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THE „PATCH-CLAMP” TECHNIQUE AND ITS APPLICATION IN INVESTIGATIONS OF THE PROPERTIES OF HUMAN T LYMPHOCYTE POTASSIUM CHANNELS

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Abstract: The first part of this review presents a historical outline on the development of experimental methods in electrophysiology starting from the first experiments performed in the 1920s and ending with the „patch-clamp” technique. Recording configurations of the „patch-clamp” technique are briefly reviewed in the second section. The areas of application of the configurations are shown.

The last section contains a short review on the available data of “patch-clamp” studies on the expression and properties of potassium channels in human T lymphocytes (TL). Problems that require further investigation are briefly presented.

Key Words: Electrophysiology, Voltage-Clamp, Patch-Clamp, Potassium Channel, T Lymphocyte.

ELECTROPHYSIOLOGY- A HISTORICAL OUTLINE

“Patch-clamp” is a modern electrophysiological technique widely used for studying ionic currents flowing through single channels and whole cell membranes. However in order to present the experimental and theoretical basis that led to the current development of the “patch-clamp”, it is necessary to go

Abbreviations: TL - human T lymphocytes, TEA - tetraethylammonium, ChTX - charybdotoxin, NTX - noxiustoxin, MgTX - margatoxin, KTX – kaliotoxin, gK – single-channel potassium conductance in picosiemens, $V_{1/2}$ – activation midpoint, K_d – dissociation constant, cAMP-cyclic adenosine 3',5'-monophosphate, Ro-20-1724 – 4-(3-butoxy-4-methoxy-benzyl) imidazolidin-2-one.

back to the beginning of the 20-th century. On the basis of the first experiments on the role of ions in cell physiology performed by Ringer in the 1880's and the studies on ionic equilibrium potentials carried out by Nernst (1888), in 1902 Julius Bernstein formulated a hypothesis that the resting cell membrane is selectively permeable for potassium ions [1-3]. This "potassium electrode hypothesis" predicts that the resting potential is equal to the equilibrium potential for potassium ions [2,3]. Moreover, Bernstein proposed that upon the excitation process the membrane selectivity for potassium ions is transiently lost due to a membrane „breakdown" which allows other ions to diffuse across the cell membrane and depolarise it. At around the same time (1905) Herrman proposed a model in which the axon is assumed to have a cylindrical conducting core surrounded by a membrane with a high electrical resistance and capacitance, such as occurs in the case of a submarine cable [4,5]. A depolarisation of one point of the cable can be propagated as an electrical current flowing to regions not yet depolarised [4,5].

Experimental studies on membrane electrical properties began in 1923, when Cole and Curtis started to measure membrane resistance and capacitance using a Wheatstone bridge. Their results showed that each cell has a high-conductance cytoplasm (30-60% of the conductance of the bathing saline) surrounded by a membrane of low conductance and an electrical capacitance of $1 \mu\text{F}/\text{cm}^2$ [1].

In 1936 Young re-discovered the squid giant axon [6]. The re-discovery provided a valuable tool for further studies on the electrical properties of cell membranes and action potential generation. In 1937 Hodgkin showed that the depolarisation wave upon generation of action potentials in nerve cells is propagated in a form of an electrical current [7]. This results generally confirmed that the Herrman's „cable hypothesis" was correct. In 1939 Cole and Curtis measured the conductance changes upon firing the action potential in the squid giant axon using a Wheatstone bridge [8]. That same year Hodgkin and his co-worker Huxley for the first time measured the full action potential of the squid giant axon using an intracellular microelectrode [9]. Their results were confirmed in further studies performed independently by Curtis and Cole (1940, 1942) [10-11]. The obtained results showed that the resting membrane potential in an axon was close to the equilibrium potential for potassium ions as the "potassium electrode hypothesis" had predicted, although it was always more positive due to a slight permeability of the membrane to other ions (especially chloride). Upon excitation membrane conductance is increased about 40-fold (but is still less than one millionth of the surrounding medium) and the capacitance is only increased by 2% [8-11]. These observations argued against the hypothesis of membrane "breakdown" during the action potential firing. Moreover, the increase in membrane conductance was accompanied by a transient membrane depolarisation to ca. +40 mV (the so-called "overshoot" of the membrane potential) [9-11]. The nature of this "overshoot" was not explained in detail and further studies were interrupted by World War II.

