SimNerv

Recordings of compound action potentials (CAP) of an isolated frog nerve in a virtual laboratory

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Aims

This practical class aims to provide a thorough understanding of the clinically relevant phenomena of extracellular recordings of compound action potentials (CAPs), based on your textbook-derived knowledge of intracellular action potentials recordings and of the ion channels and currents involved (as demonstrated by SimNeuron). Furthermore, you should get to know that your results significantly depend on the experimental edge conditions and on the recording techniques.

Clinical significance

A variety of electrophysiological measurements are used in clinical practice. The physician only performs extracellular recordings (such as in recordings of EMG, ECG and EEG). The underlying physiological processes are, in this way, only indirectly recognizable, and, importantly, the limitations and effects of the measurement technology always need to be taken into account.

Required background knowledge

The basic principles of nervous excitation; the "passive" properties of the membrane (membrane as a resistor-capacitor element); potential-dependent Na^+ and K^+ channels (their activation and inactivation); measurement and time-course of these currents (voltage clamp); membrane depolarization and initiation of an action potential; cathodic and anodic stimulus effects; the effects of stimulus amplitude and duration; extracellular and intracellular measurement of the action potential; differential amplifier; origin and shape of the compound action potential (CAP); the refractory time.

Preliminary excercises

You need to answer the following questions before starting the practical class as they deal with facts that are relevant to the practical class.

In the following, referring to Na^+ and K^+ currents, generally means those currents that are contributing to the action potential.

Q: 1. In humans, how large are the intra- and extracellular concentrations of the major ions: Na^+, K^+, Ca^{++}, Cl^-? Specify the approximate Nernst potentials!

Q: 2. Draw the profile of a measured intracellular action potential and the corresponding changes in the membrane permeability of Na^+ and of K^+. In your drawing, indicate approximate time and voltage scale. Also indicate the position of the equilibrium potentials for Na^+ and K^+.

Q: 3. What different states of the Na^+ channel can be recognised? How are these states dependent on changes in membrane potential and time? What is meant by "inactivation" and by "refractory behaviour"?

Q: 4. Draw and explain the time course of Na^+ and K^+ currents as measured in a voltage-clamp experiment, assuming a step-wise voltage change from the resting potential to about 0 mV.

Q: 5. What is meant by "driving force"? How does the "driving force" change with increasing depolarization for (i) for the Na^+ currents and (ii) for the K^+ currents?

Q: 6. How does the membrane potential change in a purely passive response to a sub-threshold rectangular current pulse that is significantly shorter than the membrane time constant? (Drawing: current pulse with the membrane potential change).

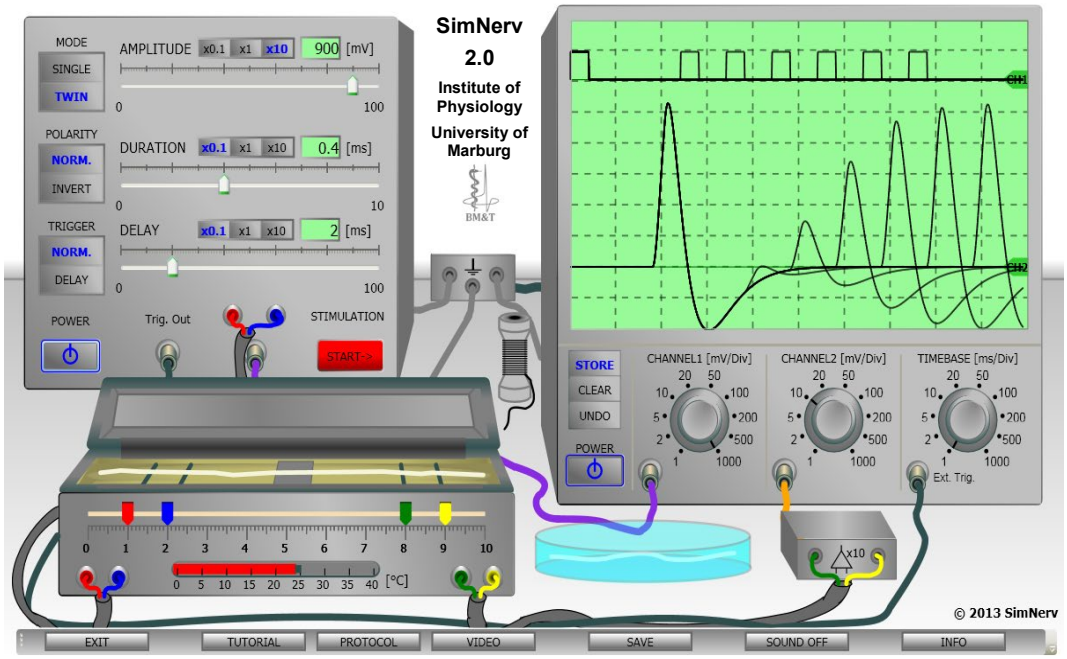
Q: 7. Explain the biphasic form of the extracellularly recorded action potential! Under what conditions does a monophasic action potential occur? How does the distance between the two recording electrodes affect the shape of the AP?

Q: 8. Under which stimulating electrode would the action potential be triggered by extracellular stimulation with a short current pulse (for example, 0.1 ms)? What would happen at the other stimulating electrode?

I. The Virtual Laboratory:

You will be working in a "virtual laboratory" in pseudo-3D display on the computer screen (see Figure) that includes all necessary devices with all major cable connections to perform experiments with extracellular recordings of compound action potentials from isolated nerve preparations: the nerve chamber, the stimulus device and the oscilloscope.

a) A brief overview on the experimental set-up



In a small bowl, you will find two ready-prepared, **isolated nerves**. Open the **measurement chamber** with click on the lid and place one of the nerves on the electrodes and then close the lid. You can now start your recordings. From here on, the performance of experiments in the virtual laboratory does not differ fundamentally from that in a real laboratory. You must pay the same degree of attention to the correct adjustment of equipment and to conduct your experiments systematically. You must document your experimental procedures and your recordings (data tables, graphs), and properly interpret the results.

In the lab, you will also find a spool of thread, with which you loop around and tie the nerve at two separate places and thus block the action potential conduction. Unlike in the real-life experiment, these blockages can be removed again without causing any lasting damage to the nerves.

The **stimulator** delivers rectangular voltage pulses that go through two cables to the two stimulating electrodes in the measuring chamber and in parallel are registered at the oscilloscope via a coaxial cable. Another cable connection ensures exact external triggering of the oscilloscope by the stimulator. The amplitude and duration of the stimulus pulses can be selected. Additionally, a time delay can be set for delivery of a double pulse (MODE switch to "Twin"); this sets the time between the start of the first and the start of the second Pulse. For single pulses a time delay can be set for between the trigger pulse and stimulation pulse but only with the trigger mode "delay" (TRIG switch). The current direction can be set using the "POLARITY" switch, with "INVERT" allow immediate switching of the polarity of the stimulating electrodes.

The **oscilloscope** is presented in a highly simplified form with the stimulus presented on channel 1. On channel 2, with an upstream differential amplifier, the CAP is presented. The difference between the measured potentials at both recording electrodes and the baseline (zero potential) of the two channels are adjustable via the slider on the right side of the oscilloscope screen. The oscilloscope works continuously in the memory mode and can also, in the "STORE" mode, overwrite previously registered curves, without deleting them. You should take advantage of this, for example, for presenting the refractory periods. In case of faulty recordings, the settings can be reversed via the "UNDO" button.

The nerve chamber can be opened and closed by clicking on the cover. For measurements, the lid should always be closed, otherwise you will catch interference voltages. The temperature can be adjusted by the scale attached to the front. The stimulus and recording electrodes in the nerve chamber can be moved, also when the chamber is closed, by moving the colored buttons at the front of the chamber. A wide earth electrode is fixed in the center of the chamber.

For your experiments take one of the two nerve preparations from the petri dish next to the chamber and place it over the electrodes. By a hold-click of the mouse on the roll of thread, you can pull a piece of thread over the nerve. When you release the mouse, you will set up a nerve block, which can be undone by pull the thread from the chamber.

b) Technical Notes

1) Noise voltages: With the lid of the measuring chamber open, noise voltages will occur. With the oscilloscope set at a suitably-chosen time base, you can observe what is referred to as the "hum", which is a 50 Hz (or 60 Hz) interference from the power grid. The so-called "noise" is due to random fluctuations from various sources, such as directly from the connected equipment or perhaps even from the preparation itself and cannot be completely avoided even with the lid closed.

2) Short circuit: If one of the two recording electrodes short-circuits to earth, there could be an amplification of the noise voltages due to the differential amplifier`s effect falling away. If one of the stimulating electrodes short-circuits to earth, there is no stimulus possible with this electrode. Also, the stimulating electrodes should be at least 3 mm, preferably 5 mm or a greater distance from each other, otherwise short circuit effects may occur that reduce the stimulus strength.

3) Stimulus time: Always work with a relatively short stimulus duration (about 0.5 to 1 ms), unless the task requires otherwise (eg when the stimulus time-voltage curve). This will avoid long delays between stimulus onset and action potential, which for example could distort measurements of nerve conduction velocity. Also, at longer stimulus durations, additional unwanted potentials occur that will complicate the interpretation of the results. In a real-life experiment, longer stimuli could also damage the nerves. In measurements on patients similar strength stimuli of shorter duration are less painful than of longer duration and lower amplitude. If a prolonged series of stimuli are required, e.g. for therapeutic purposes, as in the case of deep brain stimulation for the treatment of Parkinson's Disease, biphasic stimulus pulses are set. The depolarizing pulses are directly followed by hyperpolaring pulses thereby avoiding larger alterations of electrolyte concentrations.

