissociation of the complex, respectively (Amaiden et al., 2011; Casale et al., 2005). This suggests that a higher amount of polymerized tubulin induces that more tubulin binds to NKA. This seems to be due to an increase in acetylated tubulin, as microtubules are the preferred target from tubulin acetyl transferase (Sasse & Gull, 1988; Westermann & Weber, 2003). Lipids also affect the content of membrane tubulin. Addition of diacylglycerol (DAG) increases the content of acetylated tubulin in the membrane through a yet unknown mechanism (Monesterolo et al., 2012). Another interesting observation is that in cell cultures exposed to high concentrations of glucose, there is a stimulation of aldose reductase activity which produces a higher amount of sorbitol by reducing glucose (Rivelli et al., 2012). The increase in the amount of sorbitol results in a higher amount of microtubules, higher acetylation of tubulin and a higher amount of tubulin bound to NKA, with the consequent inhibition of enzyme activity. The inhibition of aldose reductase with quercetin causes a decrease in polymerized tubulin, in acetylated tubulin content and in the amount of tubulin bound to NKA, with an increase of NKA activity (Rivelli et al., 2012). All together these results suggest that formation of tubulin–NKA complex and consequently association of tubulin to the membrane is influenced by ability of assembly of microtubules and stability of formed structures (Figure 1). Then, there must be an increase in the assembly and stability of microtubules, in this way increment in the microtubule mass induces an increment in the membrane tubulin levels. In accordance, glucose increases microtubule assembly due to provides to cells from metabolic energy, on another hand taxol stabilize microtubules due to inhibit their disassembly, then both drugs induces increments in the microtubule mass, and both of them are related with an increment in the membrane tubulin levels, an increase in the content of tubulin–NKA complex and a higher inhibition of NKA activity. In the opposite, lack of energy (low glucose), diminution of microtubule mass induces by quercetin or disassembly of microtubules driven by nocodazole produces a lower membrane tubulin content.

The mentioned antecedents indicate that tubulin is associated to the membrane through different components that are inserted in it. We know that the different posttranslational modifications of tubulin favor the association of the protein to the membrane, but the structure of the microtubules that it interacts in the membrane it is still unknown. In the case of acetylation of tubulin, it remains in the lumen of the microtubules, therefore there are two options for the

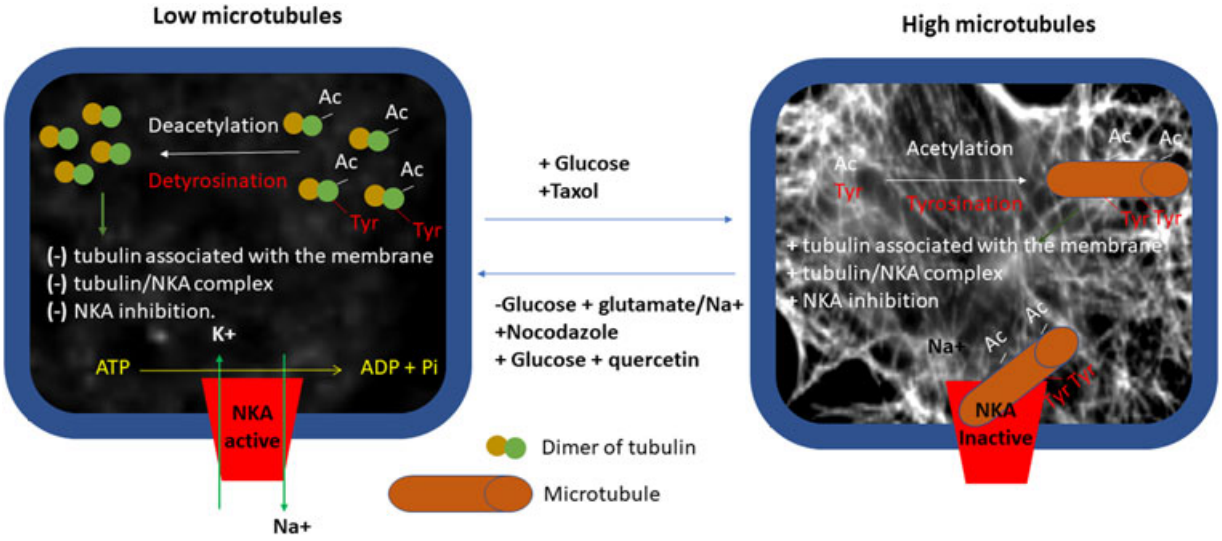

FIGURE 1 Mechanism of association of tubulin to the membrane. Increase in microtubule mass enhances association of tubulin to the plasma membrane because there is an increment in the levels of acetylated and detyrosinated tubulin, which forms a complex with NKA and inhibits its activity. This process can be reverted by replacing glucose with glutamate/Na^+ or by inhibiting polymerization of microtubules with quercetin or nocodazole. NKA: Na^+,K^+-ATPase [Color figure can be viewed at wileyonlinelibrary.com]

interaction of the microtubules with the membrane, one is that it interacts through the ends where the acetylation would be exposed and the second option is that acetylation produces conformational changes in microtubules that favor interaction. This reasoning is valid for any type of modification of tubulin; however, no data has been reported to clarify this point.