Studies on action potentials were continued after the end of the war by Hodgkin, Huxley and Katz. Their experiments carried out using an intracellular microelectrode showed that the depolarisation phase during an action potential generation was due to a transiently increased membrane permeability for sodium ions [12]. Because of a large transmembrane electrochemical gradient for sodium, the increase in membrane permeability for sodium causes a massive influx of sodium ions, which depolarises the cell membrane towards the equilibrium potential for sodium ions (ca. +60 mV). This „sodium hypothesis” first presented by Hodgkin and Katz (1949) provides a good explanation for the depolarisation phase of action potentials in many excitable systems [12].

For further studies on ionic currents underlying action potentials, a new technique was developed by Marmont (1949) [13], Cole (1949) [14], and then by Hodgkin, Huxley and Katz (1949-52) [15-18]. The “voltage-clamp” technique enabled direct measurements of ionic currents flowing through the cell membrane under a controlled (“clamped”) voltage.

The classical “voltage-clamp” setup contains two microelectrodes ([15-18] for review see 1). The membrane potential is measured by the voltage microelectrode. During an experiment the voltage stimulus is applied to the amplifier input and compared to the membrane voltage. The amplifier works in a negative feedback loop. Therefore, if the membrane voltage is different from the applied stimulus the amplifier very quickly compensates the difference by applying the error signal, which is the current applied to the cell by the current microelectrode. The current quickly changes the membrane potential to the level of voltage stimulus and keeps the value of membrane potential on this level. Thus, the current recorded in the “voltage-clamp” method is actually the current applied as an error signal needed to keep the membrane potential on the level of voltage stimulus. If the membrane voltage is stabilised, this current must be equal in size and opposite in sign to currents flowing through the cell membrane. An electrical circuit is closed by applying an additional grounded current microelectrode.

An equivalent electric circuit of the membrane of the axon contains voltage-activated sodium and potassium conductance, voltage-independent leak conductance and capacitance [1,15-18]. Currents recorded upon application of a given voltage stimulus are: sodium and potassium currents, unspecific leak current and capacitance current. Both the leak and capacitance currents are subtracted from the final record. Thus, ionic currents recorded from the axon by applying the „voltage-clamp” method contained a transient inward sodium current followed by a sustained outward potassium current [1,15-18]. After having applied an extracellular solution containing low sodium concentration, only a sustained outward potassium current was recorded, and the sodium current was estimated as the difference current [1,15-18].

On the basis of the obtained data, Hodgkin and Huxley also built a model that describes ionic permeability changes upon firing action potentials in excitable

cells - the “Hodgkin-Huxley” Model. This model applies the sodium and potassium membrane conductance as a quantitative measure of membrane permeability for sodium and potassium ions, respectively [19]. The conductance is a function of both membrane voltage and time. The model is still a valuable tool for studying action potentials in excitable cells. It is worth noting that the „Hodgkin-Huxley Model” correctly described the gating of single channels at time when nothing was known about the nature of single-channel currents.

The next important step in the development of experimental methods in electrophysiology was the recording of ionic currents flowing through single channels. It was made possible after the development the “patch-clamp” technique.

“PATCH-CLAMP” TECHNIQUE

Single-channel currents from a membrane patch of denervated frog muscle fibres were recorded for the first time by Sakmann and Neher (1976) [20,21]. They used a glass pipette with a narrow tip (3-5 μm in diameter) pressed against the surface of the fibre. After being pressed against the cell surface, the pipette tip formed a high-resistant (ca. 50 $\text{M}\Omega$) electrical contact - a “seal” with the cell membrane. Because of the small size of membrane area covered by the pipette tip, currents from only a small piece -“a patch”- of the membrane containing single ionic channels were recorded. The disadvantage of this technique was a relatively high level of background noise caused primarily by the “seal” between the pipette tip and the cell membrane.

