Review



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*Review*

# Proton Mechanisms of Neurotransmission and Calcium Signalling for Impulse Initiation, Development and Propagation

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**Abstract:** Protons are gaining increasing attention as neurotransmitters due to their extraordinary abilities to rapidly transfer electrical charge, mobilize cellular calcium and modulate ion channels. How all this is possible is currently the subject of in-depth studies and discussions concerning not only neurophysiology, but also biological materials for artificial intelligence. In this short review, some biochemical mechanisms are described by which protons, in combination with calcium, can initiate firing in sensory neurons and transmit impulse across synapses. Furthermore, mechanisms are put forward concerning how three neurotransmitters, glutamate, gamma-aminobutyric acid and acetylcholine, are able to generate protons. The results of the numerous experimental works taken into consideration indicate that protons can play a fundamental role both in the generation and in the transmission of the nerve impulse.

**Keywords:** lipid membrane; excitable cells; synaptic vesicles; $H^+$ ion; $Ca^{2+}$ signaling; calcium binding proteins; signal transduction; G-protein coupled receptors

## Introduction

The dual purpose of this review is: 1) to describe some biochemical pathways for the transmission of the nerve impulse activated by H+ and Ca2+ ions; 2) highlight endogenous sources of H+ ions, neglected until now.

The fundamental role of Na+ e K+ ions in nerve transmission was demonstrated by eighteen years of experimental work by Hodgkin and Huxley [1]. H+ and Ca2+ ions were studied less, although Hodgkin and Huxley noted the significant role of $Ca^{2+}$ as far back as 1949 (Hodgkin1976, Figure 7) [2]. Subsequent studies confirmed the fundamental role of Ca2+ in proper transmission [3–5] and showed that dysfunctions in the homeostasis of Ca2+ can cause neurodegenerative diseases. The interest in $H^+$ ions, identified below with the current terminology as "protons", picked up with technical progress in instruments after 1980 [6–8]. Nerve impulses can last no more than 10 ms, pH transients and calcium spikes less than 2 ms, hence require sophisticated instruments for their study.

It is known that both Ca2+ ions and protons are ubiquitous in organisms, at concentrations that are strictly correlated [9–11]. A widespread lasting increase in their concentration produces the pathological condition known as acidosis, whilst a local and temporary increase is used currently by cells as a signal, in physiological conditions [12,13]. The correlation between protons and Ca2+ ions is fundamental for the transmission of the signal and depends on the high degree of solubility in an acid environment of protein-calcium complexes and calcium compounds, such as carbonates and phosphates, known to be calcium-buffering molecules. In cells in a static state, most cytosolic calcium is immobilized in these compounds. When the stimulus reaches the cell membrane activating a strongly acidifying enzyme, such as an esterase, the enzymatic action produces protons and hence locally and temporarily lowers pH. The acidity dissolves the calcium compounds, which can therefore pass into solution as Ca2+, producing calcium spikes, of intensity and duration proportional with the quantity of protons freed [9,14,15]. It has been calculated that in mitochondria a fall of one unit of pH produces a 100-fold increase in the concentration of Ca2+ [16]. Clearly, the acidifying action of esterases is an important characteristic enabling the transformation of the chemical signal

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into transient electric charges and the basis for the release of Ca2+ from cellular stores and the influx of extracellular Ca2+. However, surprisingly, this characteristic has almost entirely been ignored in scientific publications [11]. Table 1 sets out some examples of esterases and the acids they produce, which can solubilize cytosolic calcium.

