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RESEARCH ARTICLE

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Extracellular potassium alters frequency and profile of retinal spreading depression waves

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Abstract The phenomenon of spreading depression (SD) was observed in chicken retina by means of optical registration via a microscope and a CCD camera applying modern methods of image processing for optimized evaluation of the wave profiles. The propagation dynamics of SD waves was investigated as a function of extracellular potassium. Two main findings were obtained. Firstly, the frequency of spontaneous wave generation increased with the increase of K^+ concentration. Secondly, there was an effect of potassium on the wave profile. In particular, the recovery zone of SD waves was shortened at increased K^+. This effect was not only due to the dispersion relation of waves in excitable media as shown by the result of the mechanically induced wave trains. Applying the basic principles of chemical excitability for the interpretation of the data led us to the conclusion that these potassium effects are due to perturbations of an autocatalytic reaction to be further explored.

Keywords Excitability · Refractory period · Intrinsic optical signal · Chicken

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Introduction

Spreading depression (SD) is a wave phenomenon that propagates in gray matter of nervous tissue with a velocity of 2–5 mm/min (Leão 1944; Bureš et al. 1984). Its spread involves a large number of cellular processes. Among them is a nearly total depolarization of the neurons and glia cells caused by a drastic ion redistribution between extra- and intracellular compartments (Somjen and Aitken 1984). These changes are accompanied by cellular swelling and by the block of action potentials in a sizable population of brain cells (for review see Somjen et al. 1992; Parsons 1998; Somjen 2001). SD waves have been linked to the pathophysiology of the migraine aura (Lauritzen 1994; Bolay et al. 2002) and may produce an expansion of a neuronal lesion after ischemic stroke (Hossman 1996).

A simplified model of a reaction-transport mechanism of SD is as follows: an initial excess of a so-called activator in the extracellular space (ECS) causes a superthreshold depolarization of the cell membrane, which leads to an autocatalytic release of the activator. Possible activators are potassium (Grafstein 1956) or glutamate (van Harreveld 1978). In both cases, intracellular K^+ is released into the ECS. This autocatalytic rise in the extracellular potassium concentration ($[K^+]_o$) is partially cleared by active uptake, extracellular diffusion, and a redistribution of K^+ through the network of glial cells. If sufficient K^+ is transported to neighboring cell groups, then the same excitation cycle is initiated there and a wave of excitation spreads via reaction-diffusion coupling through the neuronal tissue followed by a prolonged depression of neuronal activity. Spreading depression is the only phenomenon known where K^+ rises from its resting level of 3 mM much above the normally well-preserved *ceiling* level of 10 mM (Heinemann and Lux 1977) up to an extracellular concentration as high as 50 mM (Sugaya et al. 1975). Note that throughout this article we refer to 10 mM K^+ as the ceiling level, even though during SD this level is actually overshot.

In the first part of our study we have globally changed the K^+ and investigated its influence on SD wave generation. Chicken retina has become a suitable model system to study SD in neuronal tissue and was chosen for the following reasons. Retinal SD can be least-invasively observed by changes in the intrinsic optical signal (IOS) (Ames and Nesbett 1981; Fernandes and Hanke 1997; Wiedemann and Hanke 1997). The IOS of retinal SD consists of an early and late phase, together known as the "classical" IOS (Martins-Ferreira and Castro 1966; Castro and Martins-Ferreira 1985). The initial causes of the two phases are supposed to be of different nature. Both phases are ultimately related to the cellular swelling but the first coexists with the electrical signal of SD (Peixoto et al. 2001) and therefore reflects the drastic disturbances of the ionic gradients. The origin of the late phase in the IOS is not known but electrophysiological methods do not record this phase (Fernandes de Lima et al. 1993). In the second part of our study a precise analysis is given of the late phase of the IOS under varied activator concentration.

To summarize, here we show a mechanism exists that couples the concentration of an activator ($[K^+]_o$) predominantly to the late phase of the optical profile of SD and therefore to the recovery period of the tissue.

