USE OF Ca–EGTA BUFFERS FOR TRANSPOSING Ca^{2+} EFFLUX INTO pH CHANGES

Application to rod outer segment membrane vesicles

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1. Introduction

Ca–EGTA buffers are commonly used to stabilize Ca^{2+} between 10^{-8} M and 10^{-5} M [1], i.e., the order of magnitude generally accepted for the intracellular concentration of free Ca^{2+}. These buffers present 2 other interesting properties: the binding of Ca^{2+} by EGTA is accompanied by H^+ release [2] and biological membranes are weakly permeable to EGTA [3]. These properties can be used to directly measure an efflux of Ca^{2+} from a vesicular pool with a simple glass electrode.

One of the current hypotheses concerning the mechanism of visual transduction in vertebrate photoreceptors is that light could induce a release of Ca^{2+} from the rod discs [4]. Attempts to test this hypothesis have however led to conflicting results: no release [5–8] or a release of about 1 Ca^{2+}/rhodopsin [9–12] was detected using $^{45}Ca^{2+}$ and atomic absorbance methods; a rapid release of 0.03 Ca^{2+}/rhodopsin [13] was measured with arsenazo III. Apart from the study [9] in which intact rods were used, all positive reports are concerned with vesicles loaded with Ca^{2+} by sonication, which is likely to damage the integrity of the membrane.

Using the Ca–EGTA method, we have failed to detect a rapid light-induced release of Ca^{2+} from right side out vesicles from rod outer segment (ROS) membranes loaded with Ca^{2+} by dialysis; the ability of these vesicles to retain Ca^{2+} ions was studied by measuring the release of Ca^{2+} induced by addition of the Ca^{2+} ionophore X537A. As a control, the same experiments were carried out using a Ca^{2+}-selective electrode.

2. Materials and methods

2.1. *Principle of the Ca–EGTA method of Ca^{2+} titration*

EGTA has 4 acid–base groups and therefore exists in 4 differently-ionized forms which are in pH-dependent equilibrium. The proportion of these different forms as a function of pH at 20°C and in 0.1 M KCl is represented in fig.1. The equilibrium between the 4 forms of EGTA can be written as follows:

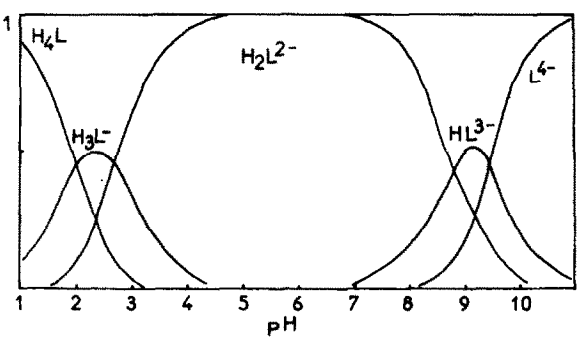

Fig.1. Proportion of the different ionization states of EGTA as a function of pH (20°C, 0.1 M KCl). The affinity constants used for the calculations ($K_{L^{4-}} = 10^{9.46}$ M^{-1}; $K_{HL^{3-}} = 10^{8.85}$ M^{-1}; $K_{H_2L^{2-}} = 10^{2.68}$ M^{-1}; $K_{H_3L^-} \simeq 10^2$ M^{-1}) are those given in [14].

$$H_4L \underset{H^+}{\rightleftharpoons} H_3L^- \underset{H^+}{\rightleftharpoons} H_2L^{2-} \underset{H^+}{\rightleftharpoons} HL^{3-} \underset{H^+}{\rightleftharpoons} L^{4-}$$

The affinity of EGTA for Ca^{2+} depends on its ionization state; the stability constants at pH 7.1 and 20°C are: $K_{L^{4-}} = 10^{6.882}\ M^{-1}$ and $K_{HL^{3-}} = 10^{3.572}\ M^{-1}$ [14]. Binding of Ca^{2+}, mainly by the L^{4-} form, shifts the equilibrium to the right. At pH 7.1, EGTA is almost entirely in the H_2L^{2-} form (fig.1); binding of 1 Ca^{2+} therefore results in the release of 2 H^+. The number of H^+ released per Ca^{2+} bound decreases with increasing pH as the L^{4-} form becomes more favoured (half-value at pH 9.15).

