On the excitation of action potentials by protons and its potential implications for cholinergic transmission

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Abstract One of the most conserved mechanisms for transmission of a nerve pulse across a synapse relies on acetylcholine (ACh). Ever since the Nobel Prize-winning works of Dale and Loewi, it has been assumed that ACh—subsequent to its action on a postsynaptic cell—is split into inactive by-products by acetylcholinesterase (AChE). Herein, the widespread assumption of inactivity of ACh’s hydrolysis products is falsified. Excitable cells (Chara braunii internodes), which had previously been unresponsive to ACh, became ACh-sensitive in the presence of AChE. The latter was evidenced by a striking difference in cell membrane depolarization upon exposure to 10 mM intact ACh (ΔV = −2 ± 5 mV) and its hydrolysate (ΔV = 81 ± 19 mV), respectively, for 60 s. This pronounced depolarization, which also triggered action potentials, was clearly attributed to one of the hydrolysis products: acetic acid (ΔV = 87 ± 9 mV at pH 4.0; choline ineffective in the range 1–10 mM). In agreement with our findings, numerous studies in the literature have reported that acids excite gels, lipid membranes, plant cells, erythrocytes, as well as neurons. Whether excitation of the postsynaptic cell in a cholinergic synapse is due to protons or due to intact ACh is a most fundamental question that has not been addressed so far.

Keywords Characeae · Acetylcholine · Acetylcholinesterase · Acetic acid · pH

Introduction

The transmission of a nerve pulse from a presynaptic to a postsynaptic cell (e.g., nerve, muscle, secretory cell, etc.) is fundamental for many physiological functions. With the exception of a class of specialized synapses (gap junctions), this transmission process is considered to be of a chemical nature. Many of our present ideas about chemical transmission have been influenced by works on cholinergic synapses (i.e., where acetylcholine (ACh) is the neurotransmitter). In short, the events taking place at such synapses are believed to be as follows: (i) A nerve pulse reaches the axon terminal and leads to the liberation of ACh; (ii) ACh translocates to the postsynaptic membrane and binds to a transmembrane protein (acetylcholine receptor (AChR)); and (iii) acetylcholinesterase (AChE) hydrolyses and thereby “deactivates” ACh.

The enzyme AChE is an integral component of cholinergic synapses (Nachmansohn 1959) where it can be anchored to parts of the basal lamina (Silman and Futerman 1987) and post- and presynaptic membranes (Nachmansohn 1959; Silman and Futerman 1987). Consensually, AChE has been attributed the role of “cleaner” of the synaptic cleft (Dale 1936; Loewi 1936; Katz 1962; Nachmansohn and Neumann 1974; Changeux and Edelstein 2005). In contrast to this presently predominant conception, earlier models of the cholinergic synapse considered AChE to be the receptor for ACh or at least part of it (Ehrenpreis 1967; Nachmansohn and Neumann 1974). Wurzel went a step further and suggested that choline (Ch⁺), which is liberated in the course of hydrolysis of ACh,

\[
\text{ACh}^+ + \text{H}_2\text{O} \xleftrightarrow{\text{AChE}} \text{OA}^- + \text{H}^+ + \text{Ch}^+
\]  (1)

could have excitatory effects (Wurzel 1959, 1967). At the same time, an involvement of the simultaneously produced acetic acid, which dissociates into acetate (OA⁻) and protons...
(H+)\textsuperscript{1} respectively, was excluded based on pharmacological reasoning (see Wurzel (1967) and discussion therein as well as Nachmansohn (1959)). Nevertheless, Kaufmann proposed a model of the cholinergic synapse in which these hydrolytically liberated protons play a key role in excitation of the postsynaptic membrane (Kaufmann 1977a, b, 1980). Dettbarn and Hoekman came to a similar conclusion when interpreting experimental results with lobster axons (Hoekman and Dettbarn 1971).

It is the aim of the present work to test these possibilities. We will focus on falsifying the following hypothesis:

(A) Hydrolysis of ACh results in substances which—by themselves—do not have an appreciable effect on excitable membranes.

To the best of our knowledge, no unambiguous evidence exists for this common assumption. Control experiments, for instance, which have shown that acetate is inactive on muscle preparations (Cowan 1936), do not appropriately address the matter. Acetate is the conjugate base of acetic acid. As such, it cannot mimic the acidification of the synaptic cleft which must occur in the course of enzymatic hydrolysis of ACh (Eq. (1)). Even if the up-to-date postulated assumption (A) held true, adequate control experiments with hydrolysis products would be very valuable because they can falsify alternative theories of cholinergic excitation such as those of Wurzel (1959, 1967) and Kaufmann (1977a, b, 1980, 1985).

