Chlortetracycline, a fluorescent probe for pH of calcium stores in cells

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Endoplasmic reticulum is the major storage site for Ca^{2+} that is mobilized during activation of cell functions. Chlortetracycline (CTC) loaded into calcium stores of fresh, isolated human blood lymphocytes was found to be sensitive to changes in H^+ concentration, in addition to its established sensitivity to Ca^{2+}. Determination of pH of calcium stores was based on dissociation of fluorescent CTC·Ca^{2+} with decrease in pH. Concentrations of H^+ at the calcium stores were calibrated to different levels by equilibrating it with different extracellular pH buffers using the H^+ ionophore, nigericin. It was possible to determine the pH of two pools of calcium stores; they were 7.2 and 6.2.

pH of cytosol and a few organelles such as endosomes and lysosomes has been determined, and has been shown to be tightly regulated by active pumps and exchange of ions[1–3]. pH of these sites and their regulation have been correlated with cell growth, metabolism, membrane trafficking, cell-volume regulation and Ca^{2+} mobilization. In this communication, we have estimated the pH of cellular calcium stores in intact live cells by a pH-sensitive, fluorescent Ca^{2+} probe, chlortetracycline (CTC). CTC was the first fluorescent probe that was reported for Ca^{2+} at the cellular calcium stores. The dye has been used in detecting release of Ca^{2+} from cellular stores[4–8], in imaging calcium storage sites in live cells[9,10] and for study of transport of Ca^{2+} from membrane vesicles[4,11]. But CTC did not come up to expectations as a probe for calcium stores because of a number of reasons. Free dye diffused across membranes[4,11]. Calcium release, but not reuptake of Ca^{2+} into stores could be detected in CTC-loaded cells[7,8]. Binding of CTC to Ca^{2+} was influenced by pH[12]. There was a small contribution to fluorescence from CTC-loaded cells by CTC·Mg^{2+} complex. This contribution by CTC·Mg^{2+} can be decreased by incubating CTC in the absence of extracellular Mg^{2+} during loading of the dye into cells[8].

Divalent cations, Ca^{2+} and Mg^{2+} enhanced the weak fluorescence of CTC[8,13]. CTC is membrane-permeable, but not the CTC·Ca^{2+} complex[4,11]. Therefore, CTC could diffuse into all compartments of a cell and form CTC·Ca^{2+} complexes at sites containing high concentrations of Ca^{2+}. The dissociation constant of CTC·Ca^{2+} in aqueous environment at neutral pH is 440 μM (ref. 13) and is very close to the latest reported concentration of Ca^{2+} in the endoplasmic reticulum[14]. When extracellular dye is washed-off, the fluorescent CTC·Ca^{2+} is retained at the calcium stores.

Fluorescence of CTC is pH-sensitive. The two fluorescence-sensitive pK_a values assigned for CTC were at 3.5 and 7.7 (ref. 12). The fluorescence at pH 3.5 and 7.7 was also Ca^{2+}-sensitive. Therefore, it could also be argued that these sites on CTC might be Ca^{2+}-binding sites[12].

Nigericin, a H^+–K^+ ionophore, has been used to equilibrate pH across membranes[15]. Nigericin has been extensively used to equilibrate pH between extracellular medium and cytosol[16,17] or cellular organelles[18]. We have used nigericin to calibrate the pH of calcium-storing organelles by equilibrating them with extracellular pH.

For the isolation of human blood lymphocytes, 15 ml of blood was drawn into 3 ml of citrate solution taken in a 20-ml syringe (1 ml of acid–citrate–dextrose was used for 5 ml of blood). Red blood cells (RBCs) were subjected to dextran sedimentation by mixing 4.5% solution of dextran 500 (MW about 500,000, Sigma) in 154 mM NaCl with twice its volume of blood sample. Lymphocytes in the supernatant plasma were subjected to ficoll-hypaque (density of 1.077, Sigma) density separation from neutrophils and contaminating RBC. Lymphocytes at the interface were drawn out, washed by centrifugation and resuspended with buffer Na-7.4 (ref. 19).