4) Biological diversity: in order to simulate the reality of biological variation, each preparation reacts differently. You will notice this if you switch to another preparation. If you leave the apparatus and return, the preparations at a new start-up will exhibit slightly different characteristics than in previous experiments.

II. Experiments

The experimental procedure is divided into the following sections:

1. The characteristics of extracellular stimulation and recording
    a. Biphasic and monophasic CAPs.
    b. The impact of electrode positions
2. Stimulus intensity and stimulus duration
    a. Size of the CAPs depending on stimulus strength (recruitment)
    b. Amplitude-duration curve: the impact of passive membrane properties (resistance and capacitance, RC)
3. The importance of Na^+ channel inactivation
    a. Refractory time - due to Na^+ channel inactivation
    b. Anode-opening excitation – relief from Na^+-channel inactivation
4. Conduction speed and temperature dependencies
    a. Nerve conduction velocity
    b. Temperature dependent changes of delay and form of biphasic CAPs

1. Particular features of extracellular stimulation and recordings

- *Experimental conditions and measurement techniques* -

During these exercises you should always keep in mind that your recordings are not measurements of intracellular action potentials, which are of about 100 mV, but extracellular CAPs of only a few millivolts. However, the extracellular CAPs of the nerves may be very similar in shape to the intracellular recordings of single action potentials. It is one of the essential aims of the practical class that you understand the differences between these two waveforms.

Exercise 1.1: Di- and monophasic CAPs

As explained above, the biphasic CAP results from the difference of two time-delayed monophasic recordings (Fig. 7). In regard to excitation what is measured extracellularly is a negative potential towards the unexcited areas. Negative potential differences, such as in EEG recordings, are usually shown on the chart recorder or oscilloscope as an upward deflection, whereas in an intracellular recorded action potential, it is a positive potential that is recorded as an upward deflection.

Also, in our experiment, the first half-wave of biphasic CAPs as well as the monophasic potentials should (with transmission to the second electrode blocked) also deflect upwards on the screen. This is ensured by the differential amplifier that is also used in the experiments in humans (nerve conduction velocity and ECG). (Move wires) Make sure, such as when swapping the two recording electrodes, therefore, that the differential amplifier inputs are set so the signal is inverted.

Exercise 1.2: The significance of electrode positioning

Due to the time differential factor of CAP recordings, the distance between the measuring electrodes has a significant influence on the shape and duration of the biphasic CAPs. You should demonstrate this by moving the recording electrodes relative to each other.

The CAP is of significantly longer duration than the single action potential of the individual fibers, since the arrival times of the individual action potentials at the recording electrodes differ due to differences in the speed of conduction of the different nerve axons. This leads to a spreading of the CAP between the early and late arriving single action potentials. If you vary the distance between the stimulus and recording electrodes, you can clearly see how the amplitude and duration of the CAPs (also with monophasic registrations) change.

You should realize that with an extracellular stimulation, the CAP is triggered at the cathode (the negative electrode). Now try to determine figure out which electrode is the cathode. You can do this several ways: you can switch the stimulation electrodes (easiest using the "Polarity" switch), move one of the electrodes, or tie the nerve between the two electrodes.

Notes for medical practice

Physiology textbooks described intracellular recording of action potentials in detail, including recording of single ion channels potentials using patch-clamp. They contain little if any information on extracellular recording of summated action potentials. The majority of physicians, veterinarians, pharmacists or other medical professionals will probably never perform or have to interpret intracellular or patch-clamp recordings of membrane potentials; such electrophysiological measurements are confined to medical research. However, electrophysiological recordings are often made in medical practice but these are always extracellular recordings of compound potentials, not only in the measurement of nerve conduction speeds but also in the electromyogram (for testing, for example, muscle weakness or the depth of anaesthesia), ECG and EEG.

Thus, in medical practice, it is extremely important to know the specifics of extracellular recordings and possibly also of the application of extracellular electrical stimuli. In extracellular stimulation and recording, the measurements are crucially influenced and possibly corrupted

by technical parameters, such as the positioning of the electrodes and incorrect device settings or connections.

2. Stimulus intensity and stimulus duration

Exercise 2.1: The relationship between CAP amplitude and stimulus strength

In a large part of the current generated by extracellular stimulation flows directly from the anode to the cathode via the low resistance extracellular spaces between the individual fibers. At low stimulus strengths, the current flow is only sufficient to trigger an action potential in those fibers than are thicker and more adjacent to the stimulating electrodes. With increasing stimulus strengths, more and more fibers are "recruited", CAP amplitude increases and, with higher stimulus, nearly all fibers will are stimulated.

Determine for your nerve preparation with a constant stimulus duration (1 ms), the stimulus intensity at which the first small CAP is detectable (=minimum threshold) and that above which the CAP amplitude increases no more (=maximum threshold). Then, by adding some intermediate CAP measurements, construct the curve for the stimulus intensity dependence of CAP for your preparation. Do not forget to include data points to cover the sub-minimal and threshold ranges.

The special feature of extracellular CAP measurements is the absence of the well-known all-or-nothing rule. This rule applies only to recordings of single nerve fibers, although not under all conditions. In extracellular CAP measurements, the CAP amplitude increases gradually from "nothing" to "everything" as more and more single fibers are recruited. The maximal value, or saturation, is reached when the stimulus is strong enough to trigger an action potential in each of the nerve fibers.

Exercise 2.2: Amplitude - duration curve (effect of stimulus duration)

The effect of a pulse stimulus is dependent not only on the amplitude but also the duration of the stimulus pulse. This time factor is relevant because, between the voltage or current stimulus and the membrane response, there is a time delay, which is mainly due to the so-called passive electrical properties of the membrane, which can be considered analogous to the presence of a parallel circuit of resistors (the ion channels) and capacitances (the membrane lipid bilayer) (see Theory section).

To demonstrate the relationship between stimulus intensity and stimulus duration, select a series of combinations of values that generate CAP values of the same amplitude. It would be suitable to select stimulus settings for the minimum threshold CAP, which from experience is about 0.5 mV (see 2.1), because this has been shown to provide the most reproducible data.

You could start with 5 ms duration, which is a rather long stimulus for the neuronal membrane, and then shorten the stimulus duration in steps of 1 ms. Up to 2 ms, the amplitude of the stimulus will require little changing for triggering the minimal CAP. At shorter stimulus duration, however, the voltage is then required to rise sharply until you absolutely no SAP can trigger more. In this area, you should choose a smaller step size in order to record this part of the curve.

You will see that, along the x-axis, the curve does not exceed a certain minimum value. This means that a minimum current or voltage is needed even such long stimuli. This minimum value is the rheobase. The rheobase may be significantly affected by external conditions, such as how well the nerve is dissected or, when measured in vivo, how good is the contact of the electrode with the skin and how much fat and connective tissue lies between the electrode and nerve. If you stimulate the nerve at double the rheobase value and determine the minimum time necessary, then one has a value, the chronaxia. The chronaxia is largely determined by the properties of the nerve and, in healthy nervous tissue, should be between 0.5 and 1 ms. The stimulus duration required for a given stimulus intensity is referred to as the time-dependence of stimulation. Thus, chronaxia is therefore the time-dependence for stimulation at twice the rheobase.

Physiological and clinical importance of time dependencies

Chronaxia measurements were once widely used in neurology as an easy way to study changes in RC properties, such as due to demyelination. But, in recent years, they have been largely replaced by measurements of nerve conduction (see point 4) and additional investigations involving use of micro-needle electrode-based electromyography.

Nevertheless, it is important to be aware of the time-dependence arising from the RC properties of the membrane. The same rules are governing alterations of nerve conduction velocity under pathological conditions (see below). Moreover, temporal summation of synaptic potentials depends to a significant extent on the thereby induced time delays of de- and repolarisation.

There are also some very practical applications arising from these time-dependencies. Thus, because of the time delay of the discharge below a minimal pulse duration or minimal frequency of alternating current, no action potential is triggered, even with large currents. This phenomenon is used, for example, in heat treatment (diathermy) and in "cautery" in Surgery. In both these examples, the thermal effect of the current are used but the utilisation of high-frequency alternating currents means that the depolarizing phase is too short to trigger another action potential. This allows large currents to be applied that are appropriate for exerting the desired thermal effects without unwanted side-effects on excitable tissues such as nerve, heart, and skeletal and smooth muscle. For heat treatment (diathermy), the currents are applied via large electrodes. At the tip of the surgical cautery, the current density is high enough that the touched tissue burns, such that severed blood vessels up to a certain are sealed any light bleeding is immediately stopped.

Here is just one final note from everyday life. If, at home, you accidentally touch a wire that is "live" with electricity, you will notice that immediately. In this case, this is not associated with heat development. It is because the frequency of the main electricity supply (sinusoidal alternating current), which is 50Hz in Europe and 60Hz in the U.S. and other countries, is ideally suited to trigger action potentials. The sensation is not comparable to any other because all nerve fibers and their endings are being activated, regardless of any sensory specificity.

