Axonal bleb recording

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Abstract: Patch-clamp recording requires direct accessibility of the cell membrane to patch pipettes and allows the investigation of ion channel properties and functions in specific cellular compartments. The cell body and relatively thick dendrites are the most accessible compartments of a neuron, due to their large diameters and therefore great membrane surface areas. However, axons are normally inaccessible to patch pipettes because of their thin structure; thus studies of axon physiology have long been hampered by the lack of axon recording methods. Recently, a new method of patch-clamp recording has been developed, enabling direct and tight-seal recording from cortical axons. These recordings are performed at the enlarged structure (axonal bleb) formed at the cut end of an axon after slicing procedures. This method has facilitated studies of the mechanisms underlying the generation and propagation of the main output signal, the action potential, and led to the finding that cortical neurons communicate not only in action potential-mediated digital mode but also in membrane potential-dependent analog mode.

Keywords: axon; patch-clamp recording; ion channel; action potential

1 Introduction

The integration of synaptic inputs takes place at the dendrites and cell body of a neuron, whereas the generation and propagation of the action potential, the main output signal, generally occurs at the axon. The roles of dendritic and somatic ion channels or neurotransmitter receptors in mediating and regulating synaptic integration have been extensively studied, and the feasibility of patch-clamp recording at these subcellular structures significantly boosted these studies. However, the properties of axonal ion channels and their contribution to the regulation of neuronal signaling remain largely unknown, due to the lack of direct

recording approaches. The thin structure of most axons in the central nervous system (CNS) prevents direct access of patch pipettes to the axonal membrane; therefore, it was impossible to probe the properties and functions of axonal ion channels and neurotransmitter receptors. A recently developed axonal recording method, known as the axonal bleb recording^[1-4], has been successfully used in studies on the physiology of CNS axons.

In the present review, we focus on the bleb recording method and findings derived from this new method. More detailed informations on axonal structure and function are available in recent reviews^[5-7].

2 Recording from special axonal structures

Before the use of microelectrodes in electrophysiological recordings, the pioneering studies on axon physiology were performed exclusively on squid giant axons because the thick electrodes could only be inserted into the largest

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cells^[8-11]. The large diameter (~ 0.5 mm) of these giant axons allowed the longitudinal insertion of electrodes (silver electrodes or capillary tubes filled with salt solution, ~ 0.1 mm in diameter) for voltage clamp experiments^[8-10]. In the early 1950s, Hodgkin and Huxley performed their Nobel prize-winning work on squid giant axons and discovered the basic ionic mechanisms of electrical excitability^[9,12]. Using the voltage-clamp technique, they measured the ionic membrane current during the action potential, and further revealed the voltage- and time-dependence of Na^+ and K^+ currents, providing insights into the properties of these currents. Using a set of empirical equations that fitted well with their experimental data, they accurately described these properties of Na^+ and K^+ currents and their roles in the generation of the action potential^[12]. The general applicability of the Hodgkin-Huxley model has been examined from different aspects in the field of neuroscience and proved to be the most successful quantitative computational model. This model thus has laid the foundation stone for modern neuroscience.

Establishment of the tight-seal patch-clamp technique by Neher and Sakmann in the 1970s allowed, for the first time, recording from single ion channels distributed in different subcellular compartments^[13]. However, this technique is not without limitations. One requirement is that the size of the target cellular structure should be larger than the tip size of the patch pipette. Therefore, most patch-clamp recordings in mammalian neurons have been made on the soma ($8\text{--}30$ μm in diameter), the largest compartment of a neuron, while studies on axons encountered technical difficulties due to their thin structure. To date, most successful axonal recordings have been applied only to special axonal terminals, such as the brainstem calyx of Held^[14-16] and hippocampal mossy fiber boutons (MFBs)^[17]. The axon initial segment (AIS, ~ 40 μm in length) in certain types of CNS neurons is large enough for patch recording. For example, the AIS of layer-5 pyramidal cells has a larger diameter ($1\text{--}2.5$ μm) than the rest of the axonal compartment ($0.1\text{--}1$ μm) and thus is accessible to patch pipettes^[18-21].