To load CTC into the calcium stores of lymphocytes, 100 μM CTC was added to lymphocytes suspended at 10 to 20 million/ml in buffer Na-7.4 with 1 mM $CaCl_2$, but without $MgCl_2$. They were incubated at 37°C for 30 min. Extracellular dye was removed by centrifugation of lymphocytes at 2000 rpm for 7 min in a swinging-bucket rotor. The supernatant was removed by decantation and the residual buffer in the tube was removed by gently tapping the inverted tube on absorbant paper. The CTC-loaded lymphocytes obtained from 15 ml of blood were then resuspended in 0.5 ml buffer Na-7.4. Less than 30 μl of the cell suspension was used for each assay volume of 2 ml.

Let us consider fluorescence assays with CTC-loaded lymphocyte in different buffers. Na-rich buffer at pH 7.4 (Na-7.4) contains 10 mM Hepes, 130 mM NaCl, 5 mM KCl and 5 mM glucose adjusted to pH 7.4. Similarly, K-rich buffers contain 135 mM KCl, 5 mM glucose and 10 mM Hepes or 10 mM Mes. When pH 6.9 or above was required, 10 mM Hepes was used and was replaced by 10 mM Mes for pH below 6.9. Autoclaved acid–citrate–dextrose anticoagulant contained 0.20 M sodium citrate, 0.14 M citric acid and 0.22 M glucose in double-distilled water. Stock solutions of ionomycin and nigericin (Sigma) were made in dimethyl sulphoxide.

Next, 0.5 to 1 million CTC-loaded lymphocytes (15 to 30 μl) were suspended in 2 ml of the required assay buffer, continuously mixed and incubated at 37°C. Excitation was at 395 nm and emission at 520 nm. Volume of

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CTC-loaded stock cell suspension was kept as low as possible to prevent any gross alteration of pH or Na^+ concentration of the different assay buffers.

Fluorescence of CTC·Ca^{2+} at the cellular calcium stores in human blood lymphocytes was sensitive to changes in H^+ and Ca^{2+} concentrations (Figure 1). Ionomycin, a Ca^{2+} ionophore, released Ca^{2+} from the stores into cytosol, resulting in decrease of fluorescence intensity. The decrease in fluorescence was due to dissociation of fluorescent CTC·Ca^{2+} to CTC, which had weak fluorescence.

pH of calcium stores was altered by nigericin added to CTC-loaded lymphocytes suspended in potassium-rich buffers at different pH. There was a decrease in fluorescence intensity on addition of nigericin, when the extracellular pH was decreased (Figure 1 *b*). The fluorescence response with nigericin increased with decrease in pH of the extracellular buffer. Extracellular sodium was replaced by potassium during the assay to prevent excessive rise in intracellular H^+ after addition of nigericin. To achieve this, small volumes (15–30 µl) of a thick suspension of CTC-loaded lymphocytes were added to an assay volume of 2 ml potassium buffers at different pH. The slow decrease in fluorescence intensity seen before Ca^{2+} mobilization or decrease in pH of calcium store (Figure 1) was due to slow diffusion of CTC out of the calcium stores.

Influence of pH on CTC and CTC·Ca^{2+} was examined in buffer solutions at pH 7.4 and 6.8 (Figure 2). There was a shift in the plot to higher concentrations of Ca^{2+} when pH was decreased. Therefore, decrease in pH decreased the affinity of CTC to Ca^{2+}. But the maximum fluorescence of CTC·Ca^{2+} remained almost the same at pH 7.4 and 6.8. Increase in pH also caused a weak enhancement of the weak fluorescence of free CTC. This was seen at 0 Ca^{2+} in Figure 2.