The minimal threshold for triggering action potentials is, for a sinusoidal stimulus, in the range of 10-100 Hz, with most pain-transmitting C fibers having slightly lower thresholds than the myelinated A fibers. This has led to already led to trials, in pain management, of targeted stimulation of myelinated A fibers, using 50 Hz (correspondsing to 20 ms period). With this alternating current, the depolarizing phase lasts 10ms, which is already in range of the minimum current (Rheobase) for triggering action potentials (see stimulus voltage-time curve). That, with a more prolonged period of sinusoidal stimuli (lower frequency), the threshold rises again is because of another property of neuronal membranes that is associated with the inactivation of Na^+ channels.

3. Na^+ channel inactivation

Exercise 3.1: Refractory period

During an action potential, the Na^+ channels initially open and then go in to a closed-inactivated state (see Figure 4). The nerve cell is then unexcitable (absolute refractory period). This is followed by a period of up to several milliseconds, as the membrane repolarizes, during which time the channels are returning to their closed excitable state. In this period, only a proportion of the Na^+ channels can be reactivated (relative refractory period). During the relative refractory period, it is still possible to open sufficient Na^+ channels to trigger an action potential but this requires a greater membrane depolarisation because the threshold for action potential triggering is increased. In addition, the amplitude of the action potential would be smaller due to the fewer available excitable Na^+ channels. With extracellular recordings of CAP, it should be noted that the end of the absolute refractory period is governed by those nerve fibers that are most rapidly re-excitable and that the end of the relative refractory period is governed by those nerve fibers with the longest period of inactivation.

For this experiment, you set the stimulus at double pulses ("Twin"), and the oscilloscope at Store mode, so that the recorded curves are viewed on the screen together. You should document your results in a table and a graph and use the data to approximately determine the absolute and relative refractory periods of the total nerve. Display these findings with a drawing to indicate the course of change in the threshold potential for action potential triggering and try to clarify the underlying ionic processes.

Exercise 3.2: Anode break excitation

While currents under the cathode cause depolarization, currents under the anode cause hyperpolarization. Nevertheless, also under the anode an action potential may be triggered. However, this AP will appear at the end of the stimulus pulse, i.e. when the circuit is opened and the current flow breaks. The action potential is not caused by the hyperpolarization, but by its cancelation, which is equivalent to a relative depolarization. Therefore such potentials are called "anode opening or break excitation" (ABE) in contrast to the "cathode closure excitation" (CCE). Hence,

This relative depolarization can only trigger an action potential when the hyperpolarization was of significant duration (a few ms). This time is needed to allow the threshold for triggering an action potential to adjust to the more negative potential. If the threshold falls below the previous resting potential, an action potential

can be triggered when the membrane potential, at the end of the current stimulus, returns to the resting potential - which is a relative depolarization.

Again, as in the membrane refractory time, the inactivation of Na^+ channels is involved, which explains the similar time dependencies. More precisely, it is in this case the removal of inactivation. At resting membrane potential, not all Na^+ channels can be opened with about 1/3 being in an inactivated state. After a sufficiently long hyperpolarization, these non-excitable Na^+ channels become excitable.

For recordings of compound activity, the amplitude of the anodic triggered CAP will increase with increasing stimulus duration. This is related to that there being more and more fibers have a sufficient number of Na^+ channels in the activated state to reach the trigger threshold. However, with extracellular stimulation of an entire nerve this can hardly be achieved in more than about half of the fibers. Therefore, the anode break excitation will never reach the amplitude of the cathode closing excitation. Please note: this relation is different with intracellularly recorded action potentials of single fibers. The anode break potential should be larger because the preceding hyperpolarization should allow a larger number of Na^+ channels to activate.

Notes on the physiological and clinical significance of the Na^+ inactivation

While action potentials after prior hyperpolarisation are larger due recruitment of additional Na+ channels, the opposite holds in the triggering action potentials after pre-depolarisation which are smaller due more Na^+ being in the non-excitable state.

A certain form of presynaptic inhibition appears to involve this mechanism. In contrast to postsynaptic inhibition by hyperpolarization, the action potential arriving at the pre-synapse after previous depolarisation will be reduced in size and so cause a decreased Ca^{++} influx and a resultant decrease in transmitter release at the synapse. This allows a much finer, gradual modulation of synaptic transmission.

The same mechanism is made use of in the clinic, as in the use of depolarizing muscle relaxants or cardioprotective solutions. The trick is that a sufficient depolarization closes all Na^+ channels, thus preventing the triggering of action potentials. If this occurs slowly enough so that only a few Na^+ channels are opened while the previously opened channels are inactivated, a complete blockage can be achieved, if required, without any interim triggering of action potentials (and muscle contractions).

Such an effect also underlies the previously-mentioned blockade of the action potential by low frequency sinusoidal stimuli. Thus, with slowly changing membrane potentials, there is sufficient time for the trigger threshold to adjust by activation and subsequent inactivation of Na^+ channels.

4. Nerve conduction velocity and temperature dependencies

Exercise 4.1: Nerve conduction velocity (NCV)

As with any speed measurement, the speed of a nerve conduction is expressed as velocity (v) equaling the distance traveled (d) over time taken (t): v=d/t. In this experiment, the "d" is determined by the distance between the cathode and the first lead electrode, while "t" is the time latency of the beginning of the CAP after the stimulus. In this way, it is the velocity of the fastest conducting fibers that is measured while that of the other fibers are overlooked due to their membrane potentials being lost in the total nerve potential.

The speed would of course only be measured precisely if the action potentials were triggered immediately with the start of the stimulus. This is certainly not the case because recharging of the membrane and the activation of ion channels causes a certain delay, the so-called "dead time". In order to keep this as small as possible, short and powerful stimulus currents should be used. You can expect a dead time of about 0.1ms. For comparison, you should also record the latency after a smaller stimulus intensity.

The dead time becomes, of course, less important the longer the conduction time. You should, therefore, adjust to the widest possible distance between the stimulating electrodes and recording electrodes. To eliminate the "dead time" you can make measurements with the electrodes at two difference distances and calculated differences in latency times as follows: v=(s1-s2)/(t1-t2). This eliminates the "dead time" since it is the same for reach latency measurement. With isolated, relatively short nerves of short latencies, such measures to improve accuracy may be offset by meter reading inaccuracies.

Exercise 4.2: Temperature effects

The effect of temperature on nerve conduction velocity can be very readily investigated only with the isolated preparation because the complete preparation can be placed in a temperature-controlled chamber. In the virtual laboratory, you can quite easily adjust the temperature and immediately record the effect, which would not be possible in reality, due of course to the time required for temperature equilibration. However, you will still observe the temperature-dependent latency shifts that occur in reality. You should try to estimate the Q_{10} from the conduction speed.

By recording biphasic potentials, there might be significantly visible changes in the shape of the summated potentials. It should be clear to you here that such changes have little to do with the temperature dependency of the single potentials but with the effect of temperature on the superposition of the two monophasic potentials. However, the form of monophasic potentials varies little relative to the difference in

time of conduction, whereas these temperature-induced changes in conduction time greatly change the shape of the biphasic potential – this is based on the same principle observed by changing the distance between the sensing electrodes.

Clinical and biological significance of conduction velocities and their temperature dependency.

The measurement of nerve conduction velocity is one of the standard tests in neurology in diagnosis of damage to the peripheral axons and altered myelination; it helps in determining the location of damage and possibly to differentiate peripheral and central lesions. The nerve, as in this experiment, is stimulated at a specific location and the CAP is detected at an easily accessible location at distance away from the location of stimulus. However, this is always easy, especially when needle electrodes but not surface electrodes are used.

Therefore, measurements are often made in the muscle innervated by the nerve. Here the total latency time will include not only the actual conduction time with the "dead time" that is referred to in exercise 4.1 but time delays due to synaptic transmission and the time for propagation of the muscle fiber potentials. Therefore, it is particularly important in such cases to stimulate at two different locations and to calculate the NCV, as described in exercise 4.1, in order to derive the actual conduction time.

In recording the so-called M-wave of the muscle, only rapidly conducting fibers of the α-motoneurons are recorded. One can, for comparison, record additional potentials, such as the H wave, which would involve afferent fibers and central spinal synapses. Overall, the measurement of NCV is of broad diagnostic potential.

In order to compare NCV values with normal healthy reference values, factors such as age and temperature need to be taken in to account. While the core body temperature is maintained relatively constant, the temperature in the periphery may vary a lot. In fact changes in peripheral nerve temperature are one of the variables used physiologically to maintain core temperature.

A measurable indicator of the temperature on the peripheral nerve is the skin temperature, which is normally well below body core temperature. Reference values in the tables of normal NCV are usually set at 32°C. So, for example, if you measure skin temperature as 28°C and a NCV of 60 m/s, a value of 4*1.5=6 m/s needs to be added, which is about 10%.

The temperature of the peripheral and central nervous systems of humans can be critical, in circumstances such as in extreme freezing weather conditions or with high fever. However, such temperature effects result more from temperature-dependent change in the form of the action potential and less with changes in NCV. In cold-blooded animals, temperature is a critical parameter that can cause such reduced NCV values that the ability to react is impaired or there is even paralysis. This means that for us warm-blooded animals on cold nights we need not fear otherwise dangerous snakes too much because, even though they can more readily detect warm-blooded prey in the cold, they move very slowly.