The calyx of Held is a giant axon terminal that was

named because of its flower-petal-like shape and the discoverer's name (Hans Held, a German anatomist, who described it in 1893). It is a large glutamatergic synapse from the axon of a globular bushy neuron in the ventral cochlear nucleus onto a principal neuron in the medial nucleus of the trapezoid body^[22]. Direct patch-clamp recordings from the calyx can be achieved in *in vitro* slices of the brainstem^[14,15]. This preparation has been increasingly used to investigate the basic presynaptic mechanisms underlying neurotransmission in the CNS, including studies of how changes in presynaptic excitability, action potential waveform or Ca^{2+} concentration contribute to short-term synaptic plasticity and the modulation of neurotransmitter release.

The hippocampal MFB is another giant axon terminal in the CNS that is accessible to patch pipettes^[17]. The mossy fibers are projection axons of the hippocampal granule cells in the dentate gyrus, sending signals to the CA3 region. The MFB is an enlarged axon terminal ($3\text{--}5$ μm in diameter) forming synaptic contacts onto CA3 neurons^[23-25]. In hippocampal slices maintained *in vitro*, it is possible to make simultaneous paired recording from the soma and the MFB of a given granule cell, or a presynaptic MFB and a postsynaptic CA3 pyramidal neuron. These recordings have been used to analyze the digital mode (mediated by action potentials) and analog mode (mediated by subthreshold membrane potential changes) of signal transmission^[26,27] and the distribution and gating properties of presynaptic Na^+ , K^+ and Ca^{2+} channels, as well as the modulation of short- and long-term synaptic plasticity. These experiments have made an important step toward understanding the mechanisms of neuronal signaling and transmission. Unlike the calyx of Held, recordings from MFBs are usually feasible in brain slices from relatively mature animals^[28].

Although recordings from these special axonal structures have revealed many important properties of the axon in signal conduction and transmission, studies of axon physiology have long been hampered by the inaccessibility of most axonal compartments (including the main axonal trunk and collaterals). Considering that the nervous system

has abundant cell types, most without patchable axonal structures (special structures are rare), a method that has general applicability for thin axons was urgently needed. Consequently, a new method^[1–4] has recently been developed for patch-clamp recording from the thin axons of both projection neurons and interneurons. We expect that this new method will facilitate studies on axon physiology.

3 Axonal bleb recording

The tip diameter of patch pipettes (1–2 μm) is usually much larger than most thin CNS axons (0.2–1 μm), making it almost impossible to obtain regular patch-clamp recordings. Interestingly, unlike the dendrites, axons usually form retraction blebs (or bulbs) at their cut ends in response to axotomy^[29–32]. During slicing procedures, axons that project from one brain region to other regions can be cut at some point on the surface of a brain slice. At the cut end, the ax-

onal membrane can reseal and form an enlarged bleb-like structure. These axonal blebs have a spherical shape (3–6 μm in diameter for pyramidal cells) and thus are suitable for patch-clamp recording. The tip size of recording pipettes is generally smaller than that of somatic electrodes. The open-tip resistance of pipettes typically ranges from 5 to 15 M Ω . Reducing the tip size can increase the rate of successful seal formation, but may lower the possibility of break-in and result in higher access resistance and sometimes membrane reseal.

Different recording configurations can be used on the axonal blebs (Fig. 1), including cell-attached, whole-cell, outside-out patch and giant outside-out patch recordings^[1–4]. For simultaneous recordings from the soma and axon of layer-5 pyramidal neurons in the prefrontal cortex^[1–4], whole-cell recording at the soma is first established with a patch pipette filled with an internal solution containing

Fig. 1. Patch clamp recording from an axonal bleb. **A:** DAB staining of two recorded pyramidal neurons. The green pipette indicates a somatic electrode filled with fluorescent dye-containing internal solution. The white pipette indicates an axonal electrode filled with standard internal solution (no dye added). Red circles indicate two axonal blebs. Scale bar, 50 μm . **B:** Schematic drawing of dual recording from the soma and the axonal bleb, recording from regular patches and giant outside-out patches (isolated blebs). **C:** Schematic drawing showing the procedures to obtain isolated bleb recording. A sharp electrode is first inserted into the cortical slice at the border of layer 6 and the white matter (top). Then, the sharp electrode is moved along the border to disconnect neuronal processes including axons (middle). Isolated bleb recording can be made from blebs located at the white-matter side of the cut (bottom). **D:** An example of isolated bleb recording (unpublished). Top, a differential interference contrast (DIC) image showing the patch pipette and the slice surface with a cut. Bottom, the fluorescent image of the same recording. Scale bar, 10 μm .