Materials and methods

Preparation of the chicken retina

Domestic chickens (6–8 days old) were decapitated and both eyes were quickly removed. The cut of the upper eyecup was made near the equator. After extraction of the vitreous humor, the posterior parts from both eyes were transferred into a chamber with standard Ringer solution, in (mM): 100 NaCl, 6 KCl, 1 $CaCl_2$, 1 NaH_2PO_4, 1 $MgSO_4$, 30 $NaHCO_3$, and 30 glucose. The pH value was maintained at 7.4 by continuously applying a carbogen gas mixture (95% O_2 / 5% CO_2). During the whole time the chambers (5 ml) were constantly perfused using a peristaltic pump at a volume flow of 2 ml/min and its temperature maintained at 29–30°C. To observe the spontaneous SD waves, the posterior side of the eyecup was glued to a glass plate and transported into another chamber. The amount of KCl was immediately changed and kept the same during the whole experiment to observe the spontaneous SD wave generation. The range of the concentration between 0 and 6 mM was varied in 1 mM steps and between 8 and 20 mM in 2 mM steps. The ionic strength of this Ringer solution was kept constant by the corresponding changes of NaCl. For each concentration value at least four experiments were performed. Mechanical stimulation of the retina in control experiments was done in the presence of $[K^+]_o$=6 mM by using a tiny glass needle (tip diameter was approximately 50 μm). The interval between stimuli was 4 (high-frequency stimulation) and 15 (low frequency stimulation) min. In the presence of elevated Mg^{2+} mechanical stimulation was done with 30 min interval.

Optical registration of the SD

The registration of the spontaneous SD waves was started immediately after the preparation and lasted 2 h during the experiments with 2–20 mM KCl, 1 h with 1 mM KCl and 30 min without KCl in the Ringer solution. The registration of the mechanically induced SD waves was started 20~min after putting it into the chamber and lasted 1 h. The IOS was detected using a setup for optical registration (Hanke et al. 1996). The changes of reflected light were observed through a stereo microscope with ten times magnification objective. The illumination was done by a cold-light source under 90° angle of incidence. The signal was recorded with a charged coupled device (CCD) camera (Hamamatsu) and camera control unit on a video recorder (S-VHS, Sony) and stored on videotapes (Sony, VHS).

Data analysis

The data analysis was done off-line with a PC with a frame grabber card (Matrox Pulsar). The evaluation of the last hour of each experiment was made to receive an intrinsic optical profile of the SD wave (in the case of 0 mM KCl it was only 30 min). For this aim we have used a software for movie digitizing that has calculated the average value of the gray level of a region of the retina of $4.6 \cdot 10^{-2}$ mm^2. The sampling frequency was 1 Hz. To reduce the noise in the original IOS, Fourier transform filtering techniques were used. We have applied a low-pass filter which allows all low-frequency components to remain unchanged while the high-frequency signals are smoothed out. In the experiments where the concentration of KCl was varied between 2 and 20 mM, a low-pass filter with a cut-off frequency of 0.028 Hz was used to analyze the first phase of the IOS (Fig. 1A). For analyzing the second phase, the cut-off frequency was 0.006 Hz. Additionally, the first phase amplitude was cut off by extrapolation (see Fig. 1B). In the experiments with 0 and 1 mM KCl in Ringer, the corresponding cut-off frequencies were 0.167 Hz and 0.022 Hz for the first and second phase, respectively.

The IOS served as a measure for investigating the dynamics in spreading depression. We calculated the amplitude and the duration of the first and second phases of the optical profile of the spontaneous SD waves and maximal derivative of the first phase leading front. For this aim the Interactive Data Language (IDL, 5.3 for Windows) was used. The amplitude was calculated as the difference between the maximal peak value and the baseline that was averaged over 50 s before the start of the leading front. The duration of the first phase as well as the second phase was taken as the width of the wave IOS on the level, which is equal to the half value of the corresponding amplitude. The amplitude of the second phase was calculated as the difference between the baseline before the leading front and its peak value (Fig. 1B).

Results

The number of spontaneous SD waves and their IOS were systematically analyzed in the presence of different K^+ concentration in the Ringer solution. To characterize the optical profile, the amplitude and duration of the first and second phases and the maximal derivative of the first phase were determined.

