Changes of cytoplasmic pH in frog nerve fibers during K^+-induced membrane depolarization

O.N. Valkina[a], O.V. Vergun[b], V.B. Turovetsky[c], B.I. Khodorov[b,*]

[a]*Ulyanovsk State Pedagogical University, Ulyanovsk, Russian Federation*
Institute of General Pathology and Pathophysiology, Russian Academy of Medical Sciences, Baltiyskaya Str. 8, 125315 Moscow, Russian Federation
[c]*M.V. Lomonosov Moscow State University, 119899 Moscow, Russian Federation*

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Abstract **Frog sciatic nerve and its thin bundles were loaded with fluorescein diacetate in order to monitor changes in cytoplasmic pH (pH_i) caused by high K^+ depolarization. Isosmotic substitution of external Na^+ by K^+ at pH_o 7.3 led to a steady concentration-dependent (20–120 mM K^+) decrease in pH_i. Elevation of pH_o from 7.3 to 8.5 prevented or even reversed these pH_i changes, indicating their strong dependence on transmembrane H^+ fluxes. The depolarization-induced intracellular acidification could not be prevented or decreased by any of the following treatments: removal of external Ca^{2+}; application of the Ca^{2+} antagonists Ni^{2+} and Co^{2+}; blockade of K^+ channels by TEA; addition to the external solution of Zn^{2+}, a blocker of putative voltage-sensitive H^+ channels. By contrast, blockade of Na^+ channels by 1–3 μM TTX prevented the effect of high K^+ concentrations on pH_i. It is concluded that the decrease in pH_i induced by a prolonged membrane depolarization in frog nerve fibers is mainly due to an enhanced H^+ influx through non-inactivating Na^+ channels.**

Key words: Nerve fiber; Intracellular pH; Na^+/H^+ exchange; Depolarization; Tetraethylammonium; Tetrodotoxin; FCCP; Sodium channel

1. Introduction

Earlier [1,2] it was shown that repetitive stimulation of frog nerve fibers combined with veratridine treatment induces a reversible decrease in cytoplasmic pH (pH_i). A comprehensive analysis of the origin of this effect led us to conclude that it results from a combined excessive influx of H^+ and Na^+ through 'hyperactivated' Na^+ channels. An increase in $[Na^+]_i$ suppresses the Na^+/H^+ exchange, which in turn enhances the cytoplasmic acidification produced by H^+ influx. The experimental data presented herein suggest that intracellular acidification in frog nerve fibers can be elicited not only by repetitive stimulation but by steady membrane depolarization, as well. In this case, however, the excessive influx of H^+ is mediated by non-inactivating voltage-sensitive Na^+ channels.

*Corresponding author.

Abbreviations: $[Na^+]_i$, intracellular sodium concentration; $[Na^+]_o$, $[K^+]_o$, $Ca^{2+}]_o$, external concentration of sodium, potassium and calcium respectively); pH_o and pH_i, external and internal (cytoplasmic) pH, respectively; CBS, control buffer salt solution; TEA, tetraethylammonium; TTX, tetrodotoxin; FCCP, carbonyl cyanide *p*-trifluoromethoxy phenylhydrazone; EGTA, ethyleneglycol bis(β-aminoethylether)-*N,N'*-tetraacetic acid; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; FDA, fluorescein diacetate; N, nerve trunk; F, nerve fiber bundle.