a fluorescent dye (normally 100 $\mu\text{mol/L}$ Alexa Fluor 488 because of its low phototoxicity; Fig. 1A, B). After 3–5 min of dye loading, the whole structure of the recorded neuron can be traced and the axonal bleb observed under a fluorescence microscope. Because pyramidal neurons normally send their apical dendrite toward the superficial layers and axons toward the deep layers and white matter, these axons are relatively parallel to each other and thus form blebs in the same side of the slice. Switching back and forth between fluorescent and differential interference contrast (DIC) images, the axonal bleb that belongs to the recorded cell can be identified. Since the bleb is located at the surface of the slice, clear images of this structure can be obtained and tight-seal patch recording easily done. One note is that the solution in the patch pipette for axonal bleb recording should not be the same as that in the somatic pipette, because the positive pressure in the pipette can cause a cloud of fluorescent dye in the recording field and thus prevent the identification of blebs. Normally, we choose either a standard internal solution (without dye; Fig. 1A, B) or one containing a different fluorescent dye (e.g., Alexa Fluor 594) to fill the axonal patch pipette. One can also tip-fill the pipette with standard internal solution and then back-fill with fluorescent solution to prevent background staining during the patching procedures. Once dual whole-cell recording is achieved, the soma or the axon can be stimulated by current injection to determine whether the two recordings are from the same cell. Stimulation at one electrode should evoke either passive or active membrane responses at both recording sites.

Regular patches can also be excised from the axonal blebs using conventional procedures (Fig. 1B). In fact, in some experiments one may want to avoid space clamp problems and influences from the somatodendritic compartments, so isolated axonal blebs (Fig. 1B–D) are ideal preparations^[1]. The isolated blebs are giant outside-out axonal patches that can be obtained by making a cut at the main axon (moving a sharp glass pipette that has been inserted into the target location) to disconnect the bleb from the main axon (Fig. 1C)^[1]. Since the recording location (distance from the bleb to the soma) is important for data

analysis, the cell can be loaded with biocytin for later DAB staining and measurement of the axon length (Fig. 1A). If applicable, z-stack fluorescent images can also be obtained during or after recording for three-dimensional reconstruction of the axon (Fig. 1D).

Axonal bleb recording has some advantages over the previous patch recordings formed at the AIS. First, the size of blebs is folds-larger than the axon, overcoming the greatest limitation – small diameter – for applying patch clamp recording to thin CNS axons. The axonal bleb recording method can be applied to the axons of various types of neurons in the CNS, including interneurons that have extremely thin axons. Interneurons are local circuit cells whose axons only innervate nearby neurons and have no predictable projection direction, however axonal recordings can still be obtained from them using GFP-expressing interneurons (unpublished data). Second, axonal blebs can form at various distances from the soma (e.g. axon hillock, initial segment, unmyelinated and myelinated compartments). These blebs are ideal preparations for studies on the properties of the axonal membrane, such as the distribution of ion channels and neurotransmitter receptors and their role in shaping neuronal signaling. Third, because axonal blebs always form at the surface of the brain slice, it is convenient to perform simultaneous somatic and axonal bleb recording. Patching the axonal blebs does not cause large movements and distortion of the brain tissue.

However, one should note that the axonal bleb is an injury site, and reorganization of the intracellular cytoskeleton participates in the formation of this structure. Since membrane proteins, including ion channels, are anchored to the intracellular cytoskeleton, the disorganization of microtubules and microfilaments may set ion channels free and ready to be drawn into the tip of a patch pipette^[19,30]. Indeed, recordings from patches excised from axonal blebs reveal a much higher current density than those obtained from the soma^[1], consistent with immunostaining results and the finding of significantly larger peak Na^+ currents in cell-attached patches from intact AIS than those at the soma after depolymerizing actin filaments^[19]. Recordings from blebs formed at the AIS also revealed that axonal

action potentials have higher peak amplitudes and faster rising phases than somatic ones^[33], indicating a high density of axonal Na⁺ channels. Therefore, the patch recording from blebs can be used to estimate the density of axonal channels when special caution is used. Whether axotomy has an effect on the trafficking of these channels and other membrane proteins at the bleb remains to be further examined. One should also note that the resealed terminal bleb can cause an increase in the length constant of the axonal cable^[3], which should also be considered when interpreting the passive properties of the axonal membrane.