Tubulin and NKA interacts in a direct manner and this interaction do not requires other molecules. Studies performed by Zampar et al. (2009) showed that cytoplasmic domains 5 (CD5) of NKA is the possible anchorage site for microtubules to the plasma membrane. It was found that: (a) incubation of tubulin with detergent-solubilized membranes induces formation of a tubulin–NKA complex which behaves as an entity of discrete molecular mass between 320 and 400 kDa; (b) in vitro polymerized microtubules are able to interact with NKA when they are incubated with detergent-solubilized membranes; (c) native microtubules purified from rat brain tubulin are associated with NKA; and (d) the CD5 cytoplasmic domain of NKA, but not CD1, 2, 3, or 6, is able to interact with tubulin. These results suggest that NKA could be an anchorage site for microtubules to the plasma membrane, through the interaction between acetylated tubulin and CD5 of NKA. This interaction site in the enzyme is very interesting because CD5 is the cytoplasmic domain that connects transmembrane domains TM8 and TM9, and it was recently reported that, together with TM10, these two domains are involved in the formation of a pocket (site A) that interacts specifically with a molecule of phosphatidylserine, three molecules of cholesterol and the FXYD subunit (Habeck et al., 2015; Habeck, Kapri-Pardes, Sharon, & Karlish, 2017). The molecular structure of NKA bound to lipids in the cytoplasmic side of site A could be relevant to the stabilization of enzyme. Then, interaction between tubulin and the CD5 of NKA could affect the binding of NKA to lipids and consequently, enzyme stability. As mentioned above, tubulin is able to regulate the activity of multiple P-ATPases. In this sense, crystallography studies done by Drachmann et al. (2014) showed that the sarcoplasmic reticulum of Ca^{2+}-ATPase contains phospholipid-binding sites whose structure and functional relevance are virtually identical to the site A of NKA reported by Habeck et al. (2015). These results suggest that tubulin could regulate P-ATPase activity by modifying interactions between enzymes and their stabilizing lipids.

3 | NKA ACTIVITY REGULATION BY TUBULIN

Due to its crucial involvement in the regulation of ionic homeostasis, NKA has been a subject of study around the world for many decades. As mentioned above, tubulin associates with NKA in a reversible fashion, acting as an endogenous inhibitor of NKA activity. In turn, this interaction can be modulated by the posttranslational acetylation of tubulin and by glucose and glutamate/Na^+ (Casale et al., 2005). The regulation of plasma membrane NKA by tubulin was described for rat brain extracts and also different cultured cells and tissues (Table 1). The inhibitory effect of tubulin was only slightly reduced with ATP in vitro at a relatively low nucleotide concentration (0.06 mM). NaCl and KCl showed no effect, while inorganic phosphate abolished the inhibitory effect of tubulin in a concentration-dependent manner (Casale et al., 2001). Inhibition of enzymatic activity by tubulin correlates with: (a) the content of the acetylated and detyrosinated isoform of tubulin (Amaiden et al., 2015; Casale et al., 2001), and (b) the amount of tubulin–NKA complex formed (Casale et al., 2001). In experiments with cultured cells, we found that all effectors which affect tubulin association with the membrane or the association of tubulin to NKA also affect NKA activity

(Table 1). This indicates that if the association of tubulin with the membrane is affected by the polymerization and the stability of the microtubules, NKA activity will also be affected. We can therefore conclude that sodium and potassium homeostasis is regulated by polymerization and microtubule dynamics and stability (Figure 1). The greater the polymerization and stability of microtubules induces more inhibition of NKA, which would lead to an imbalance in Na^+/K^+ homeostasis by accumulating Na^+ intracellularly. In opposite, lower microtubule stability leads to greater NKA activity, and consequently Na^+ would not accumulate inside the cell.

4 | EFFECT OF POSTTRANSLATIONAL MODIFICATION OF TUBULIN ON THE TUBULIN–NKA INTERACTION AND ENZYMATIC ACTIVITY

It has been suggested that posttranslational modifications of tubulin are temporary and that they act as spatial markers for the binding of different proteins to tubulin, which would confer a broad spectrum of functions to tubulin. However, the functionality of many of these modifications is not yet fully understood (Janke, 2014). Among the most studied modifications in β-tubulin we find polyamination, polyglycosylation, phosphorylation and acetylation of lysine 252. On the other hand, α-tubulin suffers polyglutamination, acetylation of lysine 40 and modifications at the C-terminal, such as the tyrosination/detyrosination cycle and the generation of Δ2- and Δ3-tubulin isoforms. All these posttranslational modifications are mediated by enzymes, some of which remain uncharacterized.