2. Materials and methods

Measurements of pH_i in frog (*Rana ridibunda*) isolated nerves and thin bundles of nerve fibers were performed by using the pH-sensitive fluorescent dye, fluorescein diacetate (FDA), and a fluorescent photometric microscope Lumam 13 (Russia) as described in detail earlier [1]. FDA was used as a pH probe because of its ability to readily cross the nerve sheaths, to penetrate into the axoplasm and to remain there for a relatively long period of time [1,2]. The isolated sciatic nerve was incubated for 15 min in the 10 μM FDA-containing control buffer solution (CBS) of the following composition (in mM): NaCl, 120; KCl, 2.5; $CaCl_2$, 1.8; HEPES, 20; pH 7.3 and room temperature (19–22°C). The 'low-Ca^{2+} solution' was prepared by adding 25 or 50 μM EGTA to nominally Ca^{2+}-free medium. During loading, FDA was hydrolyzed by cellular esterases and the resulting membrane-impermeable fluorescein was trapped inside the fiber. The fluorescence emission ratio for wavelengths 520 and 570 nm was used to calculate the absolute pH_i values from the corresponding calibration curve. The latter was obtained by the nigericine/K^+ method [3]. During each experiment, pH_i was repeatedly measured in fixed regions of the nerve trunk (N) and the nerve fibre bundle (F). The diameter of each photometric spot was 50 μM (objective ×10). In control experiments with untreated nerves, the fluorescence intensity during a 1–1.5 h monitoring period either did not undergo appreciable changes or slowly decreased, however, by no more than 25–30% of the control value (see [1]). However, the fluorescence ratio (i.e. pH_i) during this time either remained constant or diminished by only about 0.05 pH units. The $[K^+]_o$ was altered between 20 and 120 mM by isosmotic substitution of Na^+. To control for any effects of low $[Na^+]_o$, when $[K^+]_o$ was raised, in some experiments $[Na^+]_o$ was kept constant (120 mM) by addition of 60 mM KCl to CBS. The effect of the osmolarity increase caused by this procedure was estimated by adding 60 mM choline chloride to CBS. Electrical stimulation of the nerve trunk was accomplished by bipolar electrodes applied at a distance of 10–15 mm from the regions of fluorescence measurements. All the drugs used in this study were purchased from Sigma. Numerical data are presented as means ± S.E.M. of *n* experiments.

3. Results and discussion

3.1. Control measurements

In CBS, the baseline pH_i values measured in regions N and F of the nerve preparation were found to be 7.090 ± 0.004 and 7.161 ± 0.002 pH units ($n = 96$; Student's *t*-test; $P < 0.001$), respectively. The reason for this difference (revealed also in our previous studies [1,2]) is not clear. It is important, however, that in nerve fibers as well as in the majority of other cells [4] the baseline pH_i value is much higher than that predicted if H^+ ions were passively distributed. Indeed, for an average resting potential of −70 mV and a pH_o of 7.3, a pH_i value of 6.2 would be expected if H^+ ions were in electrochemical equilibrium across the membrane.

3.2. Effects of high $[K^+]_o$ on pH_i

Changing from the control solution to one containing 60 mM

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K^+ in order to depolarize the preparation produced a gradual decrease in pH_i. The rate and degree of this internal acidification increased with $[K^+]_o$ (Fig. 1a). The K^+-induced decrease in pH_i did not depend on the solution osmolarity and $[Na^+]_o$. Thus, addition of 60 mM KCl to CBS decreased pH_i by 0.140 ± 0.014 ($n = 6$) and 0.133 ± 0.032 pH units ($n = 6$) in N and F regions, respectively. In the case of isosmotic replacement of 60 mM NaCl with KCl, pH_i decreased by 0.152 ± 0.014 and 0.134 ± 0.016 pH units ($n = 17$) in N and F regions, respectively.

Repetitive stimulation (50 Hz) of the nerve in the presence of 60 or 120 mM of KCl induced an additional decrease in pH_i which partly or completely returned to the prestimulatory level after the termination of pulsing (Fig. 2). This evidently means that high-K^+ depolarization of the nerve membrane did not turn off the Na^+/H^+ exchange system responsible for the post-stimulatory recovery of pH_i in the HEPES-buffered salt solution.

Unfortunately, we were unable to measure the intracellular membrane potential (E) of nerve fibers directly and thus to relate the observed pH_i changes to E. It is known, however, that in frog nerve fibers the Na^+ channels responsible for generation of action potentials undergo a practically complete inactivation at $E = -40$ mV (for review see [5]). Meanwhile, in our experiments in the presence of 120 mM K^+, the nerve fibers retained the ability to propagate nerve impulses at least for the duration of the experiment. Therefore, repetitive stimulation of the nerve trunk produced a reversible decrease in pH_i measured at a distance of 10–15 mm from the stimulating electrodes (Fig. 2). To provide an explanation for this observation, it is necessary to accept that, owing to the numerous diffusional barriers created by lamellar connective tissue in the epineurium and perineurium, the true K^+ concentration in the pericellular space of the nerve fibers and, correspondingly, their true depolarization,

