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Effects of repetitive stimulation, veratridine and ouabain on cytoplasmic pH in frog nerve fibres: role of internal Na^+

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Changes of cytoplasmic pH (pH_i) in frog nerve fibres during repetitive stimulation have been measured using the fluorescent pH indicator dye fluorescein diacetate (FDA). Under control conditions repetitive (10–50 Hz) stimulation caused only a very small decrease in pH_i (by 0.015 0.06 pH units). Modification of Na^+ channels by veratridine (VER, 10 μM) greatly increased this stimulus-evoked (SE) internal acidification. Blockade of the Na^+-K^+ pump by ouabain (0.5 mM) enhanced the effects VER and prevented pH_i recovery after the termination of repetitive stimulation. A similar inhibition of post-stimulatory recovery of pH_i was observed after replacement of external Na^+ with Li^+, which is not accepted by the Na^+-K^+ pump instead of Na^+. These data suggest that SE intracellular acidification in nerves results from or is closely associated with an increase in $[Na^+]_i$. Treatments that promote Na^+ influx and accumulation of Na^+ inside the fibre enhance reduction of pH_i. Li^+ can be substituted for Na^+ in this process.

Nerve fibre; Intracellular pH; Veratridine; Ouabain; Stimulation

1. INTRODUCTION

External Na^+ plays a key role in the regulation of cytoplasmic pH (pH_i), providing there is a normal mode of operation of Na^+/H^+ and N^+-coupled Cl_3^-/HCO_3^- exchangers [1–4]. In contrast, the role of internal Na^+ in pH_i regulation has not yet been studied systematically, although it has been shown that activation of the ATP-fueled Na^+/K^+ pump by internal Na^+ is accompanied by an increase in the rate of proton generation and their extrusion in to the external medium [5]. It has also been established that the activity of Na^+/H^+ and Na^+/Ca^{2+} exchangers depend on a transmembrane Na^+ gradient [3,6]. The experimental data presented in this paper indicate that all the treatments of nerve fibres that increase the influx and accumulation of Na^+ (or Li^+) in the axoplasm during repetitive nerve impulse propagation promote the development of intracellular acidosis.

2. MATERIALS AND METHODS

Experiments were carried out with frog (*Rana ridibunda*) isolated nerves (n. ischiadicus), at room temperature (19–22°C). Measurements of pH_i were performed using a version of the spectrofluorimetric method described in [7] and adapted for isolated nerves by [8–10].

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Abbreviations: $[Na^+]_i$, $[Ca^{2+}]_i$, intracellular sodium and calcium (respectively) concentrations; pH_i, internal (cytoplasmic) pH; HEPES, 4-(2-hydroxyethyl)-1-piperazineethane sulfonic acid; VER, veratridine.

Fluorescein diacetate (FDA; Serva) was employed in these experiments for measurement of pH_i since it was shown to be able to cross the envelopes of the nerve trunk relatively easily and to remain in the axoplasm for a relatively long time [8–10]. In order to introduce the fluorescent probe into the fibres, the isolated nerve was incubated for 15 min in FDA (10 μM)-containing control buffer solution (CBS) of the following composition: 120 mM NaCl, 2.5 mM KCl, 1.81 mM $CaCl_2$, 20 mM HEPES, pH 7.3, at room temperature. During loading the permeable FDA was hydrolyzed by cellular esterases and the resulting membrane-impermeant fluorescein was trapped in the cytoplasm. The nerve was then washed-out with CBS, and transferred to an experimental chamber where a length of sheath of about 5 mm was removed and thin nerve bundles were pulled apart. The intensity of fluorescence was measured by using a luminescent photometric microscope Lumam I3 (Russia) equipped with a set of interference light filters. Excitation of nerve fluorescence was achieved using an halogen glow lamp, KGM 9-70, and a combination of the glass light filters, FS 1-4 and SZS 21-2. The intensity of fluorescence emission was measured at wavelengths of 520 and 570 nm, and the fluorescence ratio 520/570 was used to determine the absolute pH_i values according to the corresponding calibration curve. The latter was obtained with the help of the nigericin/K^+ method of Thomas et al. [11]. Nerve autofluorescence determined from preparations not exposed to FDA was less than 2% of the total signal.

During each experiment pH_i was repeatedly measured in the same region of the nerve trunk and nerve fibre bundles. The diameter of each photometric spot in these regions was of 50 μm (at an objective lens of 10×). In the control experiments on untreated nerves, the fluorescence intensity during an 1–1.5 h monitoring either did not undergo appreciable changes (Fig. 1a) or slowly decreased, but by no more than by 25–30% of its control value. However, the fluorescence ratio, i.e. pH_i, during all this time remained constant or diminished by only about 0.05 pH units. Electrical stimulation of the nerve trunk was accomplished using bipolar electrodes mounted in the bottom of the experimental chamber at a distance of 10–12 mm from the region of fluorescence measurements. Square suprathreshold voltage stimulus

pulses of 50-μs duration were delivered by an electrostimulator (ESL-2; Russia). A stock solution of veratridine (Sigma) was prepared to a concentration of 1.49 mM (mg per ml) with the addition of equimolar amounts of HCl. FDA was dissolved in acetone (4 mM stock solution). LiCl replaced NaCl mole for mole.