Amount, velocity and appearance of spontaneous SD waves as a function of potassium

When $[K^+]_o$ is close to the physiological level of K^+ in the extracellular space, i.e., 3 mM, spontaneous SD formation was not observed. Raising and lowering $[K^+]_o$ in the perfused solution induced spontaneously appearing SD waves (Fig. 2A, *upper*). Almost all spontaneous SD waves started to propagate from the outside margin of the retina. The more $[K^+]_o$ differs from the physiological level, the larger the number of spontaneous SD waves in a certain period of time.

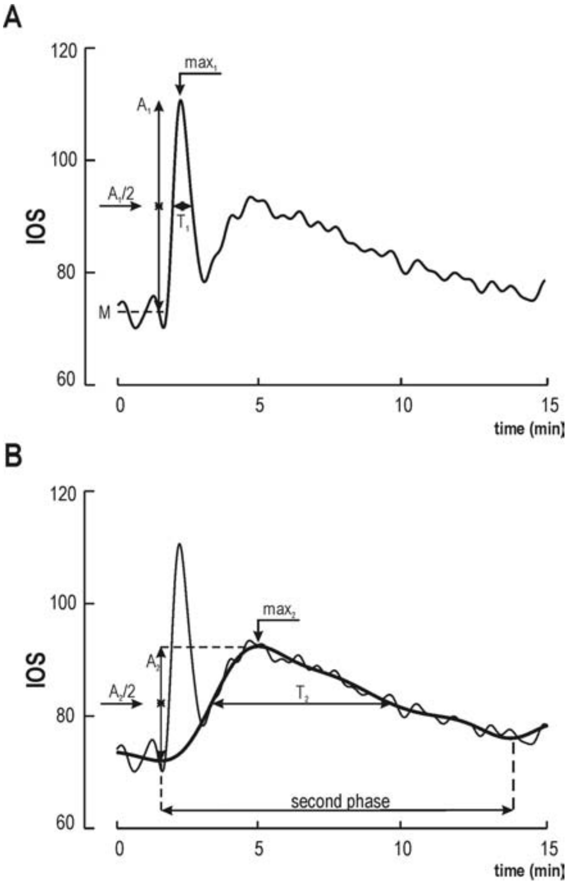

Fig. 1A, B Analysis of the IOS. **A** The definition of parameters characterizing the first phase of the IOS. The maximal value of the first peak is *max_1*. The amplitude is defined as a difference between $\max_1$ and an average value of 50 s (*M*) before the first local minimum of the wave front. T_1 is the duration at the half amplitude $A_1/2$. **B** The definition of parameters for the second phase. The maximal value of the second peak is $\max_2$. A_2 is an amplitude that was calculated as a difference between $\max_2$ and a minimal value before the start of the wave. T_2 is a duration defined as before. The presented IOS corresponds to mechanically generated SD wave in the presence of 6 mM $[K^+]_o$

Two different optical profiles were observed (Fig. 2A). When $[K^+]_o$ is maintained between 2 and 20 mM, an SD wave appears as a light milky front that propagates through a dark background. This IOS changes when $[K^+]_o$ is lowered to 1 mM. The profile of the first spontaneously appearing SD wave still has a light milky front, but the back of the IOS profile never returns to the dark background level. All subsequent waves appear now as dark waves propagating on a white background (Fig. 3A). In the absence of potassium ($[K^+]_o$=0), the dark SD waves are observed from the beginning. Under such conditions, the retina survives only for 0.5 h ($[K^+]_o$=0) to 1.5 h ($[K^+]_o$=1 mM), while at a $[K^+]_o$ level of 2 mM or higher, the retina survives for at least 3 h.