Sensitivity of CTC·Ca^{2+} to pH at fixed concentrations of Ca^{2+} was also examined (Figure 3). Concentrations of Ca^{2+} were chosen to represent those that were reported to occur at the calcium stores. Determination of concentration of free Ca^{2+} at the cellular calcium stores using different probes has reported values between 100 µM and about 1 mM (refs 14, 20, 21). When Ca^{2+} is released from cellular stores, these concentrations fall to values between 10 and 40 µM. Therefore, concentrations of Ca^{2+} were fixed between 10 µM and 1 mM for examining the pH-sensitivity of fluorescence of CTC·Ca^{2+}. In the pH range between 5.8 and 8.2, sensitivity to pH increased with increase in concentration of Ca^{2+}. This was because it was the Ca^{2+} complex that was fluorescent. In the physiological range of pH 5.8 to 7.4, maximum change in

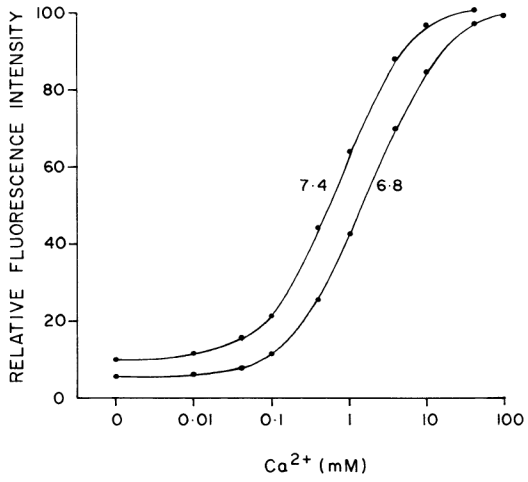

Figure 2. Influence of Ca^{2+} concentration on fluorescence of 10 µM solution of CTC in 10 mM Hepes buffer at pH 7.4 or in 10 mM Mes buffer at pH 6.8.

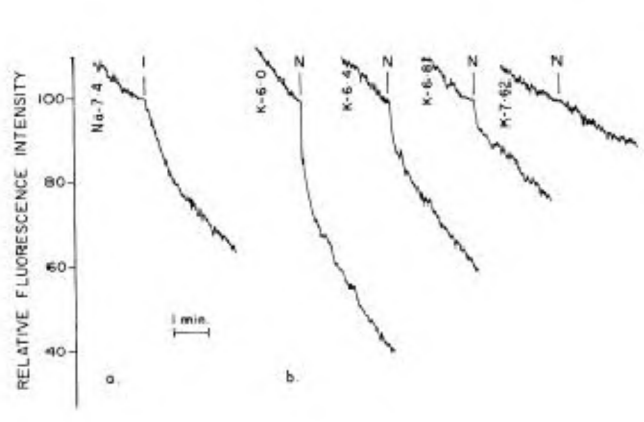

Figure 1. Fluorescence of CTC loaded into calcium stores of lymphocytes is sensitive to decrease in Ca^{2+} and increase in H^+ concentration. Ca^{2+} was mobilized from CTC-loaded lymphocytes with 500 nM ionomycin (I) and pH of the calcium store was equilibrated with extracellular pH using 7 µM nigericin (N). Excitation was at 395 nm and emission at 520 nm. CTC-loaded lymphocytes were suspended at 0.5 to 1 million cells/2 ml of buffers Na-7.4, K-6.0, K-6.4, K-6.8 or K-7.62.

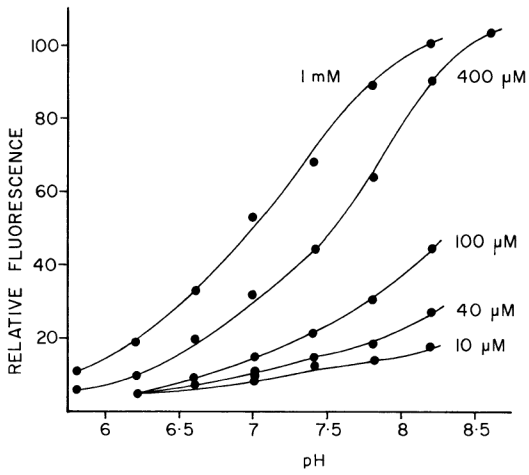

Figure 3. Influence of pH on fluorescence of 10 µM solution of CTC at fixed concentrations of Ca^{2+} (10 µM, 40 µM, 100 µM, 400 µM and 1 mM). 10 mM Hepes was used to prepare buffers at or above pH 7.0, and 10 mM Mes was used for buffers at or below pH 6.8.