Other metal ions, particularly Mg^{2+}, are chelated by EGTA [14] and can therefore interfere with the Ca^{2+} titration. It can be calculated from the stability constants that 1.23 H^+ is released per bound Mg^{2+} at pH 7.1 and 20°C. The affinity of EGTA for Mg^{2+} is however much lower than for Ca^{2+}, and part of the bound Mg^{2+} is released upon addition of Ca^{2+}, so that the no. (n) of H^+ released per Ca^{2+} added is smaller than when Mg^{2+} is absent.

If

$$\left[Ca^{2+}_{added}\right] < [EGTA] << \left[Mg^{2+}_{total}\right]$$

$$n \simeq 2 - 1.23 \frac{K'_{Mg^{2+}} \left[Mg^{2+}_{total}\right]}{1 + K'_{Mg^{2+}} \left[Mg^{2+}_{total}\right]}$$

At pH 7.1 and 20°C ($K'_{Mg^{2+}} = 53\ M^{-1}$), in 5 mM $MgCl_2$, $n = 1.74$.

The method for calculating the concentration of free Ca^{2+} in Ca–EGTA buffers, and its modification in the presence of Mg^{2+} is in [1]. At pH 7.1 and 20°C, without Mg^{2+}, $[Ca^{2+}]$ varies from 10^{-8}–10^{-5} M when the ratio $[Ca^{2+}]/[EGTA]$ varies from 0.07–0.99. Since EGTA does not readily cross biological membranes, a pCa gradient is established when Ca^{2+}-loaded vesicles are suspended in a Ca–EGTA buffer. Ca^{2+} released from the vesicles and chelated by EGTA in the external medium can thus be detected by the resulting H^+ production.

The interest of this method of Ca^{2+} efflux measurement resides in its simplicity and very high sensitivity at neutral pH and in weakly-buffered solutions. Although the Ca^{2+}-selective electrode can be used over a wider pH and pCa range, its sensitivity is usually much lower than that of the H^+ electrode, except if very low $[Ca^{2+}]$ ($< 10^{-5}$ M) can be obtained without adding complexing agents. In our experiments with ROS membranes vesicles, 0.1–0.2 nmol Ca^{2+} ($\left[Ca^{2+}_{out}\right]$ 10^{-7}–10^{-6} M) could be currently detected with the Ca–EGTA buffer method, compared to 1–3 nmol Ca^{2+} ($\left[Ca^{2+}_{out}\right]$ 10^{-5}–10^{-4} M) with the Ca^{2+}-selective electrode. A sensitivity of 1–10 nmol Ca^{2+} was reported in [15] for the Ca^{2+}-selective electrode.

2.2. *Application to Ca^{2+}-loaded vesicles from ROS membranes*

Bovine ROS membranes prepared according to [16] are used fresh or after storage in liquid nitrogen. Incubation of these vesicles with papain leads to complete disappearance of the rhodopsin band in SDS–polyacrylamide gel electrophoresis [17], indicating that the vesicles are right side out [12].

Vesicles are loaded with Ca^{2+} by overnight dialysis at 4°C against 100 mM KCl and the desired $CaCl_2$ and buffer concentration. A pCa gradient is then established by diluting the membrane suspension in at least 10-times its volume of Ca^{2+}-free medium, basically 100 mM KCl, 0–10 mM $MgCl_2$ (pH 7–7.1). Experiments are carried out at 20°C and 37°C.

When the Ca–EGTA method is used, the amount of EGTA in the external medium is calculated according to the volume of the suspension of Ca^{2+}-loaded vesicles added in order to have $\left[Ca^{2+}_{out}\right]$ 10^{-7}–5.10^{-7} M. Low concentrations of buffer (< 1 mM), EGTA (< 1 mM) and rhodopsin ($< 20\ \mu$M) are used in order to increase the sensitivity.