4.1 | Acetylated tubulin

Acetylation of α-tubulin is catalyzed by several enzymes, mainly α-tubulin acetyl transferase (α-Tat; Yuzawa, Kamakura, Hayase, & Sumimoto, 2015) and its homologue Mec-17 (Davenport et al., 2014). The opposite reaction, deacetylation, is catalyzed by histone deacetylase 6 (HDAC6) and Sirt2 (Inoue et al., 2007; G. Li, Jiang, Chang, Xie, & Hu, 2011). Acetylases have greater affinity for microtubules, whereas deacetylases act preferentially on tubulin dimers, so acetylated tubulin is enriched in long-lived microtubules whereas deacetylated tubulin is enriched in nonpolymerized tubulin. Because some cells and organisms do not possess acetylated tubulin, this isoform does not seem to be vital for cells (Jenkins, Saunders, Record, Johnson-Schlitz, & Wildonger, 2017; Perdiz, Mackeh, Poüs, & Baillet, 2011). Nevertheless, it is related to multiple important functions, such as the branching of dendrites into sensory neurons in flies (Jenkins et al., 2017), sperm motility (Bhagwat et al., 2014), and binding and activity of motor proteins such as dyneins and kinesins (Perdiz et al., 2011). Recent works have proposed that acetylation of tubulin protects microtubules from mechanical stress and thus ensures the persistence of long-lived microtubules, which are essential to support load and compression forces in cells (Bhagwat et al., 2014). Such a mechanism of resistance to stress would be the consequence of an increase in the plasticity of the microtubules through the weakening of the interactions between the protofilaments of the microtubules (Portran, Schaedel, Xu, Théry, & Nachury, 2017).

In CAD cells, which do not possess acetylated tubulin, there is no interaction between tubulin and NKA. Absence of acetylated tubulin in CAD cells responds to high deacetylase activity, because the incubation of cells with tubacin or trichostatin A (both inhibitors of tubulin deacetylase) induces the appearance of acetylated tubulin in these cells. Under these conditions, the tubulin–NKA complex is detected and NKA activity is partially inhibited (Santander et al., 2006).

4.2 | Tyrosinated and detyrosinated tubulin

α-Tubulin is encoded with tyrosine residue at its C-terminal end. After becoming associated with β-tubulin and polymerized into microtubules, this amino acid is removed to produce detyrosinated tubulin (or Glu-tubulin, so called because of exposed glutamic acid on its C-terminal end). The removal is catalyzed by tubulin tyrosine carboxypeptidase (TCP), an enzyme which has been recently characterized. Aillaud et al. (2017) and Nieuwenhuis et al. (2017) identified and characterized vasohibins as the enzymes responsible for the activity of tubulin carboxypeptidase. This enzymatic activity is due to the formation of a complex between vasohibins 1 and 2, with a small protein bound to vasohibins.

The action of tubulin tyrosine ligase (TTL) can re-add tyrosine on the glutamic acid at the C-terminus of α-tubulin, so that Tyr-tubulin is obtained again, and the tyrosination/detyrosination cycle goes on (Barra, Arce, & Argaraña, 1988). TTL acts mainly on tubulin dimers, while TCP prefers tubulin that forms microtubules (Arce & Barra, 1985; Gundersen, Khawaja, & Bulinski, 1987). The action of both enzymes results in an accumulation of detyrosinated tubulin in stable microtubules, and an accumulation of tyrosinated tubulin in dynamic microtubules. The proportion of Glu- and Tyr-tubulin in the microtubules ("tyrosination state") modulates the binding of dynamism-regulating proteins (Liao & Gundersen, 1998). Detyrosinated tubulin (Glu-tubulin) inhibits the binding of proteins with the CAP-GLY domain, a subgroup of plus-end binding proteins (+TIP; Bieling et al., 2008). Detyrosinated tubulin enhances transport mediated by kinesin-1 in neurons (Konishi & Setou, 2009) and negatively regulates the activity of MCAK, a microtubule kinesin-depolymerase (Peris et al., 2009). The interaction between microtubules and intermediate filaments and microfilaments also seems to be regulated by the tyrosination state of microtubules (Kreitzer, Liao, & Gundersen, 1999; Marcos et al., 2009). Due to the importance of the tyrosination state in microtubule dynamics, TTL activity is essential to the normal development of an organism. This could be the reason why TTL-knockout mice die a few hours after birth with an abnormal development of their nervous system (Erck et al., 2005; Peris et al., 2006). Other results, moreover, suggest that TTL is involved in cancer development, as the level of TTL decreases in cancerous cells (Kato et al., 2004; Mialhe et al., 2001).