3. RESULTS AND DISCUSSION

3.1. *Control measurements*

In the control buffer solution (CBS) basal pH_i measured in the region of the nerve trunk (N) and nerve fibre bundles (F) was found to be 7.068 ± 0.004 (n = 153) and 7.124 ± 0.006 (n = 124), respectively. This difference is statistically reliable ($p < 0.001$) and similar to that revealed earlier [9] in analogous experiments with frog nerves. Changes in pH_i measured in N and F regions during repetitive pulsing proved to be qualitatively similar, however, quantitatively pH_i responses in F were usually a bit more pronounced than in N. Repetitive stimulation at frequencies (f) of 50 Hz as a rule caused only a very small decrease in pH_i: by 0.015 ± 0.004 (n = 5) in N and 0.068 ± 0.006 (n = 5), in F. Even at f = 300 Hz, pH_i decreased by no more than 0.05–0.08 pH units.

3.2. *Effects of veratridine (VER)*

The stimulus-evoked (SE) reduction of pH_i was greatly enhanced by treatment of nerve preparations (in N and F sections) with the Na agonist, veratridine (VER) [10], which induced a considerable increase in Na^+ influx during repetitive membrane stimulation [11,12]. When applied to the resting nerve, 10 μM VER caused a gradual decrease in pH_i which varied widely in different experiments (from 0.02 to 0.2 pH units). Fig. 1b and c illustrate examples of the variations in the effects of VER. On average, the VER-induced decrease in pH_i was of 0.049 and 0.060 pH units in F and N regions, respectively (n = 76). This decrease underwent a further reduction (on average by about 0.1) during 50-Hz repetitive stimulation (Figs. 1b,c and 2b,c). After the termination of this stimulation pH_i gradually returned to its pre-stimulatory level (see Fig. 1b and 2b). Often this pH_i recovery had already begun during the continuing stimulation, as shown in Figs. 1c and 2b,c. It was logical to suppose that a reduction of pH_i induced by VER treatment and subsequent repetitive stimulation was due to an enhanced influx of Na^+ into the fiber through VER-modified Na^+ channels, and, correspondingly, that the post-stimulatory recovery of pH_i reflects active extrusion of excessive Na^+ in the external medium. To test this suggestion we have carried out experiments with ouabain, a selective inhibitor of the Na^+/K^+ pump.

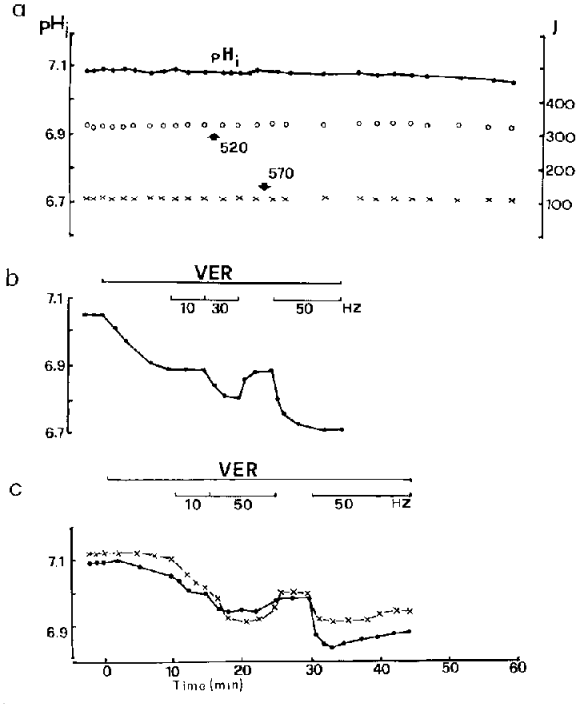

Fig. 1. (a) Control long-lasting monitoring of pH_i in non-treated nerve trunk (left ordinate); shown also are corresponding fluorescence intensities measured at 520 and 570 nm emission wavelengths (right ordinate). (b,c) Two examples of VER- and stimulus-induced changes in pH_i in the region of nerve trunk (●) and nerve fibre bundles (x). Concentration of VER, 10 μM. Stimulation frequency: 10, 30 and 50 Hz.

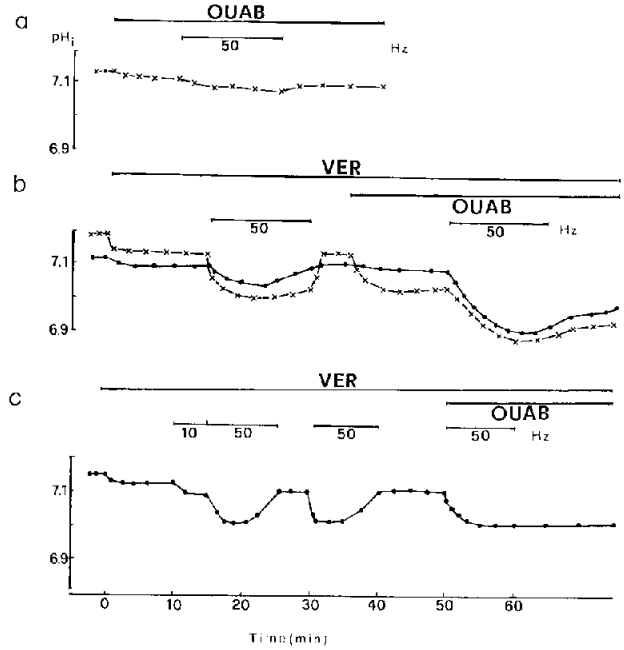

Fig. 2. Effects of ouabain (OUAB, 0.5 mM) on stimulus-evoked intracellular acidification without (a) and in the presence (b,c) of VER (10 μM). Designations are the same as in Fig. 1.