A significant improvement of the “patch-clamp” technique came with the discovery of a “giga-seal” by Sigworth and Neher (1980) [22]. They demonstrated that upon a transient application of a gentle suction to the pipette pressed against the cell surface, a tight and stable mechanical contact with a resistance of 10-100 $\text{G}\Omega$ (“giga-seal”) is formed. There are significant differences between a “giga-seal” and a megaohm seal. In the case of a “giga-seal” the membrane patch forms an Ω -shaped protrusion inside the pipette tip. The distance between the surfaces was calculated to be of the order of 1 \AA , compared to the 20-50 \AA estimated for megaohm seals [22]. The “giga-seal” formation probably represents the same phenomenon that occurs during the transfer of insoluble surface monolayers on glass substrates (Langmuir, 1938) [23].

The improved “patch-clamp” technique with the application of a “giga-seal” has several important advantages. First, the high seal resistance reduced the level of background noise by an order of magnitude [24]. It significantly improved not only the quality of the records, but also the time resolution of the method [24]. Moreover, thanks to the reduction of background noise upon “giga-seal” formation the „patch-clamp” technique uses only one microelectrode both for

controlling the patch membrane potential and for recording membrane currents [22, 24]. Finally, “giga-seals” are mechanically very stable (up to several hours). “Giga-seal” formation makes it possible to record currents not only from isolated patches of membranes on intact cells, but also from whole cell membranes and from excised membrane patches (see below).

“Patch-clamp” recording configurations

“Cell attached” configuration

The improved “patch-clamp” technique and its configurations were described in detail by Hamill, Marty, Neher, Sakmann and Sigworth (1981) and then by Sakmann and Neher (1983) [24-25].

The basic “cell attached” configuration is established after having obtained a “giga-seal” between the pipette tip and the cell membrane. Under “cell attached” conditions, the currents from a membrane patch on an intact cell are recorded.

The ability to record single-channel currents from the examined patch is limited by the background noise that contains several components. The first one is „Johnson’s noise” produced by the electrical contact- „seal” between the pipette tip and the membrane patch [24]. The second one is „shot noise” coming from leak currents flowing through the examined membrane patch [24]. Another source of noise is the recording pipette that forms an RC circuit by itself, producing large capacitance currents that have to be compensated electronically [24]. Finally, the electronic circuit is also a source of noise [24]. Because of the background noise, single-channel recording is not suitable for studying ionic channels with conductance of less than a few pS. In such cases, both the single-channel conductance and the number of channels can be estimated from the whole-cell recording by analysing fluctuations of the currents [26].

„Inside-out” configuration

The membrane patch can be excised from the rest of the cell body by withdrawing the recording pipette after having obtained the „cell attached” configuration. When the excision process occurs in a solution containing calcium ions at physiological concentrations, a tight vesicle is formed on the pipette tip [24]. The vesicle has two membranes: an inner membrane, which is the actual patch to be studied and an outer membrane, which is formed on the pipette tip when withdrawing the pipette from the cell surface. In order to be able to record ionic currents from the examined patch, the outer membrane has to be disrupted. This can be done by withdrawing the recording pipette from the bathing solution for a short time. Alternatively, a calcium-free bathing solution can be used. This makes the outer membrane of the vesicle leaky [24].

In the „inside-out” configuration the intracellular side of the excised patch is exposed to the bathing solution. The „inside-out” configuration is especially

useful for studying ionic channels that are activated by intracellular factors, such as calcium.

"Whole-cell" recording

Another version of the "patch-clamp" technique is the "whole-cell" recording method. This configuration can be obtained by disrupting the membrane patch under the pipette tip. This can be done by applying a pipette solution containing 150 mM KCl and a low calcium concentration (less than 1 μM), by applying a gentle suction or by a voltage impulse (of ca. 200 mV) applied to the recording microelectrode after having obtained the "cell attached" conditions. The disruption of the patch does not damage the seal between the pipette rim and the cell membrane [24].