Detyrosinated tubulin is the substrate of a second reaction, an elimination of glutamate at the C-terminus that generates Δ2 tubulin, or a removal of two glutamates in this extreme that generates Δ3 tubulin. Both isoforms are not substrates of TTL and, consequently, are excluded from the tyrosination/detyrosination cycle. Similarly, to detyrosinated tubulin, Δ2 tubulin is abundant in highly stable microtubules. Its functional role is not clearly understood (Magiera & Janke, 2014; Song & Brady, 2015). It has been shown that all these isotypes of α-tubulin are present in all cells and coexist in a proportion that depends on the physiological state in which the cell is found. In fact, the presence of nitric oxide in some diseases allows the nitration of free tyrosine, which can be incorporated into tubulin by TTL.

4.3 | Acetylated and tyrosinated tubulin regulate NKA activity

Both acetylation and detyrosination play important roles in the regulation of NKA. As mentioned above, our laboratory demonstrated that acetylation on Lys^{40} is essential for tubulin to form a complex with NKA (Santander et al., 2006; Figure 2). In vitro experiments using plasmatic rat brain membrane and two preparations of tubulin, one with high and another with low proportion of acetylated tubulin, show that inhibition of NKA is more efficient in presence of tubulin with high acetylation content (Casale et al., 2001). Acetylation of α-tubulin increases when human erythrocytes are incubated in a solution containing a high concentration of glucose. Under this condition, the content of tubulin–NKA complex also increases and NKA activity is inhibited (Nigra et al., 2016). A similar effect is observed in erythrocytes of hypertensive subjects, which possess a higher content of acetylated membrane tubulin compared with normotensive subjects (Amaiden et al., 2012).

Although acetylation of α-tubulin is essential to establish the interaction with NKA, detyrosination of tubulin is also important because it enhances the inhibition of NKA induced by acetylated tubulin (Amaiden et al., 2015; Figure 2). This enhancing effect of Glu-tubulin on the inhibition of NKA induced by acetylated tubulin was observed in vitro and in vivo. Partially purified tubulin preparations with the same content of acetylated tubulin produced a greater inhibition of NKA in rat brain membranes when the preparation contained a higher amount of detyrosinated tubulin. In addition, human erythrocytes isolated from hypertensive subjects were shown to have a higher content of detyrosinated tubulin and lower NKA activity than human erythrocytes from normotensive subjects.

These results suggest that both acetylation and detyrosination of α-tubulin affect the activity of NKA, so they could also influence