Choice of model system Established cholinergic synapse models (e.g., frog neuromuscular junction, electric eel or Torpedo electroplaques, etc.) contain ACh-binding proteins (Changeux and Edelstein 2005) as well as AChE (Nachmansohn 1959). Under physiological conditions, AChE activity is remarkably high in these synapses and results in quantitative hydrolysis of ACh (Nachmansohn 1959; Nachmansohn and Neumann 1974) (Nachmansohn even went so far as to state that Loewi’s experiments of detecting ACh extrasynaptically cannot be reproduced (Nachmansohn and Neumann 1974)). In most experiments on cholinergic synapses, however, AChE has been inhibited with anticholinesterases (Nachmansohn 1959). Such use of “specific pharmacological blockers” (e.g., physostigmine, diisopropyl fluorophosphate, α-bungarotoxin, etc.) in general—which it has been fruitful and central for the study of many problems in (neuro-)physiology—entails potential pitfalls. To most readers, this will be a moot point, but nevertheless, it deserves notice that (i) by adding foreign substances to the system, one creates and studies an unphysiological condition (Kaufmann 1977b, 1980); (ii) complete pharmacological knockdown of AChE activity in the ~50-nm wide synaptic cleft and experimental confirmation of the latter is not trivial (Kaufmann 1980); (iii) attribution of the term “specific” to agonistic/antagonistic properties of a substance becomes increasingly difficult when one deals with multicomponent and multiphase systems such as cells or tissue\textsuperscript{2}; and (iv) any mistakes made when assigning such qualities as specific to a substance will be carried on in logical inferences.\textsuperscript{4}

In order to test assumption (A) and in an attempt to circumvent the above problems, we considered it to be advantageous to start from a system that is excitable (in the electrophysiological sense) but does not respond to ACh. Subsequently, one can selectively introduce components (e.g., AChR, AChE, etc.) and one can observe if ACh-sensitivity arises by such manipulations. Hypothesis (A), for instance, predicts that the simultaneous exposure of such a system to ACh and AChE will have no appreciable effect because the hydrolysis products are inactive. At the same time, the current theory of cholinergic excitation requests that introduction of nicotinic or muscarinic AChRs will be necessary in order to confer responsiveness towards ACh upon a previously unresponsive excitable cell. From an evolutionary perspective, one will be in the position to address the following question: Which minimal set of components has to evolve at the interface of two excitable cells in order to allow for their communication through ACh? In principle, a similar approach has been pursued with molecular biological techniques in established expression systems (e.g., Xenopus oocytes) (Soreq et al 1982). These model systems are very valuable. Yet, it is important to recall that oocytes, for instance, possess (i) intrinsic ACh sensitivity which has to be inhibited by atropine (Kusano et al 1977) and (ii) intrinsic AChE activity (Soreq et al 1982; Gundersen and Miledi 1983).

Herein, the applicability of excitable charophyte cells for the purpose outlined above will be described. Chara braunii internodal cells, which have a long-standing history as a model system in electrophysiology, will be used to test hypothesis (A). The effect of intact ACh on the membrane potential of these excitable cells will be characterized. Subsequently, the excitatory potency of hydrolysis products of ACh, which are generated in all cholinergic synapses, will be investigated. It will be demonstrated that excitatory potency increases with progressive degradation of ACh. This result falsifies hypothesis (A) and thus casts significant doubt on a central premise of the current theory of cholinergic transmission.

\textsuperscript{2} from (Changeux and Edelstein 2005): “Initial attempts to identify the acetylcholine-binding site were hindered [because several effectors on electroplaque] also bind to […] molecules distinct from the receptor and/or have high partition coefficients in lipidic compartments”

\textsuperscript{3} from Matthews-Bellinger and Salpeter (1978): “We have found that [α-bungarotoxin] has a high non-specific affinity for many substrates, especially glass and some plastics including Teflon.”

\textsuperscript{4} The following modus ponens is an over-exaggerated example: If α-bungarotoxin binds with high affinity, the acetylcholine receptor is present. α-Bungarotoxin binds to Teflon. Thus, the acetylcholine receptor must be present on Teflon.