4 Applications of axonal bleb recording

The development of tight-seal axonal bleb recording was inspired by extracellular loose-patch recordings from the axons of cerebellar Purkinje cells^[34,35]. Because of the surrounding myelin sheath, the membrane of axonal blebs was not accessible to patch pipettes and thus only extracellular spikes were detectable. Using this axonal loose patch technique, two research groups^[34,35] found that somatic simple spikes are faithfully transmitted along the axons of Purkinje cells, whereas the complex spike waveform cannot be faithfully propagated to distal axonal compartments. Unlike cerebellar Purkinje cells, layer-5 pyramidal neurons in the prefrontal cortex emit axons that have an initial unmyelinated segment up to 400 μm long^[2,3], therefore patch pipettes can record directly from the axonal membrane.

The success of tight-seal axonal bleb recording led

to the surprising finding^[3] that, during cortical network activity (the Up states during slow-wave oscillations), subthreshold somatic depolarization spreads along the pyramidal neuron axons with a long length constant of 417 μm , meaning that steady somatic depolarization is reduced to 37% of its peak amplitude when arriving at this axonal location. Further morphological observations revealed that many presynaptic boutons are distributed within this length constant, suggesting that subthreshold membrane potential changes in the soma may regulate synaptic transmission at the axonal terminals^[3]. Indeed, dual recording from synaptically connected neighboring pyramidal neurons showed that the average synaptic strength (peak amplitude of spike-triggered excitatory postsynaptic potentials) depends on the level of the somatic membrane potential, indicating the existence of analog signaling in the CNS^[3]. Axonal bleb recording also revealed a selective distribution of K_v1 channels on the axons of pyramidal neurons (Fig. 2)^[4]. These K⁺ channels mediate the D-current^[36] that activates rapidly but inactivates slowly (with a time constant of several seconds; Fig. 2A, B). Subthreshold depolarization can cause accumulated inactivation of these channels and result in broadening of axonal action potentials, leading to an increase in transmitter release^[4,37]. Therefore, these K_v1 channels mediate the membrane potential-dependent analog signaling between cortical neurons. In addition, recent findings demonstrate that this analog communication also occurs at synaptic connections from pyramidal neurons

Fig. 2. Whole-cell recording from an axonal bleb revealed the existence of the D-current at the axon (adapted from Shu *et al.*, Proc Natl Acad Sci U S A, 2007^[4]). A: Axonal D-currents activate rapidly but inactivate slowly. B: Activation (black) and inactivation (red) curves of axonal D-currents.

to inhibitory interneurons, and regulates the timing and strength of recurrent inhibition received by neighboring pyramidal neurons^[38]. As expected, the axonal D-current plays an important role in mediating the presynaptic membrane potential-dependent modulation of recurrent inhibition, contributing to the maintenance of cortical excitation-inhibition balance^[38].

The digital form of neuronal signaling is the generation of all-or-none action potentials, which are normally initiated at the AIS followed by back-propagation toward the somatodendritic compartments and forward-propagation toward the axon terminals. Whether or not to generate an action potential is the decision-making process of a given neuron after synaptic integration, therefore, where and how the action potential is initiated are fundamental questions in neuroscience. Dual somatic and axonal whole-cell recording from neocortical pyramidal neurons revealed that the action potential initiation site is located at the distal end of the AIS, about 46 μm from the soma^[2]. If the recording is performed at an axonal bleb 96 μm from the soma, no time difference occurs between the peaks of somatic and axonal action potentials evoked by somatic current injection, indicating that the initiation site is located at ~ 48 μm from the soma. This estimate was based on the assumption that the velocities of action potential back-propagation and forward-propagation are equal. In fact, under physiological conditions, back-propagation from the initiation site to the soma is slower than forward-propagation along the axon. Considering this velocity difference, we speculated that the initiation of action potentials occurs at a site less than 48 μm from the soma. This finding is consistent with the previous electrophysiological^[20,21] and recent voltage-sensitive dye imaging results^[39,40] in neocortical layer 5 pyramidal neurons. Using dual somatic and axonal bleb recordings in hippocampal CA3 pyramidal neurons, a recent study also revealed that, in response to somatic stimulation, action potentials are initiated in the proximal region of the axon and subsequently back-propagated into the dendrites^[41]. Similar results have been obtained in neurons of the subthalamic nucleus^[42] and granule cells in the dentate gyrus^[43]. Dual recordings from the soma and axon also