fluorescence with pH was seen at 1 mM Ca^{2+}. There was significant sensitivity to pH at 400 μM Ca^{2+}. The method would be relatively insensitive to pH between 10 and 40 μM Ca^{2+} concentration.

pH of the most alkaline calcium store was estimated by the following method (Figure 4). CTC-loaded lymphocytes were suspended in potassium-rich buffers with different pH and then fluorescence intensity was plotted against time. After obtaining the initial slope, nigericin was added and the plot of fluorescence intensity was continued. When extracellular pH was 7.41, 7.3 or 7.2 there was no change in the fluorescence intensity slope on addition of nigericin, but there was a small decrease in intensity when nigericin was added at pH 7.1. Therefore, pH of a particular calcium store was decreased on addition of nigericin when extracellular pH was 7.1. pH of this particular calcium store would then be 7.2. Addition of nigericin resulted in stronger responses when extracellular pH was further decreased (Figures 1 *b* and 5).

The change in fluorescence intensity for 1 min before (x) and for 1 min after (y) addition of nigericin was measured. Difference of these intensities (y–x) was plotted against extracellular pH (Figure 5). The difference of y–x was 0 at pH 7.4, 7.3 and at 7.2. At pH 7.1 or lower, there was a negative change in fluorescence intensity after addition of nigericin. There was a second strong negative change in the slope of the plot in Figure 5 at pH below 6.2. We inferred from these observations that the pH of a second major calcium store was lowered below pH 6.2.

It was important to observe that when pH of calcium store was increased above 7.2 with nigericin (pH 7.62 in Figure 1 and pH 7.3 and 7.41 in Figure 4), there was no increase in intensity or significant change in slope of the intensity plot. This was because free CTC was very low or was absent at the calcium store.

CTC has a weak fluorescence at 520 nm and is also membrane-permeable. Complex formation with Ca^{2+} enhances its fluorescence and makes it membrane-impermeable.

$$CTC + Ca^{2+} \longleftrightarrow CTC{\cdot}Ca^{2+}.$$

Figure 4. Estimation of pH of the most alkaline calcium store. Human blood lymphocytes were isolated and loaded with CTC into their calcium stores as described in Figure 1. CTC-loaded lymphocytes were suspended in potassium-rich buffers at different pH (7.01, 7.1, 7.2, 7.3 and 7.41), and then 7 μM nigericin (N) was added.

Decrease in pH decreased the fluorescence of $CTC{\cdot}Ca^{2+}$. At pH 7.4 and 6.8, the maximum fluorescence of $CTC{\cdot}Ca^{2+}$ remained the same, but there was a change in affinity of CTC to Ca^{2+} (Figure 2). We could infer from these results that H^+ altered the fluorescence of $CTC{\cdot}Ca^{2+}$ by dissociating $CTC{\cdot}Ca^{2+}$, probably by competing with the Ca^{2+} binding sites on CTC. Deprotonation also caused a weak enhancement of the weak fluorescence of free CTC. This was seen at 0 Ca^{2+} in Figure 2.

$$CTC + Ca^{2+} + H^+ \longleftrightarrow \begin{cases} CTC{\cdot}Ca^{2+} & \text{(at saturating } Ca^{2+} \text{ and neutral pH)} \\ CTC{\cdot}Ca^{2+} + CTC{\cdot}H^+ & \text{(at intermediate } Ca^{2+} \text{ levels and pH)} \\ CTC{\cdot}H^+ & \text{(at 0 } Ca^{2+} \text{ and acidic pH)} \end{cases}$$

At 100% fluorescence intensity with saturating concentrations of Ca^{2+}, all CTC might be converted to $CTC{\cdot}Ca^{2+}$. In the absence of Ca^{2+} and at acidic pH, all CTC would be converted to $CTC{\cdot}H^+$. But at near neutral pH and intermediate Ca^{2+} concentrations, we expect that both the protonated and the Ca^{2+} complex of CTC would be formed.