III. Physiological basis

For a successful completion of these practical experiments and correct interpretation of the results, you should be aware about specific details of extracellular recording and you need to know the basics of nerve excitation, which you should have acquired from your physiology textbook, lectures, seminars and/or other practical exercises. In the following, only certain aspects of membrane electrophysiology are summarized.

1. "Passive" membrane properties

For action potential generation by activation of the underlying voltage-dependent ion channels, there must be a depolarization of the membrane potential. Experimentally, this is mostly achieved by applying a rectangular current or voltage pulse. The change in potential across the membrane is following the injected current with a certain delay due to the membrane capacitance (Fig. 2). This time delay is due to membrane capacitance (C_M), with the very thin double lipid layered membrane acting as a capacitor (Fig. 1). The time constant (T_M) of the nearly exponential charge transfer is given by $T_M=R_M*C_M$, with R_M the membrane resistance. The membrane resistance is the inverse of the membrane conductance ($R_M=1/g_M$) that is determined by the number and conductivity of open ion channels. At rest, these are mainly K^+ channels. The so-called leak conductance g_L is essentially a K^+ conductance which is the reason why the resting membrane potential is close to the K^+ equilibrium potential.

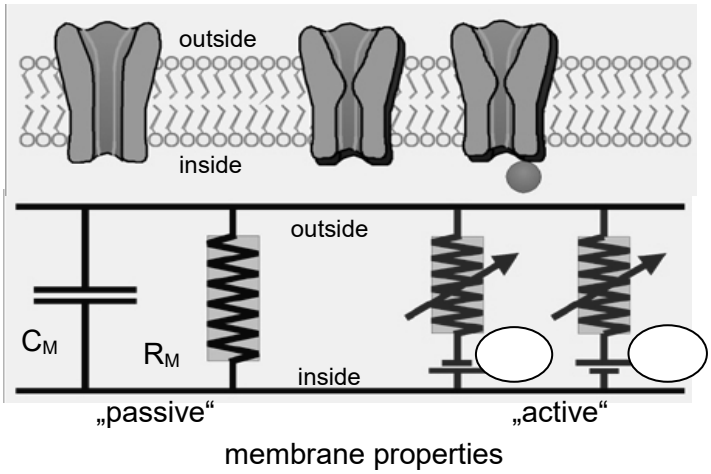


Figure 1: The double lipid layer of the membrane constitutes a capacitor (capacitance is C_M) while open ion channels introduce a certain conductitvity g_M (finite resistance R_M). Some of these ion channels are open at the resting potential (resistance, R_M). The variable resistors with batteries represent the voltage-dependent Na^+ and K^+ channels and the corresponding Nernst potentials.

These so-called passive membrane properties should well be understood because they are important for the interpretation of stimulus effects as illustrated in Figs. 2 and 3 for pulse like stimuli.

Tasks

1. Please refer to Figure 1 and indicate (in the circles) which is the Na^+ battery and which is the K^+ battery (remember, the battery symbol consists of apposing dashes, the shorter dash being the negative pole and the long dash being the positive pole).
2. Please indicate in Figure 1, assuming a resting membrane potential, the probable direction of the currents through the three resistors and the charge on the capacitor plate (direction of electric flow = direction for positive charges).
3. Please indicate in Fig. 2 the minimum current pulse duration for triggering an action potential („stimulus utilization time") when -40 mV would be the trigger threshold.
4. Please indicate in Fig. 2 how the membrane time constant can be estimated from the course of repolarization.
5. Try to explain, referring to Figs. 2 and 3, why an alternating current (AC) of 50Hz, as delivered by the grid, evokes clearly recognizable action potentials while this would hardly be the case with an AC of the same amplitude but of 50 kHz.

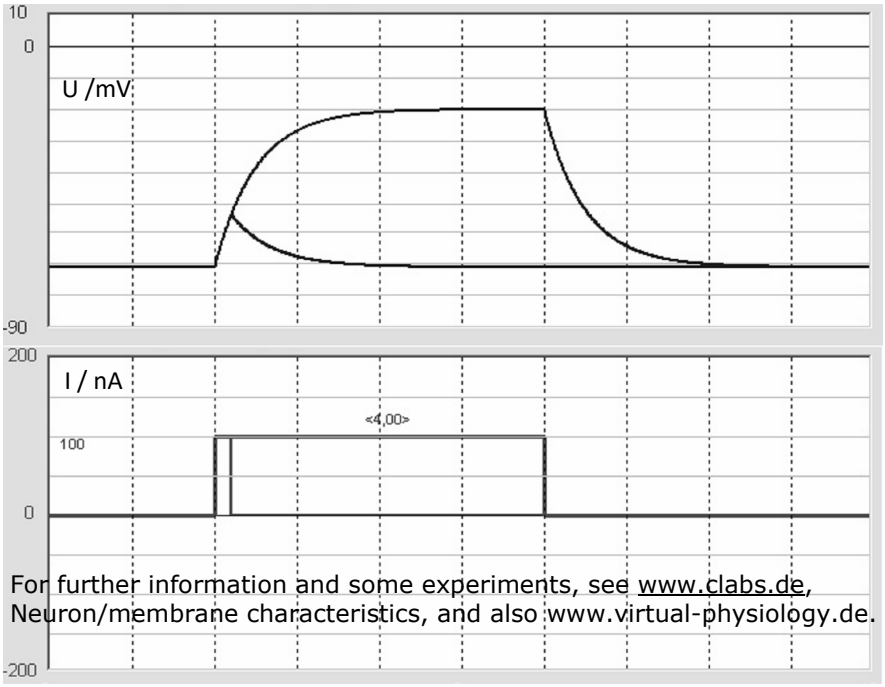


Figure 2: Charge and discharge of an RC element, corresponding to only passive membrane properties, after a long and after a short current pulse of equal strength.

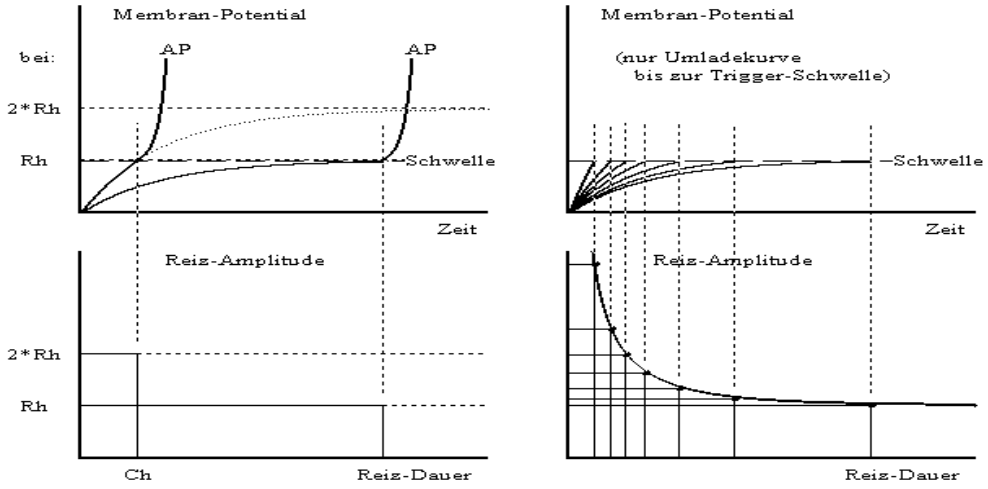


Figure 3: Strength-duration curve; utilisation time; rheobase and chronaxie: For every stimulus duration, a minimum voltage or a minimum current is required to depolarize the membrane to the trigger threshold. For shorter stimuli, without complete reloading, more current is needed. Conversely, the larger the stimulus intensity, the less time it takes for depolarization to trigger threshold (stimulus utilisation time). The minimum current that is required irrespective how long the current flows is called the rheobase (Rh). The utilization time at double rheobase strength (2 x Rh) is referred to as chronaxie (Ch). These interrelations are illustrated by the left diagrams. The right diagrams illustrate a series of membrane discharge curves up to the trigger threshold (top), induced by a series of stimuli of different duration and amplitude (bottom). By joining the stimulus coordinates, i.e. plotting the required stimulus duration vs. the stimulus strength, the strength-duration curve is formed.