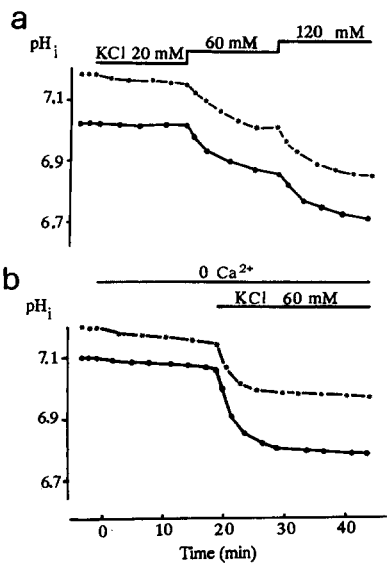

Fig. 1. (a) The K^+-induced decrease in pH_i rises with $[K^+]_o$. The successive increase in $[K^+]_o$ up to 20, 40 and 60 mM was obtained by isosmotic substitution of NaCl by KCl in a control solution (CBS) containing 1.8 mM K^+, pH 7.3. (b) Removal of Ca^{2+} from the solution (replacement of Ca^{2+} by 50 μM EGTA) does not prevent the pH_i decrease caused by 60 mM K^+. Changes in pH_i were monitored in the regions of the nerve trunk, N (●), and nerve fiber bundle, F (*).

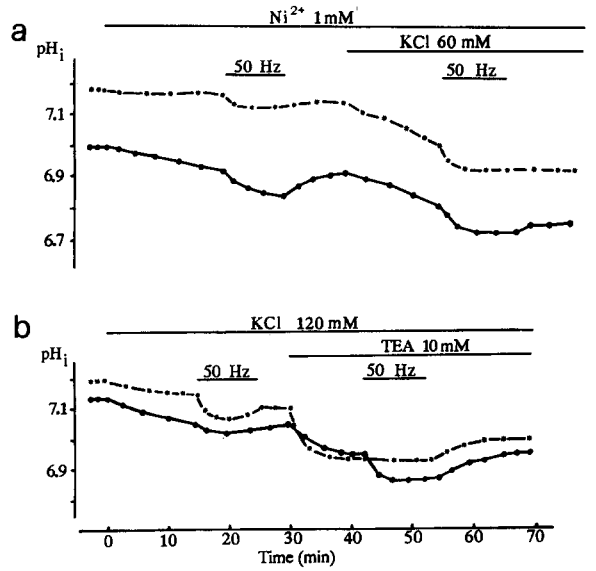

Fig. 2. (a) Ni^{2+} does not abolish the decrease in pH_i induced by K^+ (60 mM) and repetitive (50 Hz) stimulation. (b) TEA (10 mM) enhances the K^+-induced decrease in pH_i. Repetitive stimulation of the nerve causes an additional reversible decrease in pH_i. Designations are as in the legend to Fig. 1.

remain much lower than that theoretically expected for a $[K^+]_o$ of 60 or 120 mM.

3.3. Effect of removal of external Ca^{2+}

Membrane depolarization may lower pH_i through several mechanisms, one being the enhanced Ca^{2+} influx and a proton release from internal sites in response to elevation of free cytoplasmic Ca^{2+} activity [6–8]. In order to examine a possible involvement of external Ca^{2+} in the mechanism of cytoplasmic acidification induced by high K^+ concentrations, the external Ca^{2+} was replaced by 25 or 50 μM EGTA.

Substitution of Ca^{2+} by 50 μM EGTA in CBS either did not affect pH_i or induced a very small decrease – by 0.078 ± 0.017 and 0.062 ± 0.013 pH units ($n = 6$) in N and F regions, respectively. Addition of 60 mM KCl to this low-Ca^{2+} solution caused a relatively fast reduction of pH_i (Fig. 1b) by 0.220 ± 0.031 ($n = 6$) and 0.151 ± 0.014 pH units ($n = 6$) in N and F regions, respectively. These values did not practically differ from those obtained in analogous experiments with normal $[Ca^{2+}]_o$. Judging from the results obtained with frog nerve fibers and in contrast with those observed in neurons [6,7] and cardiac cells [6], the external Ca^{2+} is not involved in the mechanism of depolarization-induced cytoplasmic acidification.