\textsuperscript{1} Throughout the manuscript, the term “proton” will be used synonymously for its hydrated forms, e.g., H\textsubscript{2}O\textsuperscript{+}, H\textsubscript{3}O\textsuperscript{2+}. 
Materials and methods

Materials  Acetylcholinesterase from *Electrophorus electricus* was obtained from Sigma-Aldrich (type V-S; Lot# 021M7025V). All other reagents were also purchased from Sigma-Aldrich (St. Louis, MO, USA) and were of analytical purity (≥99 %).

Cell cultivation and storage  *C. braunii* cells were cultivated in glass aquariums filled with a layer of 2–3 cm of New England forest soil, quartz sand, and deionized water. The cells were grown under illumination from an aquarium light (14 W, Flora Sun Max Plant Growth, Zoo Med Laboratories Inc., San Luis Obispo, CA, USA) at a 14:10 light/dark cycle at room temperature (~20 °C). Prior to use, single internodal cells were stored for a minimum of 12 h in a solution containing 0.1 mM NaCl, 0.1 mM KCl, and 0.1 mM CaCl₂.

Experimental setup  A single internodal cell (3–6 cm long) was placed on a plexiglass chamber into which compartments (~5×5×10 mm; h×w×l) had been milled. Small extracellular sections (length ~5 mm) of the cell were electrically isolated against each other with vacuum grease (Dow Corning Corporation, Midland, MI, USA). The K⁺ anesthesia technique (Beilby 1989) in combination with extracellular electrodes (Dri-Ref, World Precision Instruments, Sarasota, FL, USA) was used for monitoring the cell membrane potential. Strictly, the membrane potential measured by this technique includes components from plasmalemma and tonoplast. The extracellular solutions contained 110 mM KCl in the first compartment and artificial pond water (APW) in all other extracellular solutions contained 110 mM KCl in the first compartment and artificial pond water (APW) in all other extracellular solutions contained 110 mM KCl in the first compartment and artificial pond water (APW) in all other compartments (0.1 mM KCl, 0.1 mM NaCl, 0.1 mM CaCl₂, 5 mM TRIS, and 190 mM d-sorbitol; pH set to 7.0 with HCl). The potential between the virtual intracellular electrode (KCl compartment) and an extracellular electrode was recorded with a voltage sensor (PS-2132; 50 Hz sample rate; PASCO scientific, Roseville, CA, USA) and was defined as the resting membrane potential (Vᵣ). All experiments were conducted at room temperature (20 ± 2 °C).

Test solutions  APW was acidified with HCl and acetic acid to a desired pH (monitored with a glass electrode; Van London–Phoenix Co., Houston, TX, USA). Solutions of acetylcholine chloride (10, 25, and 50 mM) and choline chloride (1, 5, and 10 mM) were prepared fresh prior to each experiment. For these solutions, APW was used as described above except that the amount of d-sorbitol was decreased to ensure a constant osmolarity of 200 ± 2 mOs mol kg⁻¹ (3D3 Osmometer, Advanced Instruments, Norwood, MA, USA). Hydrolysis of ACh (10 mM) was carried out in 20 mL APW (composition as described above; instead of 5 mM TRIS, only about 1–2 mg was added to buffer the drop in pH that had resulted from spontaneous hydrolysis of ACh). This solution was stirred in a glass beaker, and after about 10 min, AChE was added (5 μL of a stock solution in APW (~500 units per 100 μL)). At fixed points in time (corresponding to a, b, c, d, and e in Fig. 2), samples were drawn with a micropipette.

Effect of test solutions on membrane potential  APW was removed from one of the compartments, and subsequently, this section of the cell was exposed to test solution for 60 s (Figs. 1, 2, and 3) and ~2 s, respectively (Fig. 4). The resting membrane potential (Vᵣ ~ −200 mV) was subtracted from the value of the membrane potential after 60 s (Vₜₐₜ). Thus, positive values of ΔV = Vₜₐₜ − Vᵣ indicate depolarization while negative values indicate hyperpolarization. Experiments in which an action potential (AP) was triggered were not included in the calculation of averages of ΔV. Typically, a single cell was exposed to six to eight test solutions with two washing steps (with APW) and a recovery period (15 min) in between trials. The sequence of exposure of a single cell to different test solutions was varied arbitrarily and was not found to have a biasing effect on the results obtained.