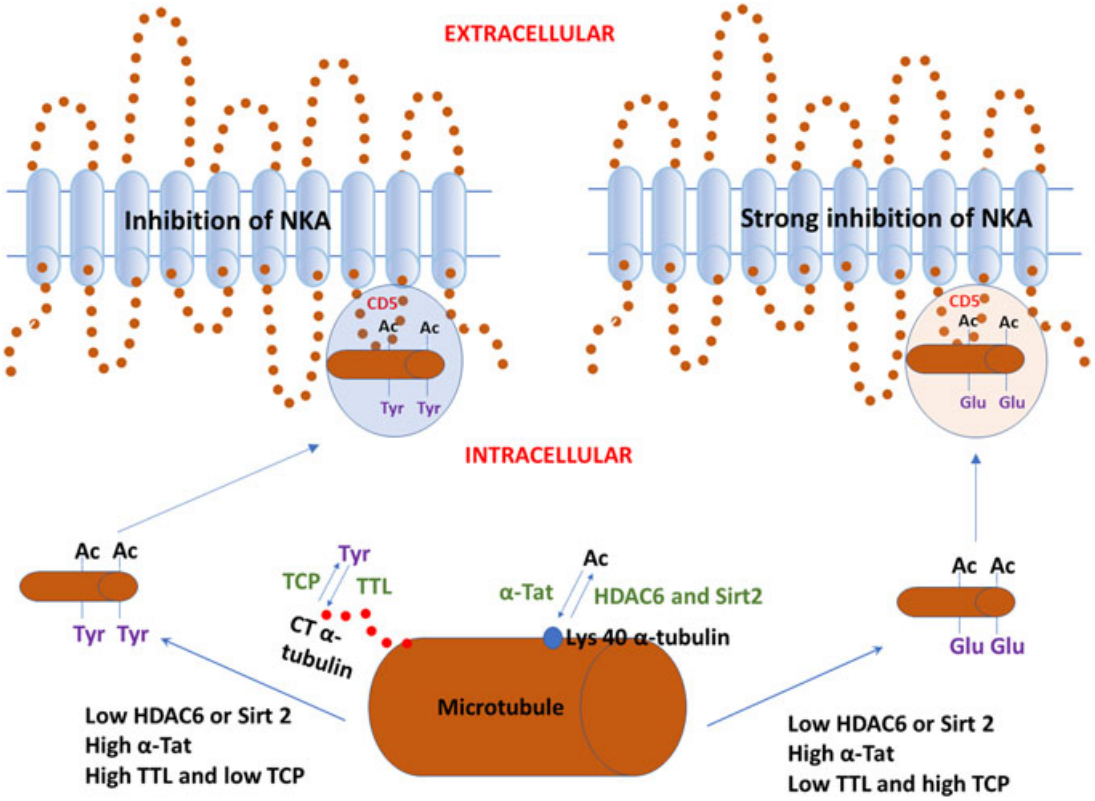

FIGURE 2 Posttranslational modifications of tubulin involved in NKA activity regulation. Tubulin can be acetylated by α-Tat and detyrosinated by TTCP. Both modifications inhibit NKA activity when tubulin interacts with cytoplasmic domain CD5 of NKA. An increase in deacetylase activities (HDAC6 or Sirt2) and a decrease in TTL activity enhance the anchoring of tubulin to the membrane and NKA inhibition. HDAC6: histone deacetylase 6; NKA: Na+,K+-ATPase; Sirt2: sirtuin 2 TCP: tubulin carboxypeptidase; TTCP: tubulin tyrosine carboxypeptidase; TTL: tubulin tyrosine ligase [Color figure can be viewed at wileyonlinelibrary.com]

TABLE 2 Regulation of NKA activity and proposed physiological role

Effector	Proposal mechanism	NKA activity	Physiological role	References
Acetylated/ detyrosinated tubulin	Direct interaction between acetylated tubulin and CD5 domain of NKA	Inhibition	Promotes anchorage of microtubules to the plasma membrane and regulates osmotic fragility and deformability of erythrocytes	Amaiden et al. (2012, 2011, 2015), Casale et al. (2001), Nigra et al. (2016), Zampar et al. (2009)
Phosphorylation	Phosphorylation of Ser, Thr, and Tyr residues of α and β-subunit of NKA by Protein kinases (PKA, PKC, and ERK1/2 activated by GMPc or AMPc)	Activation	Mainly found in skeletal muscle where function and abundance of NKA have a key role for cell physiology	Al-Khalili et al. (2004), Bertorello et al. (1991), Chibalin et al. (1992), Yeh et al. (1983)
Glutathionylation	Reversible covalent binding between glutathione and thiol residues of NKA	Inhibition	NKA fraction glutathionylated is not available for immediate activation	Juel et al. (2014, 2015), Petrushanko et al. (2012)
Gene Expression	Coupling of the transcription factor to promoter regions stimulated by hormones, growth factors, and other extracellular stimuli	High activity by increase of enzyme content	Increase in transcription of NKA when enzymatic activity is inhibited or inadequate for normal cellular function	Z. S. Li and Anghans (2015)
Endogenous and exogenous cardiotonic steroids	Direct interaction between steroid and Asn122 of NKA	Inhibition	Affect cellular growth and gene expression by activation of Src	Shah et al. (2016), Lingrel (2010)
Dysadherins FXYD2 and 5	Direct interaction between dysadherins (Ala150, Ile160, and Leu161) and NKA	Activation	Modulate cell junctions, chemokine production and cellular adhesion	Arystarkhova (2016) Lubarski Gotliv (2016)

homeostasis of sodium and potassium in the cells (Figure 1), in consistency with the hypothesis suggested by different authors that these posttranslational modifications could act in a synergistic manner, rather than independently, to regulate some functions of microtubules (Atherton, Houdusse, & Moores, 2013; Hammond et al., 2010; Jenkins et al., 2017; Kaul, Soppina, & Verhey, 2014).

5 | PHYSIOLOGICAL ROLE OF THE TUBULIN–NKA INTERACTION

Regulation of NKA activity is fundamental in every cell to maintain a gradient of concentration for Na^+ and K^+ ions across the membrane. This gradient is used by different cells to carry out diverse functions. In tubular kidney cells, the sodium gradient is used to filter waste products in the blood, reabsorb glucose and amino acids, regulate electrolytes and maintain blood pH. In the intestine, it is used for the absorption of amino acids and glucose obtained from the digestion of nutrients. In sperm, it is essential for motility and acrosome reaction. In the brain and other excitable tissues, functioning of the pump restores the ion gradient necessary to generate action potentials, and in astrocytes it is used for the uptake of neurotransmitters (Attwell & Laughlin, 2001; Clausen, Hilbers, & Poulsen, 2017; El Mernissi & Doucet, 1984; Jimenez, McDermott, Sanchez, & Blanco, 2011). In the last decades, it has been described that the function of NKA is not limited to the regulation of Na^+ and K^+ homeostasis (Table 2). NKA from skeletal muscle, for example, is key to the normal physiology of this organ (Pirkmajer & Chibalin, 2016), its gene expression participates in cell growth (Li & Anghans, 2015) and can also modulate cell adhesion (Lubarski-Gotliv, 2016). Therefore, regulatory mechanisms of NKA are very important for the correct functioning of the wide variety of processes in which this enzyme is involved.