The front propagation velocity of the SD wave depends on the $[K^+]_o$. The maximal rate of speed was measured in the presence of standard K^+ (6~mM). Both lowering and increasing of K^+ reduced the velocity

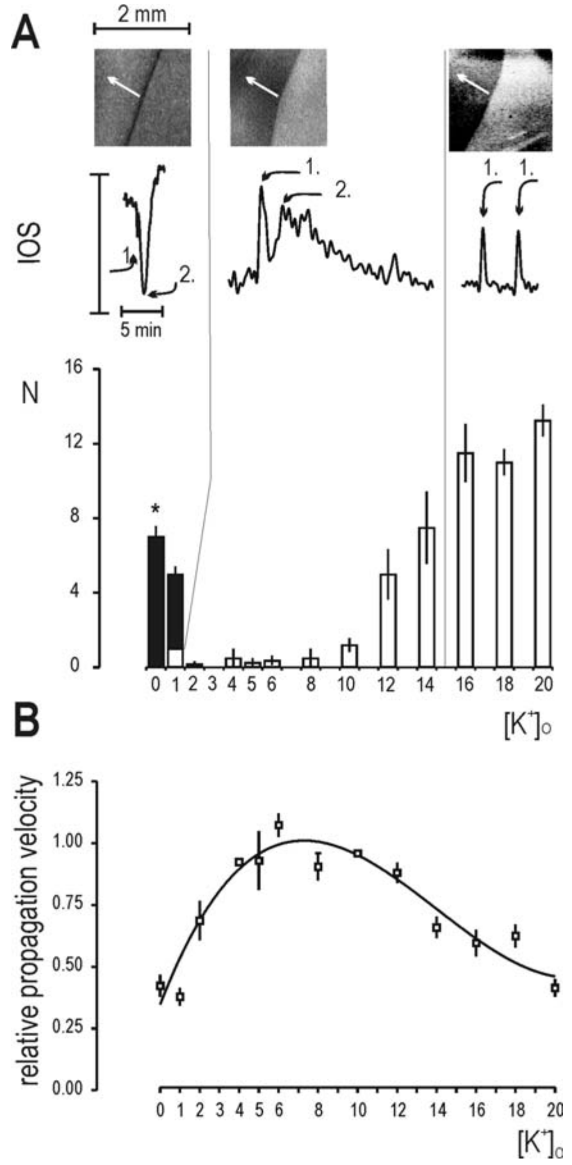

Fig. 2A, B The IOS depending on $[K^+]_o$. **A** Three different regimes are indicated by the vertical sections. (*Left*) $[K^+]_o$ 0–1 mM: The SD waves appear as a thin dark wave on a milky background. Its IOS consists of two phases. The first phase lasts only a few seconds. It is followed by a longer and larger second phase. The columns in the diagram show the total number (N) of SD waves within 1 h. *Black columns* represent dark waves. The *star* indicates experiments that were performed only within 30 min, and therefore the data was multiplied by two. (*Middle*) $[K^+]_o$ 2–14 mM: An SD wave is seen as a white front propagating through a dark background. This classical IOS consists also of two phases; however, the amplitude and duration of the second phase decreases with increasing $[K^+]_o$ and the total number of waves increases. (*Right*) $[K^+]_o$ 16–20 mM: The second phase has vanished and successive waves follow in short distance. **B** The propagation velocity of the spontaneous SD waves as a function of $[K^+]_o$. The gradual decrease of it in the presence of increased and lowered $[K^+]_o$ is detected. The data were normalized by the average velocity of SD wave in the presence of 6 mM $[K^+]_o$