Results

ACh and the Chara membrane potential  In general, limited knowledge is available about the occurrence of AChR, AChE, as well as ACh in charophyte cells (e.g., *Nitella, Chara*, etc.). AChE activity has been detected in *Nitella flexilis* homogenates, but the cellular location of the enzyme was not clarified (Dettbarn 1962). Patch-clamp studies with tonoplast membrane from *Chara corallina* indicated a certain responsiveness
to ACh (4–6 mM), which however did not bear the typical characteristics associated with nicotinic acetylcholine receptors (nAChRs) (Gong and Bisson 2002). It has also been reported that the repolarization phase of an electrically triggered AP is prolonged upon addition of >1 mM ACh in C. corallina (Gong and Bisson 2002) and Nittelopsis obtusa (Kisnierienė et al 2009). None of these studies, however, have found excitation of the cell by extracellular addition of ACh. Thus, C. braunii cells were considered to be a suitable model system for studying the problems discussed in the “Introduction.”

Hydrolysis products of ACh are excitatory In the next step, the consequences of introducing AChE to this system were tested. The rationale of the experiment was as follows: One starts in a beaker with an aqueous solution of ACh that fluctuating timecourse, was detected upon extracellular addition of 50 mM ACh for 60 s. Most importantly, however, at concentrations of ACh <50 mM, no sign of excitation in the sense of (i) pronounced depolarization or (ii) triggering of an AP was observed. Thus, C. braunii cells were considered to be a suitable model system for studying the problems discussed in the “Introduction.”

Hydrolysis products of ACh are excitatory In the next step, the consequences of introducing AChE to this system were tested. The rationale of the experiment was as follows: One starts in a beaker with an aqueous solution of ACh that
contains mainly intact ACh. Upon addition of AChE, the concentration of ACh will decrease and simultaneously, hydrolysis products (AcH, OAc\(^{-}\), H\(^{+}\), Ch\(^{+}\)) will accumulate. This process represents the ubiquitously occurring degradation of ACh in cholinergic synapses. The evolving reaction can be followed easily by use of a pH electrode (Fig. 2a). When samples (a, b, c, d, e) are drawn from the beaker at progressive stages of the reaction, their ACh content will obey the relation \([\text{ACh}]_a > [\text{ACh}]_b > \ldots > [\text{ACh}]_e\). The existing model of the cholinergic synapse clearly predicts a decrease in excitatory potency from a to e simply because the amount of intact ACh is reduced. In other words and in reference to hypothesis (A), if (A) is indeed correct for biology, then one expects that degradation of ACh by AChE will lead to inactive solutions. In the present case of Chara cells, none of the hydrolysates should elicit a response. However, the exact opposite is observed: The more ACh was degraded, the higher the activity. This was evidenced by the degree of depolarization of the membrane and by the increased likelihood of triggering of action potentials (Fig. 2c; in four out of ten cases at pH \(\sim 4.0\)). It was also found that application of a test medium acidified to pH \(\leq 3.5\pm0.2\) with HCl resulted in immediate and repeatable triggering of APs within 1–2 s (Fig. 4). This indicates that the timescales for the induction of an AP are strongly dependent on proton concentration. It is concluded from these results that protons are the main excitatory component of hydrolysates of ACh.

**Identity of the excitatory agent** It is of interest to identify if the observed excitatory effect has to be attributed to (i) choline (suggested by Wurzel (1959; 1967)) or (ii) acid (suggested by Kaufmann (1977a; 1977b; 1980)). As illustrated in Fig. 3a, choline up to concentrations of 10 mM had no detectable effect on the Chara cell membrane potential. In contrast, artificial pond water that had been acidified with acetic acid closely reproduced the depolarization that was observed with the hydrolysates (Fig. 3b). Moreover, action potentials were triggered with acetic acid-containing test solutions of pH 4.0 (in two of seven cases). Further experiments were conducted with artificial pond water that had been acidified with hydrochloric acid. These studies clearly indicated that the overall phenomenology is invariant to the type of acid employed⁶ (Fig. 3b). It is probable that the excitatory potency of an acid varies based on, e.g., its pK, solubility, etc.