As mentioned above tubulin–NKA interaction is characterized by regulating NKA activity from inside the cell, and by its reversibility. Three different systems have been described for enzymatic activation: phosphorylation, gene expression, and association with dysadherins. For the inhibition of the enzyme, three systems have also been reported: glutathionylation, association with cardiotonic steroids and interaction with tubulin (Table 2). Through all these regulation systems, the cell makes sure to have the necessary amount and activity of NKA for the appropriate maintenance of Na^+ and K^+ homeostasis. Cardiotonic steroids, which are molecules found in different tissues and species (Lingrel, 2010), interact with Asn^{122} on the extracellular side of cells and inhibit NKA. Unlike cardiotonic steroids, tubulin exerts its inhibitory effect by interaction with the CD5 of NKA on the inner side of the cells, as mentioned previously. Inhibition of NKA by glutathionylation is only seen when cells are exposed to oxidative stress, while inhibition of NKA by tubulin only requires tubulin acetylation.

Tubulin–NKA interaction has two important consequences: (a) regulation of NKA activity, and (b) anchoring of tubulin or microtubules in the cell membrane. Although the main role of the tubulin–NKA association in the cell could be enzyme inhibition, the

anchoring of tubulin/microtubules to the membrane is very important (Casale et al., 2001; Zampar et al., 2009). It has been found, in cell cultures and in erythrocytes that the tubulin–NKA interaction is involved in the regulation of mechanical processes such as deformability and resistance to osmotic stress (Amaiden et al., 2012; Nigra et al., 2016). Anchoring of microtubules to the membrane is a phenomenon that has been studied in our laboratory. When tubulin is anchored to the membrane it becomes part of a structure known as "submembranous tubulin" (Arce et al., 2008). Due to hydrophilic property of tubulin, anchoring if this protein to the membrane requires modification of its hydrophobicity. In this way tubulin interact with the membrane or with an integral membrane protein. In the first case, tubulin is subject of palmitoylation to acquire an hydrophobic character and in this way can penetrate the plasma membrane. In the second case, it was shown that tubulin has the ability to interact with a series of proteins with different functions, including NKA (Clausen et al., 2017). Here, acetylation of tubulin is paramount (Santander et al., 2006), since it must be acetylated to be able to interact with NKA and thus become anchored to the membrane.

The anchoring of tubulin to the membrane can have physiological consequences for the cells. The increase in tubulin membrane levels in human and rat erythrocytes induces a reduction in the deformability of cells (Amaiden et al., 2011, 2012) and a decrease in resistance to osmotic stress (Nigra et al., 2016). This may explain two physiological events present in diabetes and hypertension. In erythrocytes from diabetic subjects and erythrocytes from healthy subjects treated with high concentrations of glucose was observed an increase in the tubulin content in the membrane, this change was correlated with a lower osmotic resistance of this cells (Nigra et al., 2016). On the other hand, erythrocytes of hypertensive patients are less deformable and have a higher content of tubulin in the membrane. Treatment of erythrocytes of normatensive subjects with taxol, induces increase of membrane tubulin content and decrease in deformability (Amaiden et al., 2012). If the erythrocytes are treated with nocodazole, the opposite effect occurs: deformability and membrane tubulin content decrease. This shows that in rat and human erythrocytes, tubulin anchorage to the membrane mediated by tubulin acetylation has an important role in the regulation of deformability and osmotic fragility.

Results presented herein suggest that tubulin and its association with NKA have a role in the cells in three main events: regulation of NKA activity, anchoring of microtubules to the membrane and modulation of hemorheological properties such as deformability and resistance to osmotic stress in erythrocytes.

