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The Ca^{2+}:H^+ coupling ratio of the plasma membrane calcium ATPase in neurones is little sensitive to changes in external or internal pH

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ABSTRACT

To explore the effects of both external and internal pH (pH_o and pH_i) on the coupling between Ca^{2+} extrusion and H^+ uptake by the PMCA activity in snail neurones H^+ uptake was assessed by measuring surface pH changes (ΔpH_s) with pH-sensitive microelectrodes while Ba^{2+} or Ca^{2+} loads were extruded. Ru360 or ruthenium red injection showed that injected Ca^{2+} was partly taken up by mitochondria, but Ca^{2+} entering through channels was not. External pH was changed using a mixture of three buffers to minimise changes in buffering power. With depolarisation-induced Ca^{2+} or Ba^{2+} loads the ΔpH_s were not changed significantly over the pH range 6.5–8.5. With Ca^{2+} injections into cells with mitochondrial uptake blocked the ΔpH_s were significantly smaller at pH 8.5 than at 7.5, but this could be explained in part by the slower rate of activity of the PMCA. Low intracellular pH also changed the ΔpH_s responses to Ca^{2+} injection, but not significantly. Again this may have been due to reduced pump activity at low pH_i. I conclude that in snail neurones the PMCA coupling ratio is either insensitive or much less sensitive to pH than in red blood cells or barnacle muscle.

1. Introduction

Cell calcium must be kept very low to allow fast and efficient intracellular Ca^{2+} signalling. In nerve cells two ATPase pumps are involved in removing Ca^{2+} from the cytoplasm. These are the sarco-endoplasmic reticulum calcium ATPase (SERCA) which drives uptake into the endoplasmic reticulum and the plasma-membrane calcium ATPase (PMCA) which extrudes Ca^{2+} across the plasma membrane. As first shown by Niggli et al. many years ago the latter couples the efflux of Ca^{2+} to the uptake of H^+ ions [1]. The properties of the PMCA have been reviewed recently [2,3]. The PMCA is inhibited by extracellular alkalinisation and intracellular acidification [4–8]. In some cells there is a second plasma membrane Ca^{2+} pump driven by Na^+ influx rather than by ATP, the Na^+/Ca^{2+} exchanger, but in many nerve cells the PMCA is the principle Ca^{2+} extrusion mechanism at low Ca^{2+} loads [4,7,9,10].

In snail neurones the PMCA is the only mechanism for calcium extrusion from the cell body [11], even though it is sometimes described as a low-capacity system [3]. The ratio of the coupling between Ca^{2+} extrusion and H^+ uptake at normal pH levels by the PMCA remains controversial. While the early workers [1] concluded that the Ca^{2+}:H^+ ratio was 1:2, some subsequent work has suggested fewer H^+ ions are transported [12–14]. A recent review [2] states categorically that the ratio is 1:1, as does a recent physiology textbook [15]. Many biochemistry textbooks still report that the PMCA is a uniporter (see [14]). There have also been several reports that the PMCA is electrogenic, for example in hair cells [16] and in red blood cell preparations [17,18]. Electrogenicity implies that the coupling is not $1Ca^{2+}$:$2H^+$. In contrast, in snail neurones I recently found that the ratio under normal conditions is $1Ca^{2+}$:$2H^+$ [19].

The effects of extracellular pH (pH_o) on the coupling ratio have been studied in red blood cells and barnacle muscle, but not in nerve cells. In red blood cell PMCA preparations the Ca^{2+}:H^+ ratio is changed by pH_o from about 1:2 at pH 6.5 to 1:0 at pH 8.5 [20]. Similarly in barnacle muscle at external pH 6.5 the PMCA coupling ratio was 1:3, while at pH 8.2 the ratio was 1:1 [21]. The effects of changes in intracellular pH (pH_i) have not apparently been investigated in any cell. The quantity of H^+ ions pumped into neurones by the PMCA is important because many channels and carriers are sensitive to small pH changes [22,23]. The possible pH sensitivity of the coupling ratio may also have important consequences for the molecular mechanism of the PMCA.

To investigate the effects of pH on the coupling ratio in nerve cells I have extended my recent experiments [19] on the large neurones of the common snail *Helix aspersa.* Using both conventional and ion-sensitive microelectrodes I have recorded membrane potential, clamp currents, intracellular and surface pH and intracellular Ca^{2+} in intact cells. The PMCA was stimulated by depolarisation in Ca^{2+} or Ba^{2+} solutions or by direct iontophoretic

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injection of Ca^{2+}. The subsequent PMCA-generated surface pH changes (ΔpH_s) were measured. The two other processes that might change surface pH, namely intracellular pH regulation and H^+ channels, were inhibited or kept inactivated. Normal pH_i regulation was inhibited by removal of bicarbonate [24] which leaves only a relatively weak Na^+/H^+ exchanger [25]. H^+ channels were kept closed by avoiding large depolarisations [26]. To ensure that injected Ca^{2+} was not taken up by mitochondria, cells were injected with ruthenium red [27] or Ru360 [28]. This proved unnecessary for cells loaded by depolarisation. For the experiments on the effects of pH_o I measured the ΔpH_s for equal depolarisations or Ca^{2+} injections at different pH_o values. For the experiments on pH_i effects I measured the ΔpH_s induced by equal Ca^{2+} injections while changing pH_i by HCl injection or application and removal of CO_2/bicarbonate. With pH_o between 7.5 and 8.5 I have found significant changes in the PMCA-induced ΔpH_s only with injected Ca^{2+} loads. Allowing for variation in the pump rate with pH, these findings suggest that the snail neurone PMCA coupling ratio is little changed by external or internal pH.