Under "whole-cell" conditions a direct contact between the recording pipette and the interior of the examined cell is established. In this case, the membrane potential on the whole cell is set by the pipette potential. In other words, the "whole-cell patch-clamp" recording is comparable to the single-microelectrode "voltage-clamp" method. However, the ability to clamp the voltage on the membrane area in the "whole-cell" recording is limited to small cells (less than 30 μm . in diameter) and ionic currents that are not larger than a few nA [24]. Nevertheless, the "whole-cell" recording plays a vital role when studying ionic currents in small cells with a diameter of up to 10 μm , such as T lymphocytes (see below), because these cells are not accessible using the standard two-microelectrode voltage-clamp method [24]. Moreover, single-channel resolution can also be achieved in the "whole-cell" configuration [24]. Finally, the „whole-cell” recording is necessary for studying ionic channels with a low conductance (less than a few pS), because such small currents cannot be recorded properly by applying the single-channel recording (see above). In such cases, the „whole-cell” currents are recorded and both the single-channel conductance and the approximate number of channels can be estimated by analysing the fluctuations of the currents [26].

„Outside-out” configuration

The „outside-out” configuration can be obtained starting from the „whole-cell” configuration by withdrawing the recording pipette from the cell. As mentioned above, a small membrane patch is then formed on the pipette tip [24]. The formed patch containing single ionic channels is exposed to the bathing solution with its extracellular side.

The „outside-out” recording is especially useful when studying channels activated by extracellular factors, such as neurotransmitters. In such cases „outside-out” recording of single-channel currents often follows the „whole-cell” recording.

„PATCH-CLAMP” STUDIES ON POTASSIUM CHANNELS IN HUMAN T LYMPHOCYTES

Human T lymphocytes (TL) are small (diameter of 6-8 μm) and electrically non-excitable cells. Nevertheless, studies performed since the mid 1980's with the application of the „patch-clamp” technique provided evidence that many different types of ionic channels exist in human TL, including several types of potassium channels [27-29]. It is also known that potassium channels play an important role in TL cell function. The channel activity contributes significantly to cell processes such as: setting the resting membrane potential, cell mitogenesis, volume regulation [27-29].

The prevalent and best characterised ionic channels in TL are voltage-gated potassium channels termed as „n” type or, according to the current gene nomenclature, as Kv1.3. These channels were first described by Matteson and Deutsch (1984) [30] and then characterised in a further detail by several other groups of investigators [31-35, reviewed in 36]. The channel activity is induced upon membrane depolarisation to voltages more positive than -40 mV [30-36]. Activation kinetics are rapid and strongly voltage-dependent, whereas inactivation is much slower and shows no significant voltage dependence [31-36]. The channel expression is greatly diversified and is significantly increased upon mitogenic stimulation. The channels are blocked by a variety of agents including classical potassium channels blockers (e.g. tetraethylammonium - TEA), calcium channel blockers (e.g. nifedipine) and „maxi” calcium-activated potassium channel inhibitor Charybdotoxin – ChTX. Well-known specific blockers of Kv1.3 channels are: Noxiustoxin (NTX), Margatoxin (MgTX) and Kaliotoxin (KTX) (see Table 1). The channel was cloned from rat and mouse brain, mouse, rat and human TL [36]. The channel characteristics are shown briefly in Table 1.

The channel activity is modulated by numerous physiological factors such as temperature, pH, extracellular potassium ions and divalent cations [36]. The modulation of channel activity by intracellular second messengers is still incompletely investigated. For example, it is still controversial whether or not intracellular calcium applied at physiological concentrations modulates the channel activity in TL [37]. The modulatory effect of intracellular cAMP is also controversial [38-39]. In this area there is a substantial discrepancy between the data obtained for normal TL and the Jurkat T cell line [39]. Moreover, it is still unknown whether the channel activity is modulated by lipid second messengers, which are known as potent channel modulators in many excitable cells and which significantly influence the TL cell function [37]. The author of this paper is contributing to a research project in which the modulatory effects of lipid second messengers on the channel activity are studied.