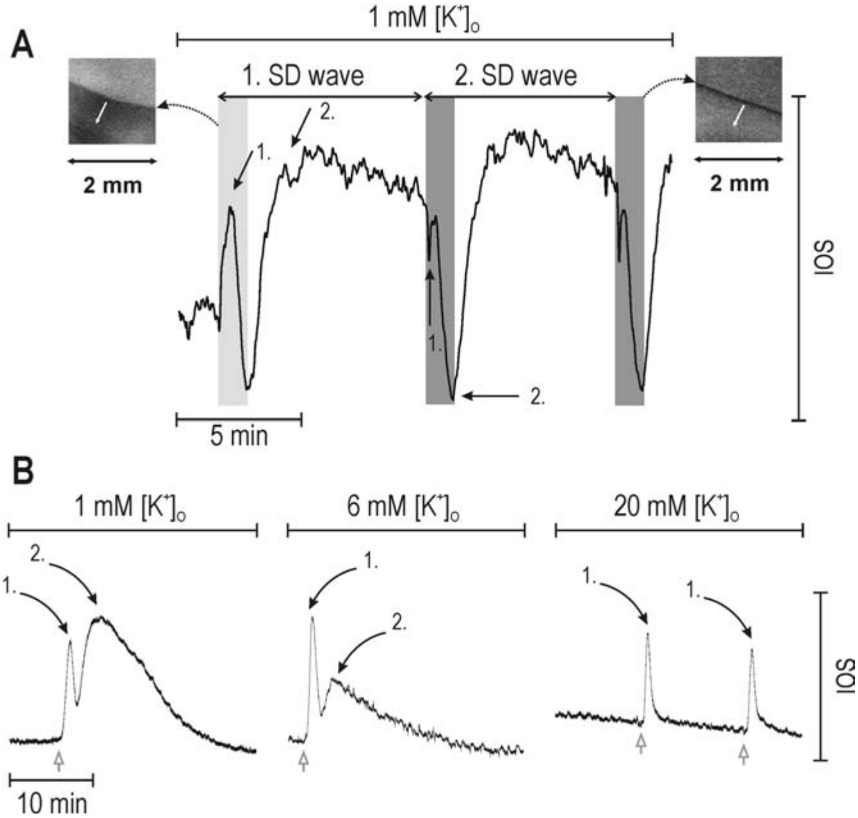

Fig. 3A, B Spontaneously appearing and mechanically generated SD waves have different optical profiles. **A** The IOS of the first three spontaneous SD waves during permanent perfusion with $[K^+]_o$=1 mM. The very first SD wave (*1. SD wave*) was always observed as a milky front spreading on a dark background (*image inset left side*). Its IOS has two maxima of the increased light scattering (peaks labeled as *1.* and *2.*). The second peak of the first wave does not recover to the base level. Already the second wave (*2. SD wave*) is visible as a dark front (*right inset*) with two peaks of decreased light scattering. All subsequent SD waves appear like the second one. **B** Three examples of *mechanically* generated SD waves in the presence of low (1 mM), standard (6 mM) and high (20 mM) $[K^+]_o$. 7 mM $MgCl_2$ was supplied in the experiments with 1 and 20 mM $[K^+]_o$ to block spontaneous SD waves and to control interval between stimulations. The smaller the $[K^+]_o$, the smaller the duration and the amplitude of the IOS second peak. During perfusion with $[K^+]_o$=20 mM, the second peak of the IOS was not visible any more. The small arrows indicate when the SD mechanical stimulation was set

gradually (Fig. 2B). The lowest value was observed in the presence of 1 and 20 mM $[K^+]_o$. This similarity indicates that both black and white fronts result from SD-like events.

Spontaneous SD waves: analysis of optical profile

Under standard conditions ($[K^+]_o$=6 mM), the classical IOS consists of two phases: a first short-lasting (52±6.7 s) and a second longer-lasting (354±47.4 s) phase. The IOS of an SD wave in the presence of low $[K^+]_o$ has an inverted profile (dark wave on a white background), but also has two phases: a first short-lasting (10.16±0.7 s) and a second longer-lasting (91.2±10.2 s, $[K^+]_o$=1 mM) (Fig. 3A).

Dark waves have decreased amplitude and decreased duration compared to white waves. The amplitude and duration of white waves at $[K^+]_o$=6 mM are 5.15 and 3.38 times larger, respectively, than dark waves at $[K^+]_o$=1 mM. However, within both regimes the amplitude and duration of the first phase depend little on $[K^+]_o$ (Fig. 4A, B). The amplitude and duration of white waves have a tendency to decrease with increasing $[K^+]_o$, but this effect is small compared to those observed for the second phase.

The second phase of dark waves increases in its amplitude but changes little in its duration when $[K^+]_o$ drops from 1 mM to zero. On the contrary, the second phase of white waves strongly depends on $[K^+]_o$ (Fig. 4C, D). The maximum duration and amplitude were observed at a $[K^+]_o$ level of 2 mM, i.e., at the transition from dark to white waves. Raising $[K^+]_o$ above 2 mM exponentially decreases the amplitude and duration of the second phase. Above the level of $[K^+]_o$=14 mM the second phase is not observable any more. At 14 mM the amplitude had a minimal value of 0.44±0.03 and the duration was 190.8±34.2 s.