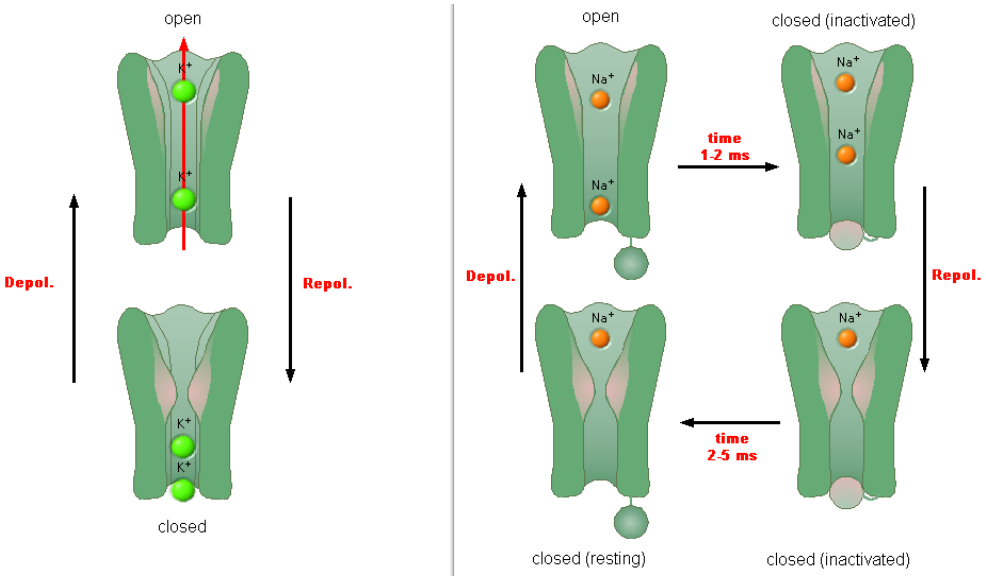
2. The opening and closing of ion channels (activation and inactivation)

The triggering and the course of an action potential is determined by the voltage- and time-dependent opening and closing of ion channels. Membrane depolarization causes Na^+ and K^+ channels to open and crucially the Na+ channels open faster. With sufficient membrane depolarization to open Na^+ channels, this leads to further membrane depolarization, which opens more Na^+ channels and so an "avalanche" of Na^+ channel opening and membrane depolarisation is formed. Due to the time-delayed opening of K^+ channels, behind the Na+ induced depolarisation, more and more K^+ channels are opening while simultaneously Na^+ channels close which leads to repolarization. When the Na^+ channels are closed while K^+ channels are still open, an "after hyperpolarization" appears. You should note that "after hyperpolarization" is not detectable in extracellular recordings. The biphasic form of the extracellular CAP is of different origin (see below).

While the K^+ channels have comparably simple voltage dependencies, opening on depolarization and closing on repolarisation, although with significant delay ("delayed rectifier"), the Na^+ channels have the special feature of automatically re-closing once they have opened. The different states of these ion channels that are contributing to action potentials in nerve fibers are shown schematically in Figure 4.

It is particularly important to be aware about the specific features of Na^+ channel inactivation because they lead to phenomena of considerable physiological and clinical relevance. You will be introduced to two of these in this experiment: the temporary non-excitability of neurons after an action potential (refractory period) and the ability to trigger the so-called anode-opening potential by the nerve membrane escaping inactivation during hyperpolarisation. Probably of even greater physiological and clinical importance is neuronal inactivation by pre-depolarization. This is a pre-requisite in the fine gradation of pre-synaptic inhibition in the central nervous system and underlies the clinical use of depolarizing muscle relaxants or cardioprotective solutions whereby the skeletal or cardiac muscle is made unexcitable by inactivation of Na^+ channels.

Typical voltage and time dependencies of the Na^+ and K^+ channels that are responsible for the shape of an action potential are illustrated in Fig. 5. In the left diagram, the curve of Na^+ channel inactivation (h) shows that, at resting membrane potential of about -70mV, only about 70% of the channels can be activated (1=100%). The other 30% are already inactivated. On depolarization to -40 mV almost all Na+ channels are inactivated. Only because the m-gate opens before the h-gate closes (see Fig. 4) and only because the Na-channels open faster than the K channels (see time-dependencies in right diagram a), is it possible for an AP to arise. Moreover, the depolarization need to be sufficiently rapid that a greater proportion of Na+ channels is temporarily opened before this this effects is counteracted by the inactivation and opening of K channels. With a slow depolarization, such as after apply depolarizing muscle relaxants, no AP is triggered. Conversely, prior hyperpolarization increases the number of Na+ channels that can be activated (see the anode-opening excitation).



epending on the
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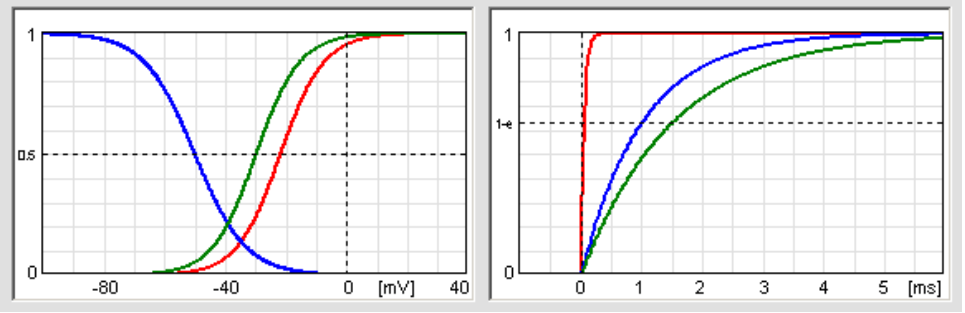


m-gate, red) and
s of K^+-channels

Questions

1. According to the above curves, what percentage of voltage-dependent Na^+ channels can maximally be opened when you trigger an action potential from a resting potential of (a) -80mV, (b) -70mV and (c) -60mV?

   (a) at -80mV: _____% (b) at -70mV: _____% (c) at -60mV: _____%

2. (a) At which of these resting potentials would the action potential be greatest? _____

   (b) At which of these resting potentials would you need the least stimulus current to trigger an action potential? _____

3. What percentage of the Na^+ channels could you open up if you depolarize the membrane to -30 mV and held this potential with a voltage clamp? _____%

4. From which minimum resting potential should you depolarise to attain this percentage of open Na^+ channels, anyway? _____mV

5. By how much would this value be reduced at a resting potential of -60mV?

3. Propagation of action potentials (nerve conduction velocity)

Nerve fibers are not very good electrical conductors. Their so-called membrane length constant, λ, which is the distance from the point of current injection at which the initial membrane potential has dropped to 1/e (e=exponential) which means to about 36%, ranges from less than 1 mm up to 5 mm. Accordingly, at a distance of 5λ, the membrane potential will be only about 1% of the initial voltage. In this way, an action potential that, for example, is triggered at a peripheral sensory cell on the little finger or big toe could never be relayed to the next nerve cell in the spinal cord.

The propagation of action potentials is only possible because the action potential is always regenerated. This involves the voltage-dependent ion channels that, in non-myelinated fibers, are distributed all along the axon or, in myelinated fibers, are concentrated at the so-called nodes of Ranvier, which are points at which the myelination (insulation) is interrupted. These nodes are positioned at short intervals of 1-2 mm along the nerve fiber. Thus, at any actual site at which an action potential

exists, the membrane potential difference to the adjacent non-excited site or next node of Ranvier is strong enough there to depolarize the membrane for the activation voltage dependent ion channels. A new and independent action potential will be generated with an amplitude that is only dependent on the number of ion channels at this membrane site.

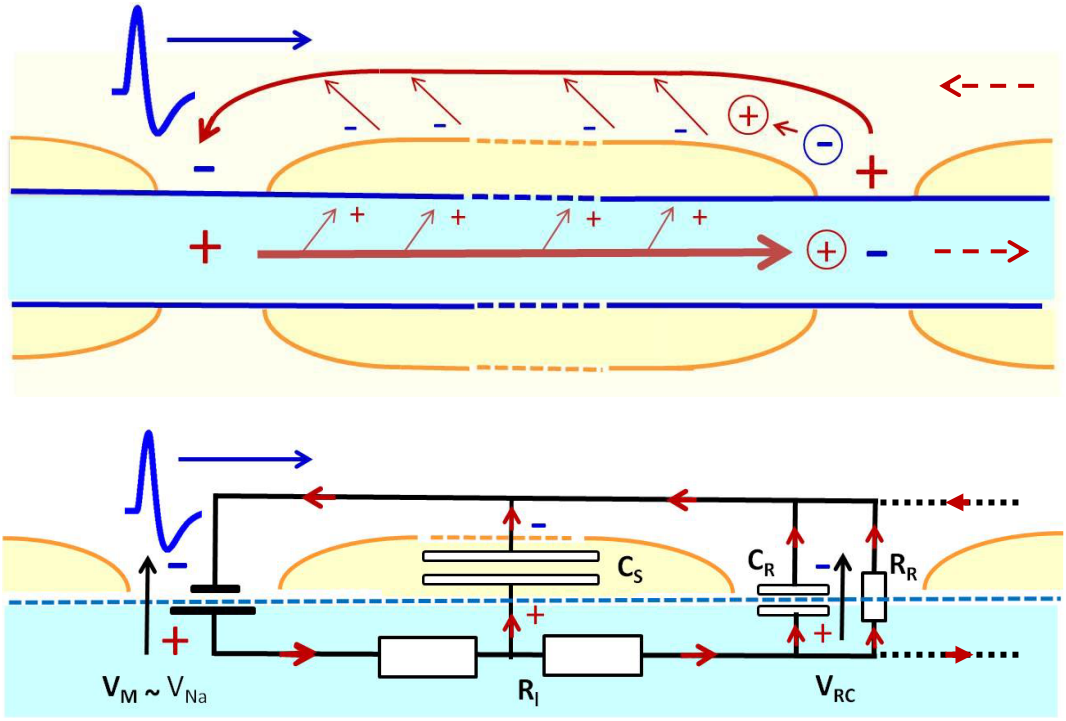


Figure 6: A schematic representation of the transmission of an action potential along a myelinated nerve fiber. For simplicity, it is assumed that one Ranvier node is excited and has a positive membrane potential (membrane potential close to Na^+ equilibrium potential) while the immediately-adjacent node is not (potential close to K^+ equilibrium potential). In both diagrams the current flows are shown (direction of positive charge). The lower figure shows the flow of current in an equivalent circuit, with each of the most important features of nerve conduction represented by the corresponding electrical circuit components. In the excited node, this is a sodium battery, while the membrane potential at the non-excited node is at the resting potential, determined by passive membrane properties. This causes a current to flow along the nerve fiber that is largely governed the internal resistance (Ri) and by the capacitive current loss during the charging of the intermodal capacitor Cs. At the next node, a part of the current will flow across the membrane, charging the corresponding local RC element. If the resultant depolarization reaches a value at which the Na^+ channels open, there will be an action potential at this node and the membrane potential at this site is then transiently determined by the local Na^+ battery (not shown).