A Ca^{2+}-selective electrode (Radiometer F 2112 Ca) is used as a control at higher concentrations of external calcium ($\left[Ca^{2+}_{out}\right]$ 10^{-5}–10^{-4} M), in order to extend the range of experimental conditions, and to detect possible artefacts due to EGTA. The sensitivity of the Ca^{2+}-electrode to H^+ is eliminated by using well-buffered solutions (Tris–maleate (pH 7–7.1) 20 mM). Large amounts of rhodopsin can be used without decreasing the sensitivity as long as the amount of external Ca^{2+} remains low.

pH and pCa changes are measured with a Radiometer pHM 64 pH meter and REC 61 servograph recorder equiped with the REA 112 high-sensitivity unit. The H^+ and Ca^{2+} signals are calibrated by injection of HCl and $CaCl_2$.

Bleaching is achieved by means of an electronic flash (Sunpak Autozoom 3400, duration 0.4 or 3 ms),

low intensity flashes being obtained by interposing neutral filters. The proportion of rhodopsin bleached by strong flashes is measured by A_{500} of aliquots of the bleached and unbleached suspension solubilized in 3% Ammonyx LO.

The Ca^{2+} ionophore X537A is used at 20–50 μM final conc. (ionophore/rhodopsin = 2–10).

3. Results and discussion

3.1. *Measurement of the release of Ca^{2+} induced by the Ca^{2+} ionophore X537A*

Addition of X537A to Ca^{2+}-loaded (1–20 mM) vesicles suspended in low [Ca^{2+}] solution produces a rapid H^+ release when EGTA is present in the external medium; the no. of H^+ released is proportional to the [Ca^{2+}] in the loading solution up to 10–15 mM, above which a saturation occurs. No effect is observed in the control experiments without EGTA. The H^+ release therefore results from the binding by EGTA of Ca^{2+} released from the vesicles by the ionophore. The ability of the vesicles to retain Ca^{2+} for a given time depends on the temperature and on the composition of the medium: smaller amounts of Ca^{2+} are retained at 37°C than at 20°C, particularly if no $MgCl_2$ is present; replacing KCl by NaCl also reduces the amount of Ca^{2+} retained.

Similar results are obtained when the X537A-induced release of Ca^{2+} from Ca^{2+}-loaded vesicles (1–5 mM) is measured with a Ca^{2+}-selective electrode in the absence of a Ca–EGTA buffer.

The fact that the vesicles can maintain a pCa gradient at 37°C indicates that the disc membrane has a very low permeability to Ca^{2+}.

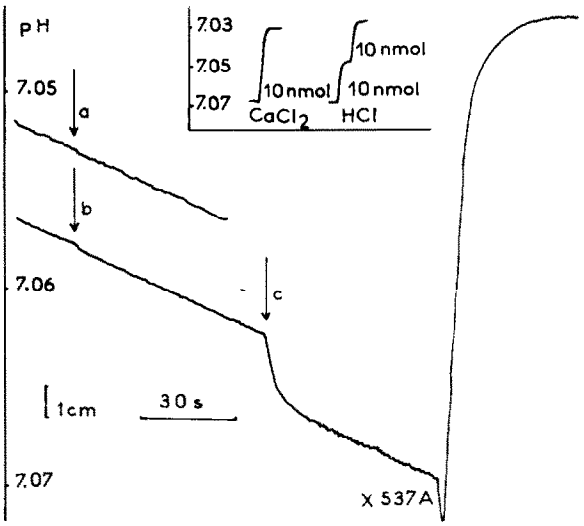

Fig.2. Light- and X537A-induced pH changes of a suspension of Ca^{2+}-loaded vesicles from ROS membranes in Ca–EGTA buffer. ROS membrane suspension 100 μl (5.8 nmol rhodopsin) loaded with 1 mM $CaCl_2$ by dialysis were injected into 1.7 ml Ca^{2+}-free solution (100 mM KCl; 7 mM $MgCl_2$; 1 mM Tris maleate; 1.2 10^{-4} M EGTA; pH 7.05; 20°C). The resulting Ca^{2+} was 1.8×10^{-7} M. Arrows indicate illumination: (a) 0.4 ms flash with a 10% density filter; (b) 0.4 ms flash without filter; (c) 3 ms flash which bleached 52% of the rhodopsin molecules (3 nmol). The signals produced by flashes (b) and (c) are similar in control experiments without EGTA and correspond to the H^+ uptake associated with the metarhodopsin I–metarhodopsin II transition. Addition of 5 μl 20 mM ethanol solution of X537A after flash (c) produced a H^+ release corresponding to the binding of about 7 nmol Ca^{2+} by EGTA; further additions of the ionophore only produced a small alkaline signal. The H^+ and Ca^{2+} signals were calibrated by several injections of 10 nmol HCl and $CaCl_2$ (10 μl 1 mM solutions). The calibrations are shown at a 10-fold reduced scale in the inset.