3.4. Effects of Ni^{2+} and Co^{2+}

Ni^{2+} (1 mM) did not produce an appreciable change in pH_i by itself. Addition of 60 mM KCl to the Ni^{2+}-containing solution caused approximately the same decrease in pH_i – on average by 0.14 pH units in N and F regions compared to that in the control medium (Fig. 2a). It is noteworthy that Ni^{2+} did not prevent the reversible reduction of pH_i induced by repetitive stimulation of nerve fibers. The effects of Co^{2+} on baseline pH_i and its changes caused by 60 mM KCl were qualitatively similar to those in Ni^{2+} (data not shown). Thus, neither the lowering

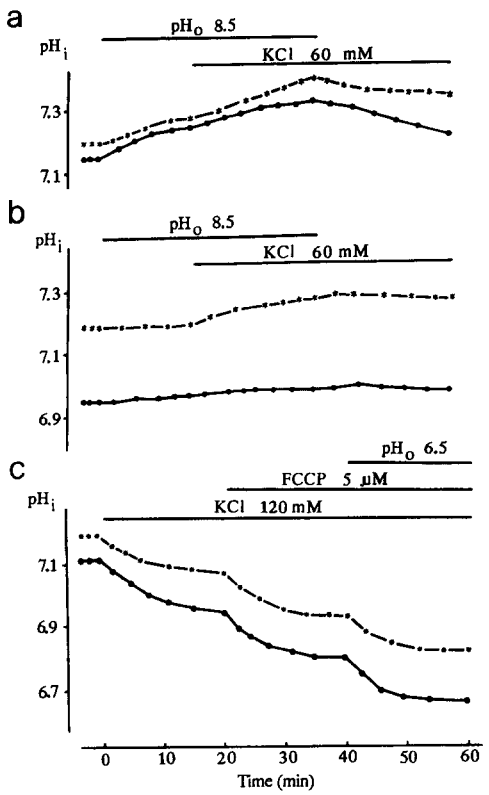

Fig. 3. (a and b) An increase in pH_o from 7.3 to 8.5 reverses the changes in pH_i induced by 60 mM K^+. (c) Membrane depolarization by 120 mM K^+ does not preclude a decrease in pH_i caused by the protonophore FCCP (5 μM). Designations are as in the legend to Fig. 1.

of external Ca^{2+} concentration nor the application of the inorganic Ca^{2+} antagonists, Ni^{2+} and Co^{2+} [9], were able to diminish the cytoplasmic acidification caused by high $[K^+]_o$.

3.5. *Effect of tetraethylammonium (TEA)*

In order to estimate the possible contribution of K^+ channel activation to the observed effects of high K^+ on pH_i, TEA, a well known blocker of these channels [9], was used. Addition of 10 mM TEA to CBS caused only a relatively small reduction of baseline pH_i: the latter decreased by 0.05–0.07 pH units in N and F regions, respectively ($n = 8$). The effect of TEA on K^+-induced cytoplasmic acidification is shown in Fig. 2b. It can be seen that 10 mM TEA considerably enhanced the decrease in pH_i caused by 120 mM KCl. Qualitatively similar results were obtained in other experiments of this series.

3.6. *The effect of alkalinization of the external medium*

To elucidate the role of external protons in the mechanism of K^+-induced lowering of pH_i, we examined the effects of alkalinization of the external medium. Fig. 3 illustrates the results of two representative experiments of this series. In Fig. 3a the increase of pH_o from 7.3 to 8.5 induced a gradual elevation of pH_i in both N and F regions of the preparation. Under these conditions, high K^+ lost the ability to decrease pH_i: instead it even accelerated and enhanced the pH_i elevation. A return to the original pH_o discontinued this cytoplasmic alkalinization, and the pH_i began to decay. Fig. 3b depicts the results of another experiment, in which the pH_i elevation from 7.3 to 8.5 induced only a relatively slight increase in pH_i. However, substitution of 60 mM NaCl with 60 mM KCl in this solution produced a clear-cut increase in pH_i, which remained at this level after returning the pH_o to 7.3 in the presence of 60 mM KCl. Similar results were obtained in another four experiments. Such effects of external alkalinization led us to conclude that K^+-induced cytoplasmic acidification in frog nerve fibers is mainly due to an increase of H^+ influx. The size of this influx is known to depend on two factors: the magnitude of the inward H^+ gradient and the membrane's permeability for H^+. Membrane depolarization decreases the driving force for the H^+ influx but at the same time activates voltage-sensitive H^+-permeable channels (e.g. H^+ and Na^+ channels), thus providing new pathways for H^+ entry. Judging by the results obtained in nerve fibers at pH 7.3, the second factor predominates. To verify this assumption we carried out some control experiments with the protonophore FCCP.