Just as there was dissociation of $CTC{\cdot}Ca^{2+}$ and decrease in fluorescence with decrease in concentration of Ca^{2+} at the cellular stores, there would also be slow decrease in fluorescence and dissociation of $CTC{\cdot}Ca^{2+}$, with slow diffusion of CTC out of the calcium stores. This was the cause of slow decrease in fluorescence intensity seen before Ca^{2+} mobilization form the stores. Decrease in concentration of CTC at the calcium stores caused reversal of the reactions illustrated above and it occurred at all pH tested.

It was observed earlier that increase in pH of calcium stores by nigericin above 7.2 (7.3 and 7.41 in Figure 4;

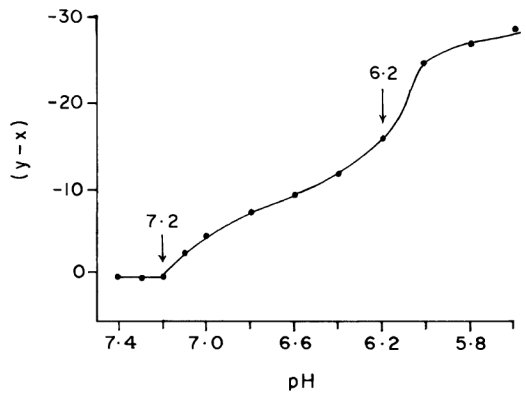

Figure 5. Estimation of pH of different calcium stores. Fluorescence intensity of CTC-loaded lymphocytes suspended in potassium-rich buffers was plotted as in Figures 1 and 3. Change in intensity for 1 min before (x) and for 1 min after (y) addition of nigericin was measured. Difference in intensity before and after addition of nigericin (y–x) was plotted against extracellular pH.

7.62 in Figure 1) did not increase the fluorescence intensity. This observation might be explained in the following way. Free CTC is membrane-permeable, but not CTC·Ca^{2+} complex. Therefore, free CTC formed at the CTC·Ca^{2+} containing calcium store of a cell would continuously diffuse out of the calcium stores resulting in very little or no CTC that may be available to bind to Ca^{2+} when pH was increased.

We could estimate the pH of two pools of calcium stores with this method. Endosomes and lysosomes have been reported to contain high concentrations of Ca^{2+} (ref. 20) and these are acidic organelles[18,22]. Endosomes have pH between 6.4 and 6.0, and pH of lysosomes is still lower[18,22]. Endoplasmic reticulum, sarcoplasmic reticulum and specialized organelles formed out of endoplasmic reticulum have been extensively reported to contain the receptor-sensitive calcium stores[20], and they are not acidic. Therefore, we might assume that the calcium store with pH 7.2 could correlate with these stores.

Though sensitivity to pH increased with increase in Ca^{2+} concentration at physiological pH values (Figure 3), very high sensitivity to pH may not be advantageous because the sudden decrease in fluorescence with decrease in pH would affect the resolution of the calibration method in cells. But very low sensitivity between 10 and 40 μM Ca^{2+} is also not desirable. Therefore, the 400 μM to 1 mM Ca^{2+} concentration, encountered at the calcium stores[20,21], that showed a moderate sensitivity to pH make the probe suitable for determination of pH of calcium stores in cells.

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Distribution pattern and heavy metal accumulation in lichens of Bangalore city with special reference to Lalbagh garden

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Occurrence of 30 species of lichens belonging to 19 genera and 15 families in 12 localities of Bangalore city is reported. The Indian Institute of Science (IISc) campus and Lalbagh garden record the maximum number of 24 and 18 species of lichens respectively, which can be directly attributed to the presence of a variety of trees in the area providing diverse substrate for lichen growth. Heavy-metal accumulation in few prominent lichens of some localities is also analysed. Cr and Pb were maximum in *Chrysothrix candelaris* (L.) Laundon, at AMCO Batteries area with 95.29 and 623.95 μg g^{-1} dry wt. respectively. Fe and Cu were maximum in *Bulbothrix isidiza* (Nyl.) Hale and *Pyxine petricola* Nyl. at IISc campus with 22721 and 338.12 μg g^{-1} dry wt. respectively, while *Lecanora perplexa* Brodo at Lalbagh garden has 531.5 μg g^{-1} dry wt. of Zn. The lichen flora of Lalbagh garden is compared to

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