indicated that cortical and subcortical axons faithfully propagate action potentials^[2,42,44], even at high firing frequencies (up to 400 Hz)^[40] and during epileptiform activity^[2].

Why does the AIS have the lowest threshold for action potential initiation? The small local capacitance of the AIS allows rapid changes in local membrane potential and action potential initiation in response to a small amount of Na^+ influx^[6,21]. Previous immunostaining and theoretical studies suggested that a high density of Na^+ channels at the AIS determines it as the initiation site^[18,19,45–49]. Since the total Na^+ channel densities are similar at the proximal and distal AISs^[18,19,46], why do action potentials prefer to initiate at the distal end? Consistent with previous reports, recordings from outside-out patches excised from axonal blebs of cortical pyramidal neurons indicate that the AIS has the highest Na^+ channel density, ~ 34 -fold greater than the soma (Fig. 3A, B)^[1]. Isolated axonal bleb recording also showed that Na^+ current density at the axon is much higher than that at the soma (Fig. 3C, D). Immunostaining of channel subtypes revealed that the high-threshold $\text{Na}_v1.2$ channels preferentially accumulate at the proximal AIS and the low-threshold $\text{Na}_v1.6$ channels at the distal AIS^[1]. In agreement with these results, patch clamp recordings from axonal blebs formed at different distances from the soma indicated that the activation threshold of Na^+ channels progressively decreases along the length of the AIS, with that at the distal end and axon regions beyond the AIS having the lowest threshold, reflecting the distribution of low-threshold $\text{Na}_v1.6$ channels^[1]. Further experimental and modeling results showed that the distal-AIS $\text{Na}_v1.6$ determines the initiation site, whereas the proximal-AIS $\text{Na}_v1.2$ promotes the back-propagation of action potentials to the soma in cortical pyramidal neurons^[1]. Schmidt-Hieber and Bischofberger^[50] performed outside-out patch recordings from somata and the axonal blebs in hippocampal granule cells and consistently found that the Na^+ current density at the proximal axonal compartment is about 5 times higher than that in the soma. Further experiments showed that axonal Na^+ currents activate and inactivate at relatively lower membrane potentials than somatic currents^[50].

Fig. 3. Na^+ current density at the axon is higher than that at the soma. **A:** Recording from a somatic patch, an axonal patch excised from near the soma (distance (d) = 39 μm), and an axonal bleb (d = 265 μm). The axon initial segment (AIS) has the largest Na^+ current. **B:** Peak Na^+ currents recorded at the soma and axonal blebs at varying distances from the soma. **C** and **D:** Similar results were obtained with recordings from a somatic nucleated patch and an isolated axonal bleb. Adapted from Hu *et al.*, Nat Neurosci 2009^[1].

In addition to voltage-gated ion channels, axons also express many types of neurotransmitter receptors^[51–55]. For example, by combining local drug application with axonal bleb recording, Christie and Jahr demonstrated that GABA_A but not NMDA receptors are expressed in layer V pyramidal neurons of the visual cortex^[56]. Similarly, Sasaki *et al.* showed that CA3 pyramidal neuronal axons express no NMDA receptors, but have AMPA receptors, and activation of these receptors results in depolarization of the axon and broadening of axonal action potentials^[57].

Taken together, axonal bleb recording can be used to probe the roles of axonal ion channels and neurotransmitter

receptors in shaping neuronal signaling. Axonal bleb recording from different neuronal cell types in the CNS will provide new insights into the properties and functions of axons.

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