This process of nerve conduction has less to do with charge propagation in an electrical conductor and is more comparable to heat emanating from one site and being powerful enough to cause ignition of an adjacent site. This analogy goes even a little further when the period shortly after

the action potential is considered. Then, there is a refractory period when the site of "ignition" contains spent fuel and is not re-flammable. However, there is also an important difference. In contrast to the need to replenish the inflammable fuel, the excitability of the nerve membrane is lost only temporarily because of the recovery from the only temporary inactivation of Na^+ channels. This happens during the refractory period of the nerve membrane, as discussed above.

Although the propagation of nerve action potentials is only possible by the involvement of active membrane processes, the velocity of propagation is largely determined by the passive properties of the axon. It is dependent on how fast the action potential reaches the next adjacent site for triggering an action potential and this, in turn, is dependent on the current flow from the excited to the non-excited site. In non-myelinated nerve fibers, the strength of the current flow is determined substantially by the internal resistance of the nerve fiber and, ultimately, on the thickness of the fiber. The thicker the fiber, the lower the resistance, the greater the current flow and the faster is the depolarization and action potential propagation.

With axon myelination and periodic exposure of the membrane at the node of Ranvier, there is considerable acceleration of action potential propagation as the action potential skips from one node of Ranvier to the next (Fig. 6, saltatory conduction). In this case, in addition to the previously-mentioned nerve fiber thickness, there are other factors to consider with regard on the nerve conduction velocity that are of particular importance in clinical diagnosis.

The most important factor is the thickness of myelination. Although even thinly-myelinated axons are completely isolated from the extracellular environment there are capacitive current losses. This relates to the membrane which, including myelination, still constitutes a capacitor that becomes charged. The thickness of the myelin sheet reduces the capacitance (enhances the distance between the "plates" of the capacitor). This reduces the capacitive current loss between one node of Ranvier and the next one (see Fig. 6) which increases the conduction velocity. Vice versa, de-myelination will enhance the current loss thereby significantly reducing the conduction velocity. This is the reason why the conduction velocity is used as an important diagnostic marker of de-myelinating diseases.

The second important point concerns the width of the nodes of Ranvier at which in myelinated fibers the depolarizing current flow is

concentrated. In the progression of neuron-demyelination diseases, also the node of Ranvier will become broader which reduces the current density. Even more important seems to be the exposure of additional K^+ channels that before might have been hidden under the myelin sheet but now can counteract depolarization.

Questions

(1) How and why does the speed of nerve conduction change ..

with increased nerve fiber thickness?

with decreasing thickness of myelination?

with wider nodes of Ranvier?

(2) How large are the approximate nerve conduction velocities of the fastest and slowest conducting nerve fibers in humans and how large are their respective diameters?

(3) Name the classification of nerve fibers according to their conduction velocities and function. How are these classifications differentiated according to Erlanger & Grasser and Lloyd & Hunt, respectively?

(4) Derive the approximate distance between nodes of Ranvier and calculate approximately over how many nodes of Ranvier will an action potential of 1 ms duration travel with a conduction speed of 50 m/s.

4. Temperature dependencies

As with every biological process, the speed of nerve conduction is influenced by temperature. In warm environment, action potentials propagate faster. This has less to do with the above-described properties of a nerve fiber but more to do with the temperature dependencies of the active membrane processes. In particular, the time constants of activation of voltage-dependent ion channels have a relatively high Q_{10} of 3, which means that with a temperature increase of 10°C, the ion channels open three times faster. Figure 7 shows the effects of such temperature dependencies in a SimNeuron simulation, with a response to a depolarizing pulse current.

4.1 Temperature Effects on Intracellular AP Recordings.

At a higher temperature, the action potential (left) shows an earlier and more rapid upstroke, reaches the peak earlier and also repolarizes earlier. The difference seems small for a single action potential but, with repetition at each of nodes of Ranvier, the effect of an increase in temperature adds up to a temperature-dependency for nerve conduction of approximately 1.5 m/s per °C.

Much more clearly recognizable are the temperature dependent alterations in the shape of the action potential. With higher temperatures, the action potential is shorter and somewhat smaller. This is due to the temperature-dependent increase in rate of activation of both the depolarizing Na^+ currents and the repolarizing K^+ currents. At very high temperatures, this could lead to the action potentials being so reduced in duration and size that they are no longer propagated.

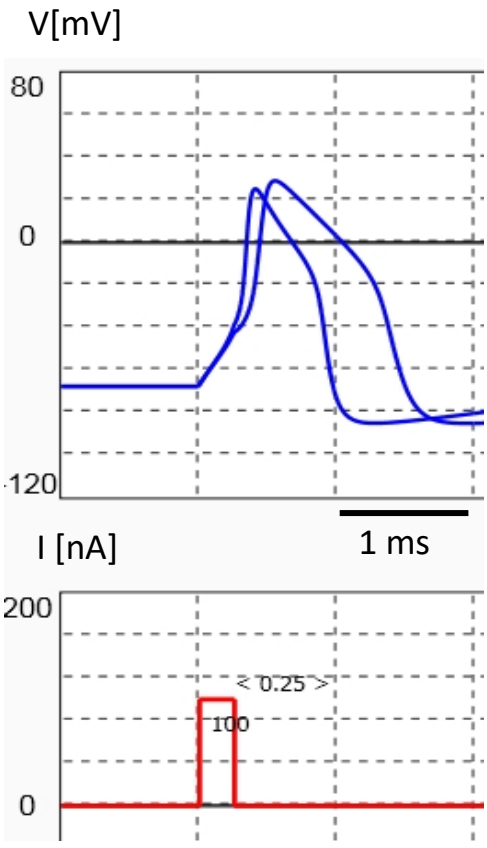


Figure 7: The temperature dependency of an action potential: with increasing temperature, the ion channels open earlier with a resultant earlier onset and shorter duration of the action potential.

Also at very cold temperatures, e.g. when the subcutaneous tissue comes close to 0°C, propagation of action potentials in peripheral nerves can be blocked. This could be related to slowing of the process of activation of Na^+ channels, so that the process of inactivation starts before an action potential can be evoked. However, these mechanisms are not yet fully explained. What is certain is that unhindered propagation of action potentials is possible only over a comparably narrow temperature range.

4.2 Temperature Effects on Extracellular CAP Recordings.

In extracellular recordings, which can be made at a greater distance from the stimulus site, temperature changes will lead to significant changes in latencies of the CAP appearance due to the altered conduction velocity. Alterations in shape of intracellularly recorded action potentials as illustrated in Fig. 7 cannot directly be seen in extracellular CAP recordings. Monophasic CAPs may be slightly larger and shorter at higher temperature because the differences in the conduction velocity of different fibres will be less pronounced.

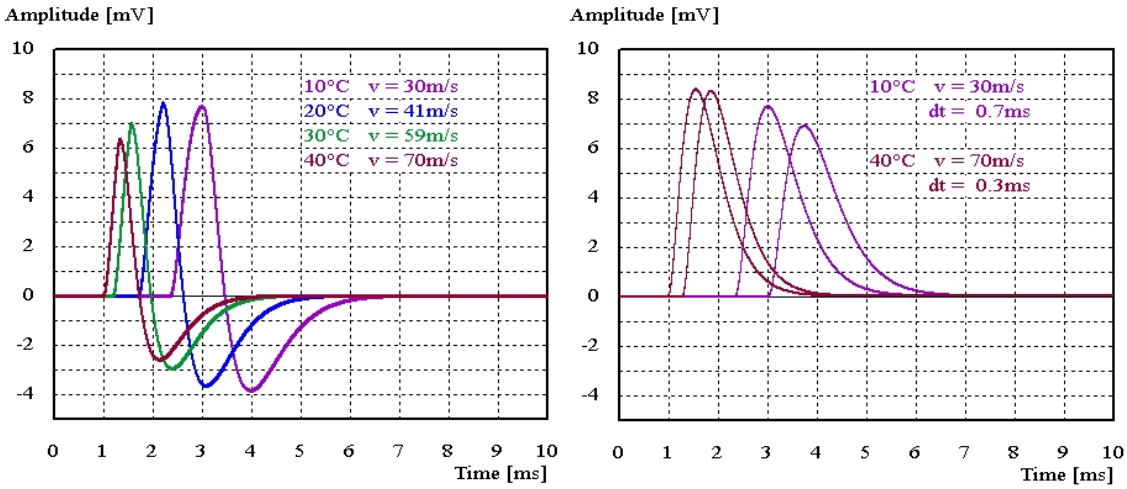


Fig. 8: Biphasic CAPs at different temperatures (left) and monophasic CAPs as appearing at the first and second recording electrode (right, only examples from 10°C and 40°C). The difference gives the biphasic potentials. Although the monophasic potentials at 40°C are higher the biphasic potential at 40°C is lower than at 10°C because of faster reversion of the upstroke by the potential by the second electrode, arriving there with less time delay than at 10°C with lower conduction velocity.