6 | RELEVANT CONCLUSIONS

6.1 | Tubulin can associate with the membrane and become a hydrophobic protein reversibly

Tubulin is found in two main places in the cells: as part of the microtubules or soluble in the cytoplasm as αβ dimer. Tubulin is soluble in the cytoplasm because it is a hydrophilic globular protein, and it exists in the form of microtubules because it polymerizes, using GTP hydrolysis energy, into a filamentous cylindrical structure that extends from centrosome and throughout the cytoplasm. The association of tubulin to the membrane further extends this structure to the membrane. This is possible because tubulin can interact with the components of the membrane by acquiring hydrophobic properties. One of the ways in which tubulin acquires hydrophobicity is acetylation (Santander et al., 2006). Unlike other forms of acquiring hydrophobicity, acetylation of tubulin has reversible character. In fact, since acetylating and deacetylating enzymes of tubulin coexist in cells, this acetylation-dependent hydrophobic property of tubulin is not constant and depends on the physiological state of the cell, mainly on the dynamism and stability of microtubules. The increase in dynamism involves the formation and disassembly of microtubules. This indicates that for there to be more microtubules there must be dynamism, in addition if the microtubules that are formed are more stable all this favors an increase in the number of microtubules, therefore to the acetylation of tubulin. In conclusion, the more dynamic and stable the microtubules, the greater the acetylation of tubulin and the greater the association of tubulin to the membrane.

6.2 | Tubulin inhibits NKA activity

Several regulatory systems for NKA activity have been described. The tubulin–NKA association is one of these systems, but it has unique characteristics, as it is a reversible system of intracellular regulation (Amaiden et al., 2011; Casale et al., 2003, 2005). In addition, because there is a tubulin analogue in the cytoskeleton of all cells, this regulation system could be functional in all types of cell and maintained throughout evolution. The inhibition of NKA by tubulin is due to the direct interaction between both proteins (Zampar et al., 2009), which requires the acetylation of tubulin, without the participation of other partners. The data suggest that the acetylation status of cells, especially for tubulin, regulates NKA activity.

6.3 | Acetylation of tubulin is essential for the association of tubulin to the membrane and tubulin–NKA complex formation

Tubulin–NKA interaction is only observed when tubulin is acetylated (Santander et al., 2006). Although tubulin acetylation can occur in tubulin dimers and in microtubules, tubulin acetyl transferases have a preference for acetylating microtubules. By contrast, the enzymes that deacetylate tubulin have more affinity for tubulin dimers. It means that acetylation of tubulin could be higher in cells with a higher content of microtubules. This implies that NKA could be more inhibited when microtubule mass increases in the cells due to greater tubulin acetylation. Therefore, NKA inhibition depends on the acetylation state of the cells but also on microtubule content, which enhances the acetylation process of tubulin.

6.4 | Detyrosinated tubulin enhances NKA inhibition produced by association with acetylated tubulin

The tyrosination state of tubulin has a role in the regulation of NKA activity. Even when detyrosination is not essential (as acetylation is) in the formation of the tubulin–NKA complex, it contributes significantly to the regulation of NKA activity. Inhibition of NKA activity is higher when the amount of detyrosinated tubulin increases. However, tubulin must be previously acetylated to form the tubulin–NKA complex. Interestingly, acetylation and detyrosination of tubulin are markers of microtubule stability, since the enzymes responsible for the generation of both tubulin isoforms have a greater affinity for polymerized tubulin. Then, physiological conditions that stimulate polymerization of the microtubules could increase acetylated and detyrosinated tubulin content, and consequently these posttranslational modifications could act in a synergistic way to induce the formation of the tubulin–NKA complex and enzymatic activity inhibition.

6.5 | Physiological role of NKA inhibition by tubulin

Findings in these last two decades related to the tubulin–NKA interaction allow us to establish a new mechanism for the regulation of NKA activity (Figure 3). Microtubule dynamics, which depend on the energy state of the cell, are particularly important in this mechanism. Metabolism of the cells is mainly oxidative in the presence of glucose, and generates ATP and GTP, which are essential for the dynamism of microtubules. GTP binds to tubulin and stimulates polymerization of microtubules that are acetylated by α-tubulin Acetyl Transferase, thus producing a high content of acetylated tubulin. The modificated tubulin migrates to the membrane and interacts with the CD5 domain of NKA, producing enzyme inhibition, which in turn results in an accumulation of Na^+ within the cell due to the prevention of sodium expulsion. When glucose is consumed, disassembly of the microtubules exceeds polymerization since there is lesser amount of GTP available. The tubulin–NKA complex is disrupted, as well, and enzymatic activity is restored, which allows the expulsion of sodium against the concentration gradient. Furthermore, when tubulin is generated as a dimer by the disassembly of the microtubules, HDAC6 and Sirt2 deacetylate tubulin, which enhances disruption of the tubulin–NKA complex due to lower amount of acetylated tubulin. This suggests that tubulin is anchored to the membrane when the cell has a high energetic state, whereas when the energy state of cells decreases, tubulin detaches from the membrane. Anchoring of tubulin to the membrane is used by cells to regulate homeostasis of Na^+/K^+ and some of the membrane properties already described.