**Discussion**

Protons excite Chara cells, neurons, gels, and lipid membranes The sensitivity of cell membranes to protonation is by no means a peculiarity of the plant cells used in this study. In fact, the typically negatively charged cell surfaces of eukaryotes (Mehrishi and Bauer 2002) make it seem unavoidable that a cell is susceptible to pH. A proton-induced increase of membrane conductance has indeed been shown to occur in neurons from trigeminal ganglia (Krishtal and Pidoplichko 1980) and neuroblastoma cells (Krishtal and

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³ It is not possible to apply pure ACh since the compound is hydrolyzed spontaneously, yet at a slow rate, in aqueous solution.

⁶ It is probable that the excitatory potency of an acid varies based on, e.g., its pK, solubility, etc.
the induction of transmembrane current fluctuations in showed that protonation-induced state changes can lead to state changes (Träuble 1977). Kaufmann and co-authors with acid or base is a potent means to induce thermodynamic namic properties of a membrane is its pK and that titration affected by pH changes. Träuble, for instance, demonstrated have been demonstrated unintentionally (Burckhardt et al 1992). It is also possible that effects of acids Dettbarn 1971), as well as Xenopus laevis oocytes (Burckhardt et al 1992). It is also possible that effects of acids have been demonstrated unintentionally in experiments involving iontophoretic application of neurotransmitters. Frederickson et al., for instance, showed that protons are expelled preferentially from norepinephrine-containing micropipettes due to their higher electrophoretic mobility (Frederickson et al 1971). The very same issue might have biased studies on cholinergic synapses. There, ACh has frequently been applied from micropipettes containing a 1M solution of ACh (Kuffler and Yoshikami 1975; Kusano et al 1982). Within seconds upon preparation, such a solution acquires a pH of ~3.25 due to spontaneous hydrolysis of ACh (observations in our laboratory). Thus, it is conceivable that the iontophoretic application of ACh (e.g., in Kuffler and Yoshikami (1975) and Kusano et al. (1982)) was to some degree an iontophoretic application of protons. Further evidence for effects of acidic pH on neurons has been reviewed recently (Akaike and Ueno 1994; Sinning and Hübner 2013). It is also well-established that model systems of biological membranes such as lipid mono- and bilayers (Träuble 1977) as well as gels (Kuhn et al 1950; Walters et al 1961) are affected by pH changes. Träuble, for instance, demonstrated theoretically and experimentally that one of the thermodynamic properties of a membrane is its pK and that titration with acid or base is a potent means to induce thermodynamic state changes (Träuble 1977). Kaufmann and co-authors showed that protonation-induced state changes can lead to the induction of transmembrane current fluctuations in protein-free lipid membranes (Kaufmann and Silman 1983; Kaufmann et al 1989). It was also reported that rapid application of acid droplets onto a charged lipid monolayer leads to excitation of pulses that propagate over macroscopic distances (Griesbauer et al 2012). Another beautiful example for protonation-induced state changes is Kuhn’s artificial muscle model, which exhibits reversible macroscopic shortening and extension upon acidification and alkalinization, respectively (Kuhn et al 1950; Walters et al 1961).

**Bulk versus interfacial protonation** In most experiments aimed at understanding the effects of pH on cells and tissue, protons are typically applied via the bath or at considerable distances from the excitable membrane. This “bulk” approach, which was also pursued in the present study, seems rather crude in comparison to the refined mechanisms that have evolved in nature. In biological systems, the majority of water exists at hydrated interfaces (membranes, polymers (such as cytoskeletal filaments or DNA), etc.). Enzymes located at such an interface (e.g., AChE, carbonic anhydrase, ATPase, etc.) have the potential to rapidly switch its thermodynamic state by catalytic activity. This is best illustrated in the system which has been addressed in the present work: the cholinergic synapse. The latter can be considered a hydrolytic “hotspot” for ACh due to the remarkably high local acetylcholinesterase activity (Nachmansohn 1959). Under physiological conditions (=no anticholinesterase present), a considerable fraction (Rosenberry 1979; Wathey et al 1979; Bartol et al 1991) or almost all of the ACh (Kaufmann 1977b, 1980) liberated in a synapse will be rapidly degraded before it has the opportunity to interact with other components (e.g., an ACh-binding protein, AChR). An order of magnitude estimate based on data from rat diaphragm (Kaufmann et al 1989). It was also reported that rapid application of acid droplets onto a charged lipid monolayer leads to excitation of pulses that propagate over macroscopic distances (Griesbauer et al 2012). Another beautiful example for protonation-induced state changes is Kuhn’s artificial muscle model, which exhibits reversible macroscopic shortening and extension upon acidification and alkalinization, respectively (Kuhn et al 1950; Walters et al 1961).