These variations are almost negligible compared to biphasic recordings for which the distance between the first, typically the inverting electrode, and the second, non-inverting electrode, plays a major role. Increasing the temperature has the same effect as bringing the electrodes closer together. It is the time delay in which the CAP arrives at two recording electrodes which essentially determines the shape and size of a biphasic

response. The potential upstroke arising from the CAP at the first electrode is turned into a downstroke when the CAP reaches the second electrode which happens earlier at higher temperatures when the conduction velocity is faster, eventually before the maximum value of the monophasic potential is reached.

Questions

(1) What factors determine the nerve conduction velocity and how are these affected by changes in temperature?

(2) How are the temperature-dependent changes in the shape of an intracellular recording of an action potential explained?

(3) If in a person with a skin temperature of 27°C, the speed of nerve conduction is measured as 60 m/s, what would be the speed of nerve conduction in this person if the temperature had the reference value of 32°C?

5. Special features of extracellular stimulation and recording

5.1. Differential Amplifier – bipolar recording

The course of an intracellularly-recorded action potential is explained in physiology textbooks. It is derived from changes in ionic conductions (see above). The course of an extracellularly-recorded CAP, which - as in this experiment – is recorded from an isolated nerve, is very similar to an intracellularly-recorded single AP. But it has other underlying causes and is highly dependent on the recording conditions, particularly the position of the recording electrodes. This is because it involves bipolar conductances being measured, via a so-called, "differential amplifier", as the potential difference between two points.

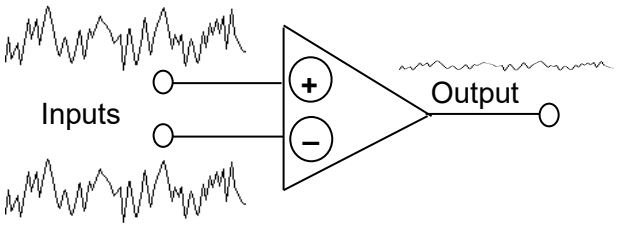


Figure 9: The function of the differential amplifier: the elimination of interference voltages. As the interference voltages of two inputs become similar, they will be almost completely eliminated.

The **differential amplifier** generates the voltage difference between two recording electrodes, one connected to a non-inverted (+) and the other one to an inverted (-) input. If the two inputs simultaneously deliver the same signal, then the output of the differential amplifier will be zero. In this way, the differential amplifier acts to eliminate or minimize the ever-present interference potentials, especially the network "hum" (see figure 9). It is for this reason that practically all measurements of electrophysiological signals (such as EMG, EEG, ECG) involve a differential amplifier.

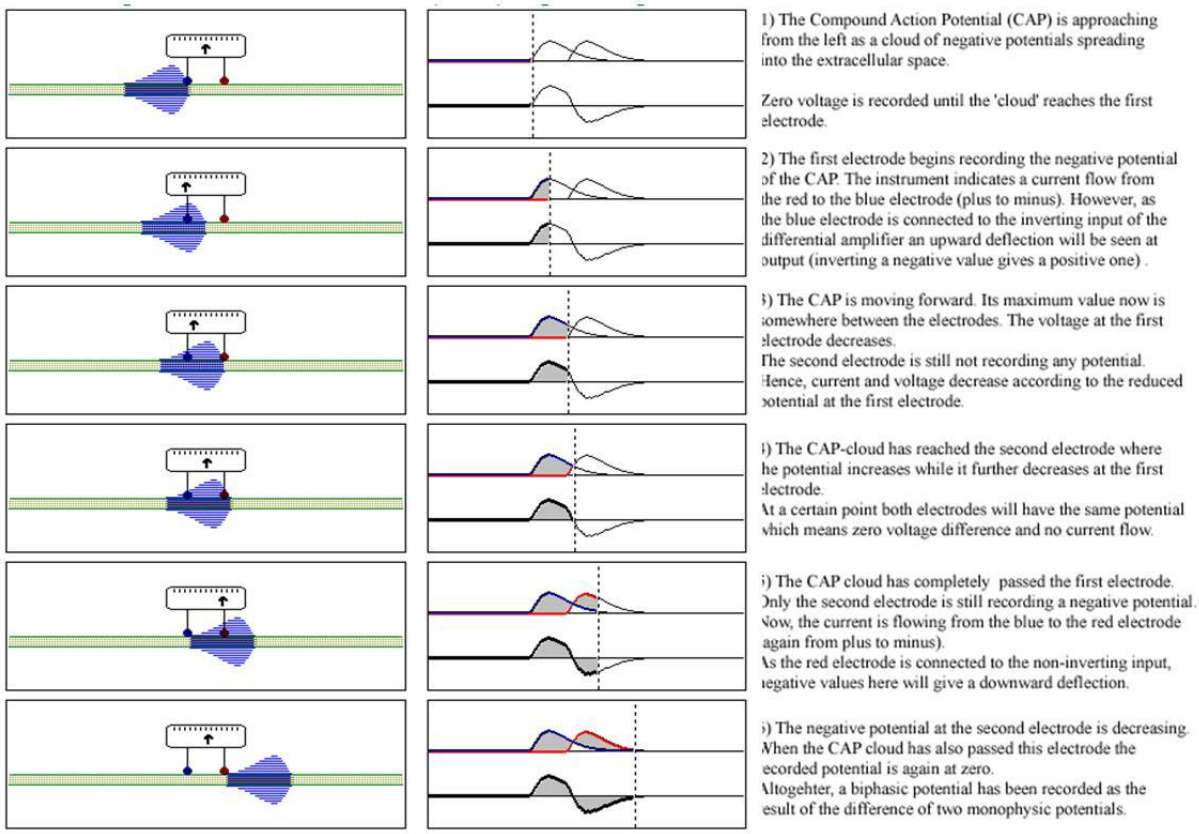
If the two inputs to the differential amplifier are of the same amplitude, the output signal will clearly also be zero. There must be some asymmetry in the nerve conductions for the nerve CAP to be registered first at one electrode and then, with a time delay, at the second electrode. Figure 10 shows schematically how each phase of an CAP is generated.

Questions:

1. In what way would you expect the CAP to change if the electrodes are (i) moved closer together, (ii) moved further apart, or (iii) switched? – see experiment 1.3.

2. How would the CAP change if the nerve was tied between the 2 electrodes? – see experiment 1.1?

Figure 10: The different phases of a CAP during its passage along the nerve as a cloud of extracellular negativity (a "depolarization cloud") (left) and the time-course of the signals at the inverting (blue) and non-inverting (red) input electrode and at the output of the differential amplifier (right traces).



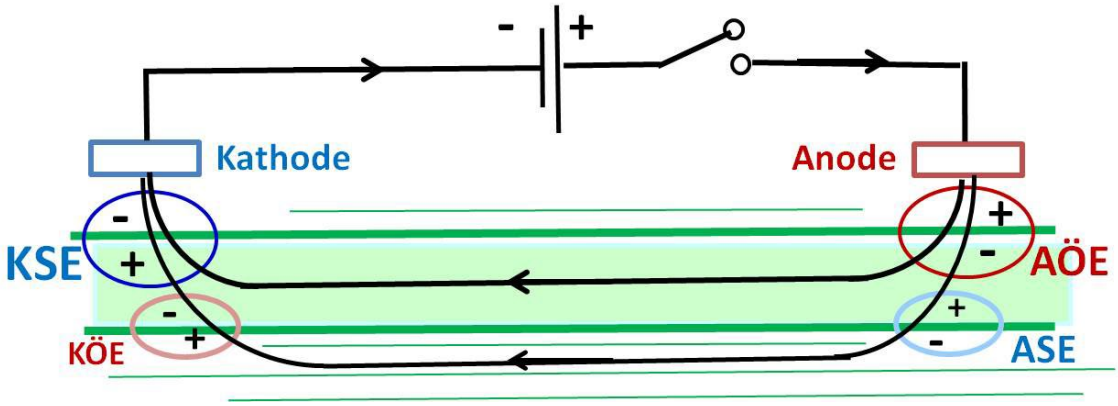
5.2. Extracellular stimulus application – „Pflueger`s Twitch Rule"

The occurrence of a nerve action potential is essentially due to the opening of voltage-dependent sodium channels, which, in turn, depends on a sufficient membrane depolarisation.

On current flow, when the circuit is closed, membrane depolarisation occurs at the cathode (negative electrode): extracellular positive charge is removed while positive charge is transferred to the intracellular side. This results in a **cathode-closing excitation** (**CCE**).

At the anode, the opposite occurs. The nerve fibers become hyperpolarized by the current flow. However, previously-inactivated sodium channels in the range of hyperpolarization can now go into an activatable state and can be opened upon depolarisation. Such a relative depolarization appears when the current flow is shut off - eventually leading to an **anode-opening excitation (AOE)** if a sufficient number of previously-inactivated channels can be opened.