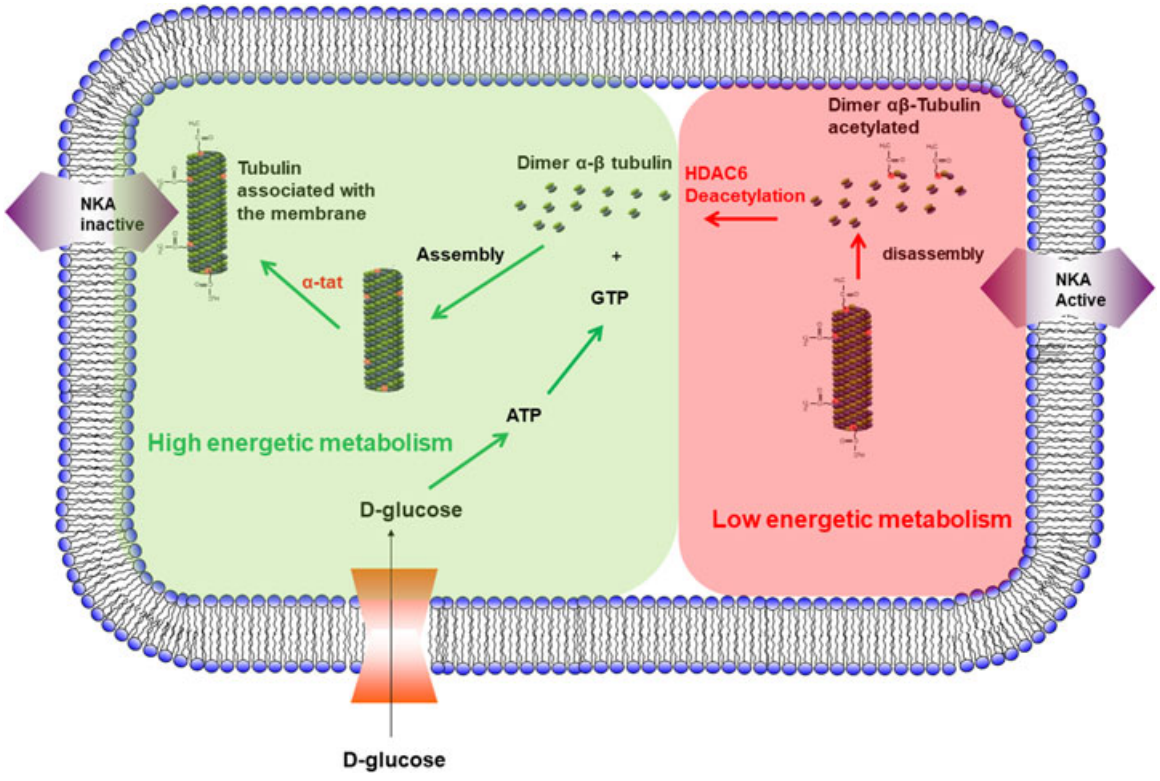

FIGURE 3 Physiological mechanism of NKA inhibition by tubulin. When cells are exposed to high levels of glucose, this is metabolized with production of GTP. GTP is necessary for the polymerization of microtubules, which enhances tubulin acetylation, anchorage of tubulin to the membrane and NKA inhibition. When fuel is consumed, the absence of GTP promotes the depolymerization of microtubules and consequently the deacetylation of tubulin, the detachment of the membrane and finally the activation of NKA. ATP: adenosine triphosphate; GTP: guanosine-5'-triphosphate; NKA: Na^+,K^+-ATPase [Color figure can be viewed at wileyonlinelibrary.com]

7 | PERSPECTIVES

The new regulatory mechanism of the NKA activity described in this review allows us to identify the anchoring site of the microtubules to the membrane, which gives a new perspective in the understanding of modifications of important cellular properties, such as deformability and resistance to stress osmotic in erythrocytes. In addition, the regulation of Na^+/K^+ homeostasis, also dependent on the tubulin–NKA interaction, opens new perspectives in different pathologies in which the homeostasis of these ions is unbalanced. In hypertension, this mechanism is important because the decrease in the content and the enzymatic activity of TTL in hypertensive erythrocytes (Amaiden et al., 2015) increases the level of detyrosinated tubulin, which has an inhibitory effect on NKA activity, according to the mentioned results. This would explain why the cells of hypertensive individuals accumulate Na^+. On the other hand, a high content of glucose in the insulin-independent tissues of diabetic subjects forces the cells towards a high metabolism of energy and generates a constant association of tubulin to the membrane, which would explain the low NKA activity and the intracellular accumulation of Na^+, all of which are characteristic of diabetes. This shows that the regulatory mechanism of NKA activity by tubulin and its association with the membrane are future targets of studies to regulate the rheological properties of membrane and the correct homeostasis of N^+/K^+.

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