2. Methods

2.1. General

Experiments were done on large (150–250 μm diameter) neurones in isolated sub-oesophageal ganglia of the common snail, *H. aspersa* [8,19,29]. Cells were voltage-clamped to −50 mV using two microelectrodes. The PMCA was stimulated either by depolarisations in Ca^{2+} or Ba^{2+} snail Ringer's solution, or by iontophoretic injection of Ca^{2+} ions. The changes in surface pH (ΔpH_s) resulting from the subsequent extrusion of Ba^{2+} or Ca^{2+} were measured using pH-sensitive microelectrodes which were pressed against the surface of the chosen cell. The effects of changing pH_o were assessed by changing the superfusate pH using a mixture of three different buffers (with different dissociation constants) to minimise changes in buffering power. To reduce pH_i, H^+ ions were injected by iontophoresis, while to increase pH_i the preparation was first equilibrated with bicarbonate-buffered saline and then returned to normal snail Ringer's solution. The consequent loss of accumulated intracellular bicarbonate as CO_2 caused a pH_i increase of 0.4–0.5 units. In some experiments changes in intracellular Ca^{2+} were followed with a Ca^{2+}-sensitive microelectrode (CaSM).

2.2. Preparation

An aestivating snail was killed by rapid removal of the circumoesophageal ring of ganglia, and the large cells on the dorsal side of the suboesophageal ganglia exposed as previously described [19]. All experiments were carried out at room temperature, 18–23 °C, starting at least 1 h after the dissection.

2.3. Solutions

The normal snail Ringer solution contained (mM): 80 NaCl, 4 KCl, 7 $CaCl_2$, 5 $MgCl_2$, and 20 Hepes, titrated with NaOH to pH 7.5. Solutions of pH 6.5, 7.5 and 8.5 with a low buffering power had 2 or 5 mM of Pipes, Hepes and Taps, with additional NaCl to maintain tonicity. pH 9.5 Ringer was buffered with 20 mM CHES. Ba Ringer solutions had the same ionic composition but with $BaCl_2$ replacing $CaCl_2$. The CO_2 Ringer solution was the same as normal except that it had 20 mM $NaHCO_3$ instead of Hepes, was bubbled with 2.5% CO_2 in air and contained 0.1 mM NaH_2PO_4.

Ruthenium red was dissolved in 0.1 M KCl at 10 mg ml^{-1} [31], and Ru360 was made up at 500 nM in 0.1 M KCl with 0.1% Fast green FCF to make it visible when injected. Aliquots of 0.1 ml of the solution were kept frozen until use. Ruthenium red injection pressure-injection electrodes often blocked, so Ru360 was preferred in later experiments. Both compounds had the same effects.

2.4. Microelectrodes

Conventional micropipettes were pulled from 1.2 mm filamented borosilicate glass tubing and backfilled with 1 M CsCl for passing clamp current or recording membrane potential. Microelectrodes for iontophoretic injection were filled with 0.1 M $CaCl_2$ with tips broken by touching a pin in the bath to give resistances of 5–10 MΩ. For H^+ injection microelectrodes were pulled from quartz tubing filled with 1 M HCl and left with tips intact.

Intracellular CaSMs were made from quartz glass and pHSMs from borosilicate glass [30,31]. For surface pH, both liquid ion sensor and Hinke-style glass pH microelectrodes [32] were used. The latter had the advantage of recording from a large area, and being less likely to penetrate the cell membrane.

2.5. Data collection and analysis

Potentials from the conventional microelectrodes, CaSMs and pHSMs were recorded as voltages referred to membrane potential, with the voltages from the pHSMs converted to pH before display. Potentials from the surface pHSMs were referred to the bath potential. Results were discarded if on withdrawal of an electrode its potential in Ringer solution had changed by more than 7 mV.

Potentials from the voltage-recording microelectrodes were led via preamplifiers in the Faraday cage to an 8-pole Bessel filter and recorded at 20 Hz on a PC via a CED micro 1401 interface and Spike 2 data collection program (Cambridge Electronic Design, UK). The clamp current was recorded at 100 Hz as above.

Figures were prepared from the CED data after loading into Microsoft Excel. Spikes in the V_{Ca} or pH records generated by electronic pickup were partially erased, and the clamp current records were in some cases restricted in range. Data are presented as means ± S.E.M. of n observations. The statistical significance of observed differences was determined by a paired two-sample two-tailed t-test for means. Differences between means were considered significant when $P < 0.05$.

3. Results

3.1. Ca^{2+} uptake by mitochondria or extrusion by the PMCA

The PMCA is continually active, maintaining the normal low $[Ca^{2+}]_i$ in the face of a continuous influx even in a cell voltage-clamped at −50 mV. To investigate the coupling between Ca^{2+} extrusion and H^+ uptake I have chosen to stimulate the PMCA with a brief, repeated, additional load of Ca^{2+} or Ba^{2+} ions. I assume that the coupling does not change with rate of activity. A Ca^{2+} load can be delivered in several ways. The simplest way is by a brief depolarisation to open Ca^{2+} channels, while a second more controllable method is by iontophoretic injection of Ca^{2+} ions [32].

In initial experiments I measured the intracellular pH change resulting from the uptake of H^+ while an injected Ca^{2+} load was extruded. With a number of assumptions, including that all the injected Ca^{2+} was extruded, I was able to assess possible changes in coupling ratio. This last assumption, however, proved untenable, as was indeed suggested by earlier work with pressure-injected Ca^{2+} [33]. My later experiments confirmed that some of the injected Ca^{2+}, but not Ca^{2+} entering through channels, was taken up by mitochondria, as shown in Fig. 1. In this experiment I recorded both pH_i and V_{Ca} (the potential from the CaSM referred to the membrane potential) and stimulated the PMCA by both depolarisations and Ca^{2+} injections. The first three depolarisations each caused an