A second type of voltage-gated channels in TL are channels of yet unknown type (see Table 1). These channels were preliminarily described by Lee and co-

workers (1992), who demonstrated that upon application of 50 nM ChTX to completely block Kv1.3 channels a small fraction of the current with an apparently slower inactivation kinetics was still observed [33]. The effect of ChTX was reversible. The ChTX-insensitive potassium conductance was voltage-dependent, but it was activated at more positive membrane potentials (see Table 1) [33,40]. The currents were blocked by TEA and nifedipine [33,40]. The channel contribution to the total whole-cell potassium current in TL varies between 20% and 60% with a mean value of 39% [39]. The channel properties are still incompletely characterised and require further investigation.

Tab. 1. Characteristics of human T lymphocyte potassium channels

Channel type	Number of channels per cell	gK [pS]	Activation mechanism	Blockers
Kv1.3	~ 300 - resting TL ~ 600 - activated TL	10	Membrane depolarisation $V_{1/2} = -40$ mV	TEA, nifedipine, ChTX, NTX, MgTX, KTX
Kv ?	?	10	Membrane depolarisation $V_{1/2} = +50$ mV	TEA, nifedipine
K _{Ca} "intermediate"	~ 10 - resting TL ~ 500 - activated TL	11	Intracellular calcium $K_d = 425$ nM	TEA, ChTX, clotrimazole
K _{Ca} "mini"	~ 400 only in Jurkat T cell line	4-7	Intracellular calcium $K_d = 400$ nM	TEA, apamin, scyllatoxin
K(?)	?	10	Membrane stretch (?) Intracellular cAMP	TEA

Two types of calcium-activated potassium channels are also expressed in TL (Table 1). The first type are recently cloned voltage-independent, intermediate-conductance ChTX-sensitive channels termed as IKCa1 first described by Grissmer and co-workers (1993) [41] and then by other authors [35, 42-46]. The channel expression is abundant only in activated TL, where the currents were first recorded in the "whole-cell" mode with the intracellular (pipette) solution containing 1 μ M. of free calcium [41]. Under such conditions, calcium-activated currents appeared additionally to the Kv1.3 currents [41]. The currents

were not gated by the membrane potential [41]. Studies performed with an application of the “inside-out” configuration provided further evidence that the channel activity was strictly calcium-dependent within the range from 38 nM to 2.3 μ M. [42]. On the other hand, the channel expression is negligible in resting cells (see Table 1). The activity of this channel is mediated by calmodulin [44-45]. The channels are resistant to NTX, MgTX and KTX and are inhibited by a specific blocker clotrimazole (see Table 1). The second channel type expressed abundantly only in Jurkat T cell line, first described by Grissmer and co-workers (1992) [47] and then by Hanselmann and Grissmer [48] and cloned very recently [49], these are also voltage-independent channels with much lower conductance, resistant to ChTX and sensitive to apamin and scyllatoxin (see Table 1).

More studies are required to elucidate mechanisms of channel modulation by physiologically relevant factors such as temperature, pH and intracellular second messengers.

Finally, voltage- and calcium-independent potassium channels of yet unknown type are also present in human TL. These channels were first observed by Lee and co-workers (1992) as non-inactivating voltage- and calcium- independent potassium channels that are spontaneously and transiently (for not longer than a few minutes) activated immediately or soon after “giga-seal” formation [33]. The channels may therefore be activated by a membrane stretch. Further experiments performed by Oleson and co-workers (1996) showed that the channel activity is significantly and transiently increased when intracellular cAMP level is raised, probably due to the direct action of cAMP molecules on the channels [50]. The channels are insensitive to ChTX [50] (see Table 1). The channel characteristics are still incomplete.

In conclusion: the substantial progress that has occurred in the area of developing experimental methods applied in electrophysiology in the 20-th century provided the basis for the recent development of the “patch-clamp” technique. This technique is a valuable tool for studying ionic channels in TL both on the macroscopic and microscopic level. Much data concerning TL potassium channel properties is already available. However, many problems concerning the activity of potassium channels in TL still need more investigation.

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