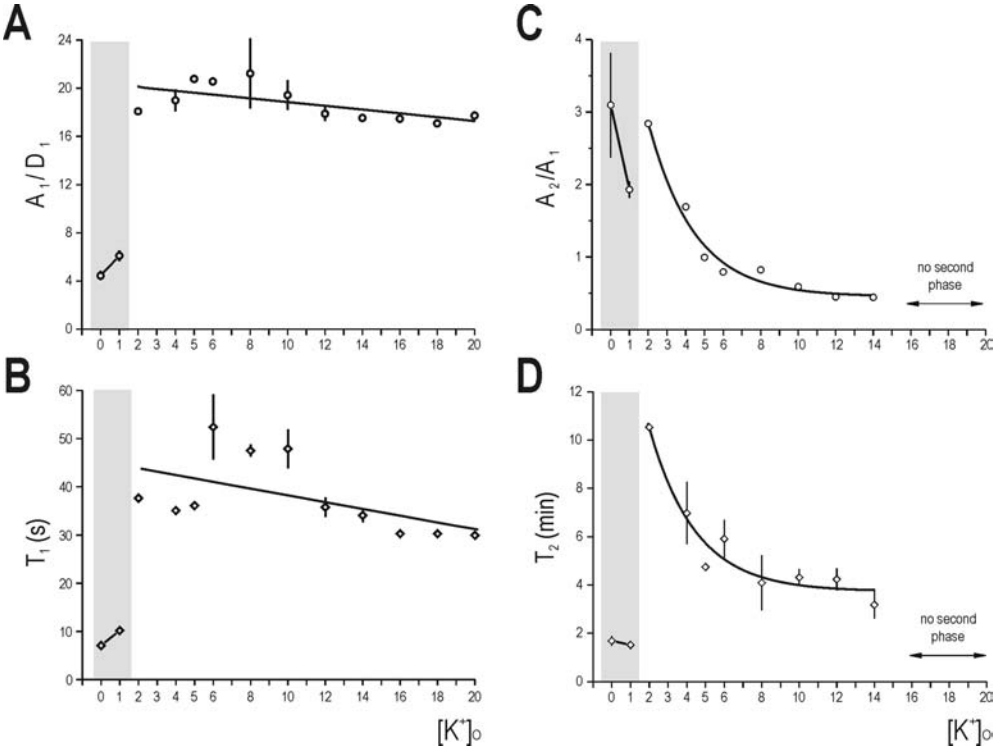

Fig. 4A–D Normalized amplitude and duration of both phases of an SD wave. Numbered tics on the abscissas correspond to [K$^+$]$_o$ values in the experiments. Note that at [K$^+$]$_o$ 3 mM no data point is shown because of the absence of spontaneous SD waves. The shaded area marks the [K$^+$]$_o$ regime between 0 and 1 mM [K$^+$]$_o$ in which dark SD waves were observed. **A** The amplitude of the first phase A_1 was normalized by its maximal derivative D_1. The normalized amplitude is little affected by [K$^+$]$_o$. A linear fit of the data shows a slight decrease. **B** The duration of the first phase T_1 shows a greater variance, but still is little influenced by [K$^+$]$_o$. **C** The amplitude of the second phase A_2 was normalized by the amplitude of the first phase A_1. The normalized amplitude decreases exponentially with increasing [K$^+$]$_o$ and vanishes when [K$^+$]$_o$ is over 14 mM. **D** The duration of the second phase T_2 depends on [K$^+$]$_o$ and shows an exponential decrease. When [K$^+$]$_o$ exceeds a level of 14 mM, the duration of the second phase can not be determined any more

Mechanical generation of SD waves with low and high frequency at standard potassium

The observed effects could mirror a dispersion relation rather than be a direct effect of potassium. Dispersion means that waves occurring in wave trains with high frequency affect each other depending on the distance of consecutive waves (Brand et al. 1997). To determine whether the second phase disappears merely as a result of the decreased spatial distance between consecutive waves at higher levels of [K$^+$]$_o$, we performed experiments with repetitive mechanical stimulation every 4 and every 15 min in the presence of 6 mM [K$^+$]$_o$. The lower frequency stimulation is but little higher than the frequency of spontaneous SD waves caused by 6 mM [K$^+$]$_o$. There is no marked difference in amplitude and duration between these two situations (Table 1, *first two rows*). At the higher frequency of mechanical stimulation, the amplitude of the second phase is lowered. However, the magnitude of the observed effect could not account for the much larger decrease when [K$^+$]$_o$ is raised to 20 mM (Table 1, *last two rows*).