**Fig. 4** Instantaneous triggering (within ~2 s) of APs in a Chara braunii cell upon exposure to acidified (HCl) but not neutral or alkalinized APW (NaOH). Mean pH threshold in $n=4$ cells±StDev 3.5±0.2

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7 The interested reader is referred to evidence in Wunderlich et al. (2009) and Heinrich (2010) that contradicts the concept of ion translocation through protein channels.

8 Area of endplate, ~7000 μm²; volume of endplate, ~450 μm³; catalytic site density, ~2500 sites μm⁻²; hydrolysis rate, 0.3 molecules catalytic site⁻¹ msec⁻¹; ACh release, 3·10⁷ molecules pulse⁻¹ endplate⁻¹ (the latter value from Potter (1970) has been quoted frequently despite the fact that the calculation and assumptions taken were not detailed in the paper). Moreover, it is not clear if residual AChE activity (after eserin treatment) was accounted for. Generously assuming 99 % of inhibition, this still leaves a hydrolysis rate of ~5·10⁹ molecules msec⁻¹ endplate⁻¹. In Potter (1970), ACh release was determined in the bath ~300 s after repetitive stimulation. During this timespan, ~1.5·10¹⁰ additional molecules of ACh could have been hydrolyzed per endplate and would not appear in a detection essay. Thus, the actual concentration of ACh per pulse per endplate could easily have been 3·10⁷ molecules—an order of magnitude larger than assumed—or even higher (as argued by some, 0.1–1 mM; see discussion in Ehrenpreis (1967)). Hydrolysis of ~10⁷ ACh molecules will, thus, take ~1–2 msec under physiological conditions. In the presence of cholinesterase inhibitors, the reaction is not stopped but simply extended in time to ~10–100 msec. These order of magnitude estimates are in good agreement with experimentally obtained timescales of excitatory postsynaptic potentials in the absence and presence of anticholinesterase (compare Fig. 6 in Katz (1962)).
(Potter 1970; Salpeter and Eldefrawi 1973) indicates typical timescales for hydrolysis of most of ACh within \(\sim 1–2\) msec. If one treats the synaptic cleft as an aqueous bulk volume into which reaction products are released, this corresponds to a drop of pH from \(~ -7\) to \(-4.5\). However, such an analogy unlikely captures the conditions in the synapse, which is a small system with a high surface to volume ratio (Bal et al. 2012). It is conceivable that protons, instead of being released into an aqueous bulk phase, are directly buffered by the negatively charged hydrated interfaces into which AChE is embedded. Such an AChE-induced increase in local proton concentration has indeed been reported to occur on artificial colloidon membranes (Goldman et al. 1965), \textit{E. electricus} membrane fragments (Silman and Karlin 1967), and “modified” electroplaque cells (Podleski and Changeux 1967). Moreover, it has been shown (del Castillo et al. 1967; Kaufmann and Silman 1980) that incorporation of AChE renders lipid bilayers sensitive for ACh (which was attributed to local protonation of the interface (Kaufmann and Silman 1983)). Cell membranes of erythrocytes from several species (human, guinea pig, dog, etc.) also contain AChE. When exposed to ACh, these cells are progressively permeabilized due to enzymatically catalyzed acidification (Holland and Graham 1955). Although biological effects due to interfacial protonation by AChE seem to be rather common, they have neither been considered in quantitative models of the cholinergic synapse (Rosenberry 1979; Wathey et al. 1979; Bartol et al. 1991) nor in the respective literature (e.g., Changeux and Edelstein (2005) and Sinning and Hübner (2013)).

**Cholinergic transmission and the nature of receptors** The current theory of cholinergic transmission has been elaborated in remarkable electrophysiological and molecular detail (Changeux and Edelstein 2005). Nevertheless, it is important to challenge its underlying assumptions and to point out evidence that is missing. Herein, it was demonstrated that intact ACh does not particularly affect an excitable plant cell. However, catalytic hydrolysis of ACh generates protons, which depolarize \textit{Chara} cells and excite action potentials. Such sensitivity of biological membrane towards protons is not a peculiarity of plant cells but a more general phenomenon well documented in the literature (Hoekman and Dettbarn 1971; Frederickson et al. 1971; Gruol et al. 1980; Krishtal and Pidoplichko 1980; Akaike and Ueno 1994; Sinning and Hübner 2013).