3.2. *Absence of a rapid light-induced release of Ca^{2+}*

The effect of flashes of light causing from 0.1% to > 50% bleaching is studied, and the presence of Ca^{2+} inside the vesicles after bleaching is checked by addition of the Ca^{2+} ionophore X537A.

The only light-induced pH change observed is a H^+ uptake signal (fig.2), which is similar whether EGTA is present in the external medium or not, and therefore corresponds to the protonation of the protein associated with the metarhodopsin I–metarhodopsin II transition. The no. of H^+ bound/rhodopsin bleached, measured after strong illumination (3 ms), is 0.5 ± 0.2 (maximum deviation, 16 expts) in the presence of a Ca–EGTA buffer as well as in the control experiments. It can therefore be estimated that < 0.1 Ca^{2+} is released per rhodopsin bleached. Although the precision of the measurement is lower for low intensity flashes, it seems unlikely that a more important release may occur when few rhodopsin molecules are bleached, since the duration of the flash is too short to allow any inhibition mechanism to take place. It can also be noted that the no. of H^+ bound/rhodopsin bleached in these experiments is very close to the value reported [18] using unloaded vesicles.

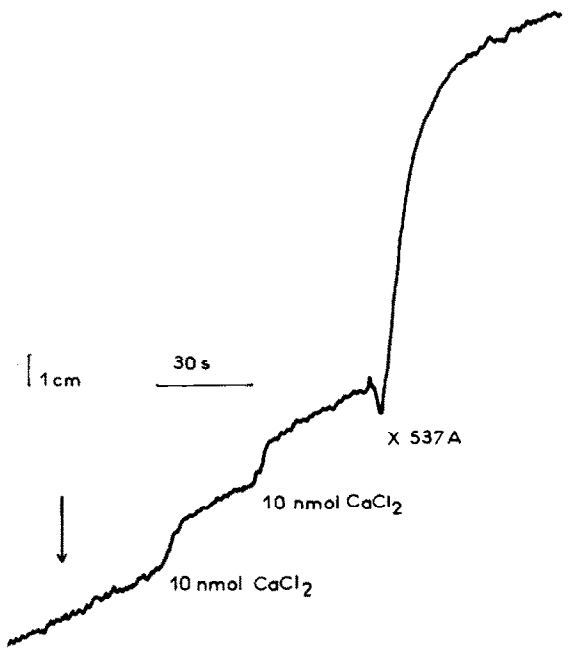

Fig.3. Effect of light and X537A on the pCa of a suspension of Ca^{2+}-loaded vesicles from ROS membranes: Ca^{2+}-selective electrode recording. ROS membrane suspension, 100 μl (87 nmol rhodopsin) loaded with 5 mM $CaCl_2$ by dialysis were added to 10 ml Ca^{2+}-free solution (100 mM KCl; 10 mM $MgCl_2$; 20 mM Tris–acetate (pH 7) at 37°C). The resulting $[Ca^{2+}]$ outside the vesicles was $\sim 5 \times 10^{-5}$ M. The arrow indicates illumination with a 3 ms flash which bleached 64 nmol rhodopsin. Addition of X537A (10 μl 20 mM ethanol solution) after bleaching released about 100 nmol Ca^{2+} (8.5 min after diluting the external Ca^{2+} concentration). Note that the experimental conditions (temperature, $[Ca^{2+}]$, amount of membranes) are different from those of the experiment reproduced in fig.2.

Similarly, no light-induced release of Ca^{2+} is detected with a Ca^{2+}-selective electrode in the absence of a Ca–EGTA buffer (fig.3).

In conclusion, it appears that under the conditions described, in right side out vesicles loaded with Ca^{2+} by dialysis, rhodopsin does not act as a Ca^{2+} channel nor as a 'one-shot' carrier upon illumination. If the function of rhodopsin is indeed to release Ca^{2+} from the discs, it is necessary to postulate that an important cofactor is missing.

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