Table 1 Comparison of IOS parameters between mechanically stimulated and spontaneously appearing SD waves. The first column (*Stimulus*) distinguishes between mechanical and spontaneous wave generation. The frequency of mechanical stimulation was chosen to mimic the frequency of spontaneous SD wave appearance in the presence of 6 and 20 mM [K$^+$]$_o$. In the first two rows, both with low frequency of SD wave generation, there is no difference between mechanical and spontaneous stimulation in the second phase. (Because of the low total number of spontaneous SD waves and the large variation of the interval of successive waves, the smallest value was chosen for the comparison.) The last two rows compare mechanical and spontaneous stimulation at high frequency

Stimulus	[K$^+$]$_o$	Int, min	A_2/A_1	T_2
Spon	6	16.25	0.79±0.05	5.9±0.08
Mech	6	14.86±0.16	0.73±0.08	4.62±0.38
Mech	6	4.00±0.04	0.53±0.04	2.14±0.07
Spon	20	4.42±0.25	–	–

Mech mechanical wave generation, *Spon* spontaneous wave generation, *Int* average interval between two successive waves, A_2/A_1 changes in amplitude of the second phase, T_2 duration of the second phase

Mechanical generation of SD waves in the presence of low and high potassium

Mg^{2+} is known to lower excitability and even block the generation of SD waves. Thus, it was possible to perform mechanical initiation of SD waves with controlled time interval even in the presence of high $[K^+]_o$. Mechanical stimulation in the presence of low (1 mM) and standard (6 mM) K^+ was done with 30 min interval; that is longer than the duration of the second phase. All generated waves had "classical profile" with two phases and a white front of propagation. The gradual decrease of the second phase was observed (Fig. 3B). In contrast, spontaneous SD waves in the presence of 1 mM K^+ had a black front and the inverted optical profile (Fig. 3A) Stimulation of the retina in the presence of 20 mM $[K^+]_o$ led to SD appearance with only the first phase (Fig. 3B). So, in the presence of $[K^+]_o$=20 mM the optical profile of the mechanically generated SD did not differ from that of the spontaneously appearing SD waves.

Discussion

There are three main findings in this study. First, the more extracellular K^+ concentration differs from its physiological level, the greater the number of the spontaneously appearing SD waves in a period of time. Second, the dynamics of the first phase of the intrinsic optical signal (IOS) are mainly independent on potassium, while the second phase exponentially decreases at higher K^+ concentrations and disappears at levels of more than 14 mM. Third, there is a drastic difference between mechanically stimulated and spontaneously appearing SD in the presence of low (1 mM) $[K^+]_o$ due to the different time interval between SD waves.

The spatio-temporal dynamics of spreading depression waves are very similar to that of reaction-diffusion waves, e.g., the mutual annihilation of colliding waves or the formation of rotating spirals from open wave ends (Müller et al. 1985; Dahlem and Müller 1997). It has been shown that reaction-diffusion waves occur in different systems, as for example in biological (Lechleiter and Clapham 1992; Steinbock et al. 1993; Wussling et al.1997), chemical (Zaikin and Zhabotinskii 1970; Winfree 1972) or physical ones (Jakubith et al. 1990). Although the particular mechanisms of wave generation and propagation are completely different, the basic principles are the same: the coupling of an autocatalytic reaction (e.g., an all-or-none reaction regulated by activator and inhibitor) with diffusion in an open system. Accordingly, the waves have some common properties, as for example suprathreshold excitation, refractoriness, and excitability. The propagation dynamics of the waves should be strongly affected by the spatio-temporal dynamics of an activator and an inhibitor of the autocatalytic reaction.