The results presented herein falsify hypothesis (A), and taken together, we find ourselves confronted with the critical question: Did nature really come up with two entirely different mechanisms for excitation of cells by ACh, one where AChE inactivates and one where it activates, or are we under a misapprehension? We suggest that our current conceptions of cholinergic transmission need to be revisited to clarify this issue. To facilitate the latter, we propose the following falsifiable hypotheses:

1. **Transmission in cholinergic synapses is due to a state change of the postsynaptic membrane induced by protonation (as proposed herein and by Kaufmann (1977a; 1977b; 1980; 1985)).**

   This hypothesis will be falsified if application of protons (pH range \(\sim 7–4\)) fails to depolarize typical postsynaptic membranes. It will also be falsified if the protonation state of a postsynaptic membrane does not change during cholinergic impulse transmission.

   Furthermore, lack of falsification of the following hypothesis would cast significant doubt on our proposition:

2. **A membrane that contains AChR proteins can be excited by ACh while—at the same time—the membrane is not protonated.**

   Experiments challenging this hypothesis will be even more convincing if purified AChR proteins are used and if esterase activity can be eliminated. However, in any case, it is essential that the membrane’s protonation state be monitored. The latter may not be assumed constant based on indirect arguments, such as the presence of anticholinesterase or buffer substances in the medium. We suggest that \textit{Chara} cells could be used for such experiments, since their “baseline” response to ACh is low (see Fig. 1) and since the expression of nAChRs seems feasible (Lühring and Witzemann 1995).

   Finally, some consequences of our study are as follows: (i) A sufficient set of components for cholinergic transmission are ACh, AChE, and a charged hydrated interface (this argument is based on parsimony\(^9\) and does not preclude the realization of more complicated systems\(^10\). (ii) In the current cholinergic theory, acetylcholinesterase performs work “against the receptor” by rapidly degrading its ligand. The inherent inefficiency of this kinetic competition would be resolved if one assumed that AChE generates the excitatory agent (Kaufmann 1980). (iii) The postsynaptic membrane is specific for acetylcholine because of the presence of highly specific enzymatic activity. (iv) Currently and conventionally, the term “receptor” is used for a specific molecule that exerts a function upon binding of a ligand. We would like to shortly elaborate on an alternative to this molecular viewpoint to which we have been motivated by Einstein’s approach to thermodynamics (Einstein 1913). As has been documented above, simple (pure lipid membranes, gels, etc.) as well

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\(^9\)From I. Newton’s Rules of Reasoning in Philosophy: “We are to admit no more causes of natural things than such as are both true and sufficient to explain their appearances. Therefore, to the same natural effects we must, so far as possible, assign the same causes.”

\(^10\)For instance, butyrylcholinesterase or spontaneous hydrolysis could contribute to the liberation of protons from ACh.
as compositionally complex hydrated interfaces (biological membranes)—systems with entirely different molecular composition—are highly susceptible to pH. Thus, it seems intuitive to consider a system (e.g., a part or the entire cellular membrane including lipids, proteins, carbohydrates, attached extracellular matrix, hydration layers, etc.) and not a single molecular entity as the receptor. Single molecules are part of this system and should not be seen as isolated entities. In other words, we deal with a receptive system, which reveals its functions in a particularly impressive manner upon drastic changes in its thermodynamic state, i.e., near transitions. Therefore, we propose that the thermodynamic state diagrams of a system determine its ability to receive stimuli and to exert functions (state-function relationship). Protonation\(^\text{11}\) of a cell membrane (=receptive system), for example, will induce a state change. In particular, when near a transition, this new state of the system will manifest dramatically changed biological functions (e.g., permeability [Kaufmann et al 1989, Wunderlich et al 2009; Heimburg 2010], affinity for ions (e.g., H\(^+\), Ca\(^{2+}\)), catalytic activity (Kimelberg and Papahadjopoulos 1974), etc.). Yet, neither reception nor function is created by single molecules in an inert matrix. They arise as inseparable consequences of the receptor being a thermodynamic system which experiences state changes.

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\(^{11}\) In fact, any variation of a thermodynamic variable (temperature; dissolution of, e.g., ethanol; change of ion concentrations; mechanical extension; etc.) to which the system is susceptible

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