**Table 1.** Examples of esterases, as possible sources of protons and intracellular Ca2+ spikes.

| enzyme | substrate | acid product | reference |
|---|---|---|---|
| phospholipase $A_2$ | phosphatidylcholine | arachidonic acid | Sun [17] |
| phospholipase C | phosphatidylinositol 4,5-bisphosphate | acid IP3 | Molinari, Figure 1A [18] |
| phospholipase D | phosphatidylcholine | phosphatidic acid | Cazzolli [19] |
| ecto-ATPase | ATP | ADP + acid phosphate | Vultaggio-Poma [20] |
| phosphodiesterase | cAMP | acid AMP | Delhaye [21] |
| phosphodiesterase | cGMP | acid GMP | Delhaye [21] |
| cADPR cyclase | cADPR | acid ADPR | Young [22] |
| acetylcholinesterase | acetylcholine | acetic acid | Fillafer [23] |

It is important to underline that hydrolysis of any ester that yields an acid with pKa < 6 will release a proton at pH 7. While phospholipases (i.e., $PLA_2$, PLC, PLD), triphosphatases (i.e., ecto-ATPase) and phosphodiesterases are acidifying enzymes, phosphomonoesterases (phosphatases) are not, due to the unfavorable pKas of phosphoric acid. Therefore, contrary to what was stated in my previous articles [11,18], the ability of phosphatases to lower pH and consequently mobilize calcium stores is questionable.

With an atomic mass about 23 times lower than sodium and a radius of about 0.08 nm, the proton is the smallest and most mobile ion in existence. In its hexahydrate form, proton has a radius of about 0.25 nm [24], against 0.95 nm of $Na^+$. Its level of permeability through the phospholipidic membrane is controversial, however it is ≤ that of $Na^+$ [25]. The elemental charge of the proton is the same as for other individual monovalent cations, at $1.602 \times 10^{-19}$ C. Anyway, protons can transport the charge much more quickly [26,27], via proton-hopping [24,28]. In addition to reacting with water, they can interact with amino acids and proteins and modulate [29] a large variety of channels and receptors, such as Voltage Gated Calcium Channels (VGCC/CaV) [30,31], Store Operated Calcium channels (SOC) [32], calcium-activated potassium channels ($K_{Ca}$) [33,34], inward rectifier potassium channels (Kir) [35,36], voltage gated proton channels (Hv) [37], proton gated Acid Sensing Ion Channels (ASIC) [38–40], multimodal Transient Receptor Potential channels (TRP) [41,42], and G-protein Coupled Receptors (GPCR) [43]. The interaction depends on the species, the extracellular or intracellular position of the protons, their concentration and the type of channel [44]. Many channels, including ASIC and TRPV1, mainly trigger activation, others such as VGCC [45] and TRPV5 [46] have a control or inhibitory function.

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To sum up, protons are tiny ionic particles that in an aqueous environment are acidic and highly mobile, able to rapidly transfer positive charges and to temporarily modify pH, Ca2+ concentration, electrical potential and the protein structure, as a result activating numerous receptors. Due to these extraordinary chemical and physical properties they are used in the preparation of organic electro-conductive materials [47,48] and are attracting increasing attention as neurotransmitters [12,13,49–58]. They have been shown to have an essential role at the synaptic level [58–62] and it has been posited that they are responsible for conduction in axons [57]. Some authors have also posited a significant role in the transmission and modulation of the signal in the nervous system generally [13,38,63]. However, the endogenous sources of the protons have yet to be determined. There are four candidates: Na-H exchangers, V-ATPases, carbonic anhydrases and AE3 chloride-bicarbonate exchanger [12,13,64,65], which however appear to be insufficient [65]. Specifically, Soto et al. [13] rightly observe: "*A problem of classifying protons as neurotransmitters is related to the fact that its regulated release is always a co-release with classical neurotransmitters*". In addition, some criticisms have been levelled against the theory of Hodgkin and Huxley; for example, it does not explain the origin of the firing of neurons [66]. X-ray crystallography and cryo-electron microscopy have revealed the structure of many ionic channels in the inactivated/open state and in some cases the amino acid residues involved in gating [67]. However, a knowledge of the structures of the intermediate states at the atomic level is required in order to better understand the origin of the movement of charges in the gating mechanism [68]. These problems could be overcome more simply if neurotransmitters and second messengers [69] were included among the possible sources of protons, given that these molecules can generate protons, i.e., new mobile charges as described in paragraph 2 of the discussion and protons can trigger firing and open channels, as described in paragraph 1.