Potassium is known to be an affector of SD, however, its impact for the autocatalytic reaction is unknown. We used this ion to perturb the process of wave generation and propagation in order to proof its role as an activator or inhibitor. The increased excitability at higher potassium concentrations points to a role of this ion as an activator for the autocatalytic reaction. An increase in the activator concentration leads to a reduction of the threshold for excitation and thereby to an increased excitability. In the case of the retina, this potassium effect may result either directly from some K^+ activated enzyme reactions or indirectly via coupled transport/enzyme reactions. Since the production/activity of the activator in an autocatalytic reaction is coupled with some time delay to the production/activity of the inhibitor (Tyson 1988), we would also expect effects of potassium on the refractory zone of the SD waves. This zone is located in the wave back. In fact, it is the second phase in the back of SD waves that is strongly reduced at increasing potassium concentrations. The shortening of this phase coincides with the increase in frequency of wave generation.

One reason for an increased frequency, i.e., reduced wavelength, is related to a shortening of the refractory zone in the wave back due to reduced production of the inhibitor. Since the second phase is the only observed parameter that is reduced at increased frequencies of SD waves, it may be related to the refractory zone (Brand et al. 1997). In order to test whether potassium is responsible for this effect, we made a control experiment where we initiated the SD waves mechanically by means of a tiny glass needle. For this, the extracellular potassium concentration was held constant at 6 mM, whereas the frequency was changed. Table 1 summarizes the results. In fact, an increase in the frequency of mechanically stimulated waves (reduction of the period from 14.8 to 4 min) leads to a marked shortening of the second phase of more than 50%. Spontaneous generated waves at 20 mM extracellular K^+ concentration appear with a similar frequency (period 4.4 min, see Table 1), but they do no longer produce a second phase. This clearly demonstrates, that the reduction in the second phase at higher potassium concentrations is not only due to a reduction in the wavelength.

When K^+ exceeds 14 mM, the global swelling of the retinal tissue could mask the appearance of the second phase. In this case the physiological processes associated with the second phase would be still present, but due to the already swollen tissue they are no longer visible. Two experimental findings argue against this possibility.

Firstly, when the spontaneous generation of SD waves was blocked by Mg^{2+} ions, the mechanical generation of SD waves still led to the gradual decrease of the second phase with the increase of $[K^+]_o$ (Fig. 3B). Moreover, all SD waves had a white front (in contrast to the spontaneous SD waves in the presence of $[K^+]_o<2$, Fig. 3A). This clearly demonstrates the impact of K^+ on the second phase.

Secondly, the SD wave that was generated within the second phase of a preceding wave has a dark front. This corresponds to the inverted optical profile of SD waves in the presence of 1 mM K^+ (Fig. 3A). Similar results have been obtained by Martins-Ferreira and Castro (1966). In the presence of 20 mM $[K^+]_o$, regardless of the interval between waves, mechanically generated and spontaneously appearing waves had a white front. Although corresponding optical profile had only the one peak, it was not inverted such that a black wave appears (Figs. 2A and 3B). That means the SD waves propagated through the recovered tissue, and it rules out the presence of the second phase.

As previously mentioned, SD wave propagation is an autocatalytic reaction in which super-threshold production of an activator leads to delayed synthesis/activation of an inhibitor. Therefore, our interest is focused on those physiological/metabolic processes that are affected by increased potassium concentrations. One such process might be the dramatic changes in the energy metabolism associated with the breakdown of ion gradients during SD wave propagation.

The energetic profile of the refractory zone is dominated by glycolytic activity (Mayevsky et al. 1974; Harris et al. 1984; Mayevsky 1984; Scheller et al. 1992; Gault et al. 1994) and it is likely that the inhibitor of the autocatalytic reaction is connected to this pathway. For example, K^+ can change the K^+/ Na^+ ATPase activity and thereby the energy demand. Under conditions of increased $[K^+]_o$ K^+/ Na^+ ATPase does not work against such a great gradient as when $[K^+]_o$ is at physiological level. As a result, the increased $[K^+]_o$ leads to a reduction of the ATP consumption and a faster ion gradient recovery after SD wave propagation, i.e., a reduction of the second phase.

Future experiments will be directed toward the identification of the particular process that is responsible for the refractoriness of the spreading depression phenomenon.

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