Hence, an AOE can clearly be distinguished from a CCE because it is always related the end of the stimulus and it will only appear if the stimulus is of a certain duration of a few milliseconds. This is because recovery from inactivation, as also seen in the refractory period, needs some time. Moreover, the AOE of compound action potentials will hardly reach the amplitude of CCE, presumably because not all fibres have sufficient Na channels below their resting potential that can be activated after hyperpolarization to generate an action potential. By contrast, in intracellular recordings of single fibres an action potential that is generated at the end of an hyperpolarizing stimulus will typically be bigger than the one which is directly induced by depolarization, simply because additional Na-currents can be activated to contribute to the action potential.



Under certain conditions two more components of extracellular action potentials on extracellular stimulation can be observed. These are the so-called "anode-closing" excitation (ACE) and "cathode-opening" excitation (COE). The occurrence of these components of extracellular nerve membrane excitation has less to do with ion channel dynamics but with particular electrical current flows that are evoked during the extracellular excitation of the total nerve fiber (see Figure 11).

In addition to the main currents flowing along an individual fiber, entering at the anode and flowing out at the cathode, parts of the current simply cross the nerve, i.e. flowing out at the anode, eventually re-entering at the cathode. With regard on these parts of the current stimulus, the cathode becomes a virtual anode while the anode becomes a virtual cathode.

Obviously, as only part of the current entering a fibre at the anode is also leaving the fiber, it requires much more current to evoke a ACE than a COE. Therefore an ACE is only observable when the the regular cathode-evoked CAP is blocked or when the anode is significantly closer than the cathode to the recording electrode.

The cathode opening excitation, COE, is even more difficult to resolve; it is actually a

anodic potential that, in additions to sufficient current strenght, requires sufficient time to recover from inactivation through a prolonged stimulus.

The following relative strength relationship was established as a rule already in 1859 by Eduard Pflüger, after measurement of nerve activation-induced muscle twitch and is now known as "Pflüger`s Twitch Rule" (see Figure 12):

CCE > AOE > ACE > COE

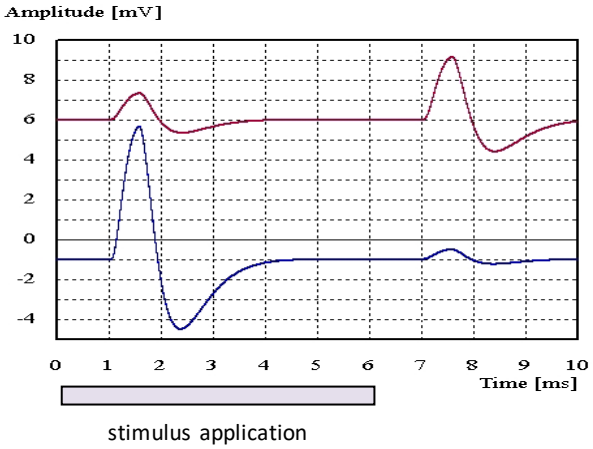


Figure 12: Recording of cathode (CCE, COE) (blue tracing) and anode (AOE, ACE) potentials with opposite current directions and blockade of nerve conductance between the stimulus electrodes. A clearly above-threshold stimulus of sufficient duration (6 ms) was set in order that an open-stimulus could evoked. The baseline was set at -1mV (for the blue trace) and, for recording, the red curve was shifted to +6mV.

5.3. Superimposition of different potentials

If a CAP is evoked at each stimulation electrode and transmitted, a complicated form of CAP may be recorded if the 2 CAPs arrive partly or completely coincidently at the recording electrode. This could happen if long stimuli at a relatively short interval are applied, with the ACE of the first CAP and COE of the second CAP superimposing (Figure 13, black tracing). Then, the now smaller second CCE (shown separately as the blue curve) would be further reduced in size, as nerve fibers have become refractory because of the AOE (see the separate red curve).

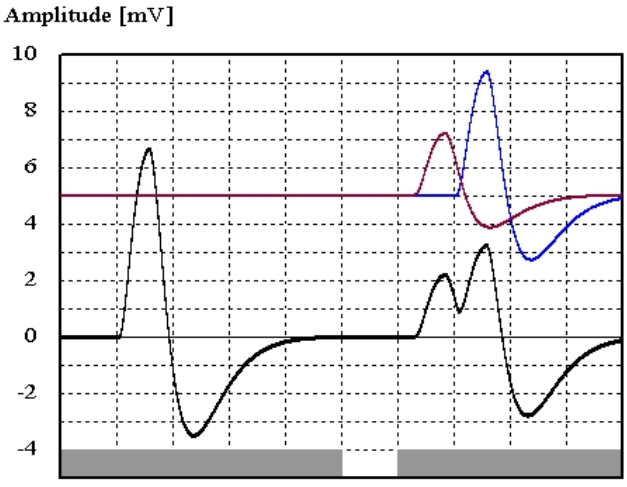


Figure 13: Separate tracing of AOE (blue tracing) and second CCE (red tracing) and their superimposition (black tracing). The bar indicates the stimulus pulse.

5.4. Variability of recordings depending on stimulus strength

In contrast to an AP measured intracellularly, the CAP is not an all-or-none phenomenon but increases with increasing stimulus strength, as more and more nerve fibers become stimulated.

The maximal stimulus threshold is reached when all nerve fibers are stimulated and, then, any further increases in the strength of stimulus above this threshold produce an identical CAP. Repeated stimuli above the maximal stimulus threshold leads to continuous generation of largely identical APs (see Figure 14, black tracing).

With stimulus strengths below the maximal threshold, the picture is quite different (blue and red tracings, Figure 14). In this case, a significant proportion of nerve fibers will be just on the AP threshold and the smallest, uncontrolled change to the nerve fiber, may evoke an AP or not.

Therefore, repetition of a stimulus below the maximal threshold will evoke a series of CAPs that are similar but with significant variability in form. This variability increases the further the stimulus strength is below the maximal, as fewer nerve fibers are excited and the minimal stimulus threshold is approached.

A further characteristic of such CAPS, is an increased latency, which is because of the greater time required at low stimulus strengths for membrane depolarization to

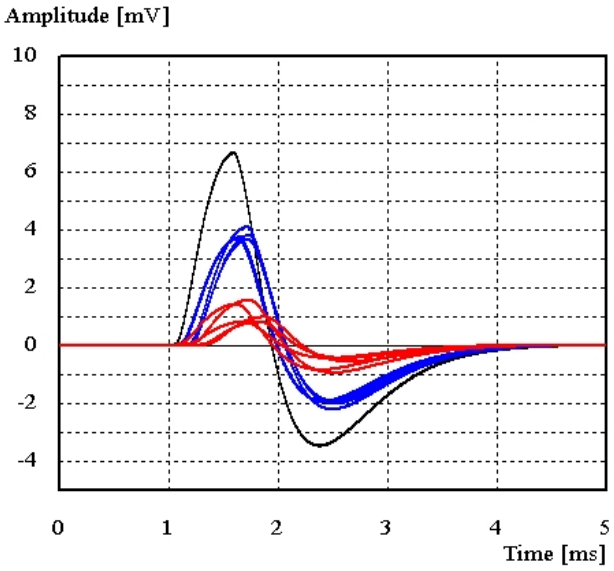


Figure 14: Compound action potentials (CAPs) recorded on repeated application of identical stimuli under 3 different stimulus conditions a) above the maximal stimulus strength threshold (black), b) close to the minimal stimulus strength threshold (red) and c) in between these thresholds with about a half of fibers excited (blue).

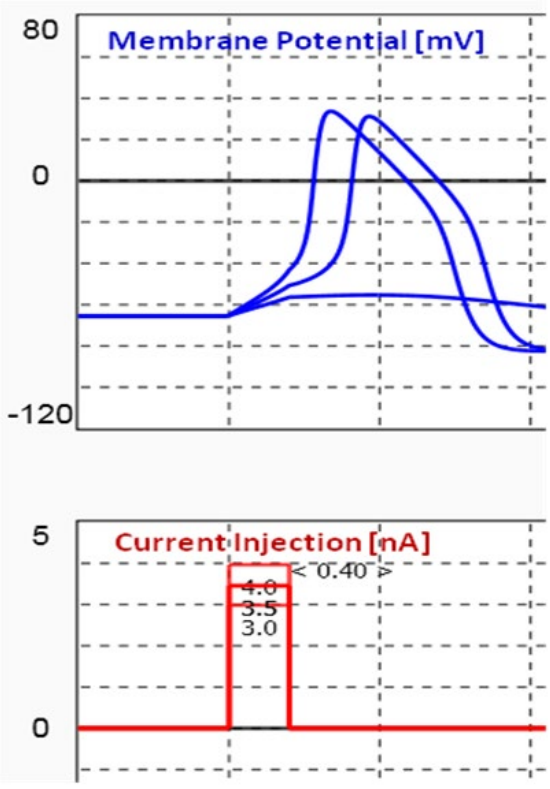


Figure 15: Tracings of intracellularly-measured AP after stimuli of different strength showing prolonged latency with decreased stimulus intensity until only local potential changes can be observed (recordings from SimNeuron).

reach a potential that evokes an AP. This effect (seen in Figure 14) is also seen in intracellular measurement of an AP (and is presented in SimNeuron and illustrated here in figure 15).

The latency between starting to apply the stimulus and the occurrence of an AP is referred to as the stimulus-response time. In clinical neurophysiological testing, such as in measurement of the nerve conductance velocity, the stimulus-response time must be kept to a minimum. In such tests, it is important that stimuli are used that are set well-above the maximal threshold so as to minimize such extra latencies that could lead to error in clinical measurements.