## Methods

A review and critical assessment was made of the scientific publications dealing with the topic between 01.01.1950 and 25.05.2023, all available online.

## Results and Discussion

### *1. Pre-Synaptic Transmission of the Impulse in Sensory Neurons*

Protons can contribute to the generation and transmission of impulses in sensory neurons via biochemical mechanisms that differ in modality and effects [70].

For the perception of tastes there are substantial differences between vertebrates and insects [71].

In the specific case of neurons sensitive to a sour taste, it has been shown in mammals that protons can directly cause firing by opening OTOP1 [72], TRP [73,74] and perhaps ASIC [75] type channels. OTOP1 channels induce a change in the potential of the membrane, directly importing protons into the cytosol [73,76]. The resulting fall in cytosolic pH solubilizes calcium-buffering molecules and reduces the action of Kir channels [36] and this may cause further depolarization [76]. Any activation of ASIC and TRPV1 channels can produce proton-induced calcium influx [44]. The increase in Ca2+ concentration in the cytosol modulates calcium-activated potassium channels [77,78].

The pathway is more complex in the case of sensory neurons with GPCR-type metabotropic receptors at the distal termination of the axon; these are very common in mammals [79,80] for the transmission of visual stimuli [81], smell [82,83], nociceptive stimuli [84] and taste, limited to taste/flavour perceptions of sweet, bitter, umami and kukumi [85–87]. In these cases, the biochemical mechanism begins with the activation of a phospholipase C (PLC) [88,89]. The PLC hydrolyzes the phosphatidylinositol (4,5)-bisphosphate of the neuron membrane, this reaction for several enzyme isoforms is pH and Ca2+ dependent [90–92]. It is important to note that the reaction can be acidifying and autocatalytic, because inside the neuron hydrolysis produces inositol 1,4,5-trisphosphate (IP3) and protons [18,55], which in turn free Ca2+ [93,94], solubilizing the calcium-buffering molecules hence fostering a gradual increase in enzymatic activity. The acidifying action has been confirmed experimentally at the presynaptic termination [95–97]. The protons and IP3 released from the PLC by

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the enzymatic action of hydrolysis produce a threefold increase in cytosolic Ca2+ concentration due to: 1) the lowering of pH and resulting solubilization of the calcium-buffering molecules, 2) Ca2+ release from endoplasmic reticulum stores, 3) Ca2+ influx by stimulation of the SOCs. Since the activation of the SOCs induces the depolarization of the neuron membrane [98], the influx of Ca2+ can be seen as the first step in depolarization. A second step may follow rapidly with the closing of Kir-type channels and the opening of low threshold VGCC/CaV channels [99–102] permeable to Ca2+, TRP [12,42,55,103] and ASICs [104] channels permeable to Ca2+ e Na+ [105]. The opening of the channels enables the influx of further Ca2+ and Na+. The above studies jointly demonstrate that protons, together with Ca2+ ions, can start the process of depolarizing the membrane not only in neurons sensitive to a sour taste, but also in many other neurons with GPCR-type receptors.

It is likely that the three ions, H+, Ca2+ and Na+ contribute in differing degrees to depolarization until the threshold value is reached. When the threshold value is exceeded Voltage Gated Sodium Channels (NaV) open, generating the action potential [1,106]. This produces the exocytosis of the vesicles and release of the neurotransmitters into the synaptic cleft [107,108]. In the following repolarization phase the NaV channels close and the Kv [1,109,110], $K_{Ca}$, Kir and Hv proton channels [37,111] open enabling the efflux respectively of the K+ ions and the protons leading to the immobilization of Ca2+ and the return to static conditions. Pumps and exchangers are secondary contributors to the entire process.