3.3. *Blockade of Na^+/K^+ pump by ouabain*

Ouabain (0.5 mM), upon its external application to the resting nerve, caused a gradual small decrease in pH_i. Even after a 30-min ouabain treatment, reduction of pH_i in N and F regions did not exceed, respectively, 0.03 and 0.08 pH units (n = 12). Repetitive stimulation

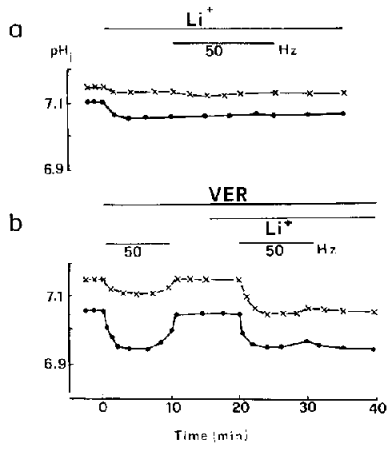

Fig. 3. Effects of replacement of external Na^+ by Li^+ on the stimulus-evoked decrease in pH_i in the control (a) and VER (10 μM)-containing solutions. Designations are the same as in Fig. 1.

(50 Hz) of ouabain pretreated nerves usually caused only a small additional decrease in pH_i (on average by 0.075 pH units), similar to that observed in control experiments with non-treated nerves (Fig. 2a). However, when applied to the VER-pretreated nerve (10 preparations) ouabain enhanced the pH_i decrease during repetitive stimulation, and, most importantly, it abolished almost completely both the post-stimulatory recovery of pH_i and its spontaneous recovery during repetitive stimulation (Fig. 2b,c). This is just wat we had expected, assuming that the SE-induced decrease in pH_i results from increasing $[Na^+]_i$. It is also clear that recovery of pH_i after the termination of repetitive stimulation and its spontaneous recovery during a continuing pulsing (see Figs. 1 and 2) are of the same origin: both result from extrusion of Na^+ from the fibre by the Na^+/K^+ pump.

3.4. *Effect of replacement of external Na^+ by Li^+*

Li^+ is known as an excellent substitute for Na^+ in spikes and after-potential generation both in normal and VER-treated nerve fibres ([13,14], our unpublished observations). Li^+ also replaces Na^+ in Na^+/H^+ exchange [1,4]. However, unlike Na^+, Li^+ cannot be extruded from the cell either by the Na^+/K^+ pump or by the Na^+/Ca^{2+} exchanger [6]. In our experiments, equimolar replacement of external Na^+ by Li^+ did not appreciably affect either the basal pH_i or its change during repetitive stimulation. However, in the presence of VER, Li^+, like ouabain, prevented the post-stimulatory recovery of pH_i (Fig. 3b). This evidently means that reduction of pH_i can be induced not only by an increase in $[Na^+]_i$ but also by accumulation of Li^+ inside the fibre.

Now the question should be posed: how can an increase in $[Na^+]_i$ induce intracellular acidification? There exist several possibilities for interpretation of this effect. Some of them, however, can be discarded right away taking into account the results of the above experiments. Thus it is quite clear that a SE-induced decrease in pH_i cannot be explained by activation of the Na^+/K^+ pump associated with an increase in the rate of ATP turnover and enhanced proton generation [5]: we have seen that inhibition of this pump by ouabain did not impede, rather it enhanced and stabilized the pH_i reduction (see Fig. 2b,c). Another possible explanation of SE changes in pH_i could be that an increase in $[Na^+]_i$ enhances the exchange of internal Na^+ for external Ca^{2+} which may compete with H^+ for some common intracellular buffers (see [15] for references). This, although at glance an attractive hypothesis, is, however, disproved by our observation that replacement of external Na^+ with Li^+ enhances and stabilizes the SE decrease of pH_i in the presence of VER (see Fig. 3b), although the Na^+/Ca^{2+} exchanger does not accept Li^+ instead of Na^+. Therefore, we believe that the most probable reason for the stimulus-induced decrease in cytosolic pH_i is the inhibition of the Na^+/H^+ antiporter which, under normal conditions, prevents the spontaneous acidification of cytoplasm caused by a continuous influx of H^+ down their electrochemical gradient [1–4]. This problem, as well as a possible contribution of H^+ influx through the open Na^+ channels, in the mechanism of SE intracellular acidosis, will be considered in more detailed in a separate paper.

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