Addition of 5 μM FCCP to CBS induced a gradual reduction of pH_i. This effect could be enhanced by a decrease in pH_o to 6.5, indicating that the FCCP-induced acidification resulted from the H^+ influx. Membrane depolarization by 120 mM K^+ did not impede these effects of FCCP: the latter caused an additional decrease in pH_i (Fig. 3c). Finally the elevation of $[K^+]_o$ from 2.5 to 120 mM against the background of FCCP-induced internal acidification caused an additional decrease in pH_i (data not shown). These results clearly indicate that even at $[K^+]_o$ of 120 mM the electrochemical gradient for H^+ remained high enough to provide an enhanced H^+ influx. This conclusion agrees well with that made earlier (see section 3.2) concerning the relationship between $[K^+]_o$ and the true K^+ concentration in the inter-fiber clefts of the nerve.

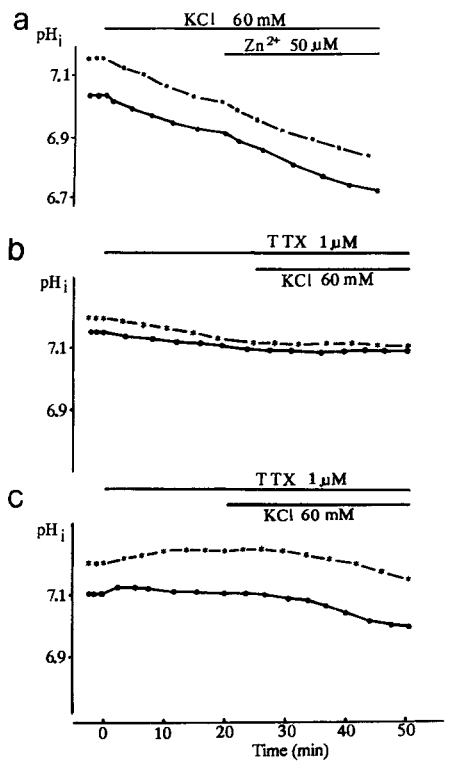

Fig. 4. The blocker of voltage-sensitive H^+ channels, Zn^{2+}, does not diminish the K^+-induced decrease in pH_i (a). By contrast, the blockade of Na^+ channels by TTX prevents (b) or considerably attenuates (c) the effect of 60 mM K^+ on pH_i. Designations are the same as in Fig. 1.

In subsequent experiments we attempted to identify possible pathways for an enhanced H^+ influx during a steady membrane depolarization caused by high $[K^+]_o$.

3.7. The effect of Zn^{2+}

Zn^{2+} is well known as a potent blocker of voltage-sensitive H^+ channels in a variety of cell types [10]. However, in our experiments ($n = 7$) 50 μM Zn^{2+} failed to prevent or attenuate a decrease in pH_i induced by 60 mM K^+. Moreover, addition of 50 μM Zn^{2+} to a high-K^+ solution caused an additional decrease in pH_i, as shown in Fig. 4a. Judging from these results in frog nerve fibers, the putative voltage-sensitive H^+ channels are not responsible for K^+-induced intracellular acidification.

3.8. Blockade of Na^+ channels by TTX

Voltage-sensitive Na^+ channels of nerve fibers are known to be highly permeable to H^+ [12]. In order to elucidate the possible involvement of these channels in the mechanism of depolarization-induced intracellular acidosis, we used TTX (1–3 μM). When applied to the nerve preparation alone, 1 μM TTX produced only a very slight change in the baseline pH_i. However, in the presence of 1 μM TTX, 60 mM K^+ usually failed to induce cytoplasmic acidification. Fig. 4b and c illustrate examples of this protective effect of TTX.

Qualitatively similar results were observed in another six experiments with 1 μM TTX and four experiments with 3 μM TTX. This led us to conclude that voltage-sensitive Na^+ channels provide a major pathway for the H^+ movement responsible for the depolarization-induced decrease in pH_i in frog nerve fibers. As far as classical Na^+ channels responsible for action potential generation undergo fast inactivation during a maintained membrane depolarization, we have to conclude that the persistent H^+ influx during a steady membrane depolarization is mediated by non-inactivating Na^+ channels revealed in various cells [13], including frog nerve fibres [14].

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