In the eye, the activation of GPCRs via the PLC/IP3 pathway occurs by means of the cells containing melanopsin, such as the ganglion cells of the iris, whilst the cells of the retina containing rhodopsin and the cells of the auricular cochlea follow a different pathway, via PDE/cGMP [112,113]. In this case, the protons are generated by the hydrolysis of cGMP and the dissociation of acid glutamate, as described below in paragraph 2. The role of protons in hair cell transmission is currently under debate [114].

In relation to the sensory neurons that transmit mechanical stimuli, it is believed that in mammals these neurons generally respond via mechanoelectrical channels [115]. The physical stimulus induces the opening of ionic channels enabling the influx of Ca2+, depolarization and the generation of the action potential. The mechanisms for the activation of the channels are not clear [116]. In some cases, ASIC channels [117] or GPCR receptors [118] are involved. It has been shown that the G protein-coupled receptor OGR1 (GPR68) responds to mechanical stimuli and to protons via the PLC/IP3 pathway [119,120].

To sum up, for the above sensorial neurons, with ionotropic channels of the OTOP, TRP, ASIC type or metabotropic channels of the GPCR type, protons are essentials for increased cytosolic Ca2+ concentration. With limitation to these cases is it therefore possible to respond to the criticisms of the Hodgkin and Huxley theory and to affirm that protons, inducing with Ca2+ the first depolarization step, via proton-influx and/or proton-induced calcium influx, may be at the origin of firing.

*2. Synaptic Transmission of the Impulse*

In two prior publications we have described how protons may be generated in different cells by second messengers with the chemical structure of an ester or anhydride, such as ATP, IP3, NAADP, cADPR, cAMP or cGMP, by the hydrolytic action of specific enzymes [11,18].

The hydrolysis of an ester or anhydride always produces an acid, in these cases phosphoric acid or a derivation, which can rapidly dissociate, freeing protons. Table 1 in the introduction provides some examples of the hydrolysis of esters in organisms. Schematic representations of the reaction are available in many cases, for example for: ATP (Feng, equation 5),[121] IP3 (Huang, Supplementary Information, Figure S1),[55] cAMP (Barbosa, Figure 3) [122] and cGMP (Rybalkin Figure 1) [123]. However, it is not easy to find the complete representation, because most texts inexplicably fail to mention protons.

Neurotransmitters include compounds with an acid or ester type structure that can therefore generate protons. Below, three fundamental neurotransmitters are considered, released in the ribbon-type synapses by vesicle exocytosis: acetylcholine (ACh), gamma-aminobutyric acid (GABA) and glutamate (Glu). ACh is an ester, GABA and Glu are acid molecules. It is worth clarifying something

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regarding the latter: glutamate is the name given to a neutral salt and this can lead to confusion. In fact, for the acid strength GABA and Glu are very similar amino acids: they have respectively 4.0 and 4.3 pKa. Therefore, in vesicles where the pH is acidic [124–128], they are both partially undissociated, in the protonate form; therefore, for the sake of coherence, like GABA, Glu should be called acid glutamate. When they are released in a neutral or slightly alkaline environment, such as the synaptic cleft in the static state, these undissociated acid molecules tend to dissociate, each in its respective anion and a proton, as shown in Table 2.

**Table 2.** Protonated and deprotonated states of acid neurotransmitters.

| VESICLE LUMEN | | SYNAPTIC CLEFT |
|---|---|---|
| acid glutamate | ⇌ | $glutamate^- + H^+$ |
| γ-aminobutyric acid | ⇌ | $\gamma\text{-aminobutyrate}^- + H^+$ |

Therefore, it is evident that vesicle exocytosis produces inter-synaptic acidification [13,58,127,129–132] through the release of protons due to the acid content of vesicles and that the two acid neurotransmitters Glu and GABA may be, in this case, the principal source of the protons. The importance of this source is shown by the fact that the organism consumes energy to recycle Glu and GABA in the vesicles sufficiently rapidly to reuse them [133–136].

Regarding the ACh, which has the molecular structure of an ester, the protons are released by the acetic acid produced by the hydrolytic split of the ester by the cholinesterases: acetylcholinesterase and butyryl-cholinesterase. The reaction is very rapid and produces choline and acetic acid. For a long time, it was believed that the acetic acid and choline, constituting the ACh, were neurologically inactive molecules. It is still believed that the activity of ACh concerns the entire molecule because the limited use of anticholinesterases inhibits the response in direct proportion to the inhibitor dose and the response increases with the accumulation of Ach [137]. From this standpoint, cholinesterases have the sole function of rapidly eliminating the ACh, after its action. Today, we know that both constituents, choline and acetic acid, carry out a specific neurologically significant action [74,138] and that acetylcholinesterase may be indispensable for the action of Ach [23,60]. In addition, it has been posited that cholinergic transmission is due to the protonation of the postsynaptic membrane, caused by the acetic acid derived from the hydrolysis of Ach [23].

If the hypothesis that ACh can also act via its constituents were confirmed, it would be easier to clarify a number of questions that have been perplexing for some time. In addition, the fact that the three neurotransmitters ACh, Glu and GABA can release protons explains the observation of Soto et al. regarding co-release, as cited in the introduction.

The protons released by Glu, GABA or ACh acidify the inter-synaptic space and can activate acid-sensitive receptors at the postsynaptic termination together with specific receptors for Glu, GABA and ACh. There are numerous proton-sensitive receptors in the postsynaptic termination [139], both ionotropic such as ASICs [39,117], TRPV1 [41,140–142], CaV3 [143] and metabotropic, of the TASK type [144] and GPCRs [43]. The proton activation of the postsynaptic receptor can foster the opening of ionic channels [103,145], depolarization and the generation of a new action potential, enabling the impulse to continue [23,59,146].

Furthermore, many ligand receptors, specific for Glu, GABA and ACh, of the GPCR type, such as Group1 Glu [147,148], GABAb [149], nicotinic $\alpha 7$ [29,150] and muscarinic M1, M3 and M5 [151,152] receptors are activated by protons generated by PLCs. Ionotropic GABAa are also activated by the PLCs [153]. On the contrary, most ionotropic postsynaptic receptors of glutamate are inhibited by the protons, particularly AMPARs [154], Kainate receptors [155] and NMDARs [156,157].

To sum up, the protons may act at the synaptic level in various ways and via a large number of receptors. However, since protons are highly mobile and reactive but have low specificity, it is logical to attribute to protons mainly the quantitative aspects of the mechanisms of neurotransmission, whilst the qualitative aspects could be modulated by variations in the frequency, intensity and

duration of the proton impulse, by a parallel series of events such as variations in the concentration of Ca2+ and other ions such as Zn2+ and Mg2+, the type of other neurotransmitters involved, the receptors activated, their interrelations and their responses. In line with the general principle of co-release and co-transmission [158,159].

## Conclusions

The introduction points out the interdependence of protons and Ca2+ ions due to their chemical properties and it is useful to bear this in mind when seeking to understand the role of these ions in neurotransmission. The following paragraphs cite numerous experimental works the result of which, when taken together, provide an answer to the dual aim of this paper and support the hypothesis that protons may play a fundamental role both in the generation and the biochemical transmission of the nerve impulse. Specifically, paragraph 1 of the discussion describes how protons are able to trigger the depolarization of sensorial neurons by directly opening ionotropic channels and to activate GPCR receptors, via PLC/IP3 and the mobilization of Ca2+, thereby contributing to the generation of the action potential and the exocytosis of the vesicles. Paragraph 2 describes the mechanisms by which neurotransmitters in the vesicles, such as Glu, GABA and ACh, are able to become the sources of protons, generating them and, via the protons, fostering the transmission of the impulse through the synaptic cleft to the postsynaptic termination and beyond. To conclude, the role of protons in neurotransmission may be more important that has so far been believed and may in the future lead to many surprising discoveries.

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