Loading pyranine via purinergic receptors or hypotonic stress for measurement of cytosolic pH by imaging

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Gan, BING SIANG, Eric Krump, Lamara D. Shrode, and Sergio Grinstein. Loading pyranine via purinergic receptors or hypotonic stress for measurement of cytosolic pH by imaging. Am. J. Physiol. 275 (Cell Physiol. 44): C1158–C1166, 1998.—Although used extensively for the measurement of intracellular pH, derivatives of fluorescein such as 2',7'-bis(2-carboxyethyl)-5(6)-carboxyfluorescein (BCECF) have suboptimal sensitivity and can generate toxic photoproducts. These limitations can be overcome using the pH-sensitive fluorescent dye 8-hydroxypyrene-1,3,6-trisulfonic acid (pyranine), which has improved spectroscopic properties. However, the use of pyranine has been limited by the difficulties encountered in delivering this highly hydrophilic dye to the cell interior. We describe a strategy for intracellular delivery of pyranine based on the reversible activation of purinergic P2x7 receptors, which allow permeation of the dye to the cell interior. When loaded into J774 or RAW cells by this method, pyranine is not only more sensitive than BCECF (the dynamic range is ~7-fold greater), but is retained better and is less toxic. Pyranine was distributed throughout the cytosol but was not detectable in endomembrane compartments. Repeated illumination resulted in blebbing and loss of functional responsiveness of cells loaded with BCECF, whereas comparably irradiated cells loaded with pyranine remained healthy and responsive. Pyranine can also be loaded into cells not expressing P2x7 receptors by brief exposure to a hypotonic solution. The properties of cells labeled by this method are similar to those loaded via purinergic receptors and compare favorably with those of BCECF-loaded cells. Pyranine thus provides a useful alternative to fluorescent derivatives for the measurement of intracellular pH, particularly when using the high excitation intensities required for microscopic digital imaging.

P2x7 receptor; 2',7'-bis(2-carboxyethyl)-5(6)-carboxyfluorescein; pyranine; intracellular pH; fluorescence ratio imaging

A VARIETY OF PROCESSES, including protein synthesis, cytoskeletal dynamics, cellular proliferation, and differentiation, are stringently controlled by the cytosolic pH (pHi). Because of its importance, the regulation of pHi has become a subject of great interest, and several methods have been introduced for its measurement.

These include pH-sensitive microelectrodes, determinations of the partition of isotopic weak acids or bases, and NMR (17). However, fluorimetric procedures have become the method of choice for the measurement of pHi for the following reasons: 1) they are extremely sensitive and therefore applicable to small samples, including single mammalian cells; 2) their response time is rapid; and 3) they can be virtually noninvasive, allowing for repeated and even continuous monitoring of pHi over extended periods of time.

Fluorescein derivatives have been used most extensively for measuring pHi. Their fluorescence displays an exquisite sensitivity to pH and has a high quantum yield. Moreover, most commercial fluorescence microscopes are equipped with the appropriate optical components to detect the emission of fluorescein. Fluorescein itself, however, is not appropriate for the measurement of pHi: its pKa is too acidic (~6.5), and it cannot be selectively targeted to the cytosol. These disadvantages have been largely overcome by progressive modifications of the basic fluorescein moiety (3, 17). Improved retention and more selective targeting to the cytosol were accomplished by inclusion of additional carboxylates following their esterification (19). Deesterification by cytoplasmic enzymes releases the charged, less permeant form of the probe in the cytosol. In addition, introduction of additional carboxylic groups to the basic fluorescein structure resulted in an elevated pKa, more appropriate for the measurement of pHi near neutrality. The prototypical compound 2',7'-bis(2-carboxyethyl)-5(6)-carboxyfluorescein (BCECF)-AM has been used extremely in recent years for both macro- and microfluorescence determination of pHi (4, 15, 16).

Despite its extensive use, BCECF suffers from a number of shortcomings. First, intracellular hydrolysis of the ester bond has been shown to cause significant intracellular acidification over time (1). Second, neither the excitation nor the emission spectra of BCECF shift with pH, limiting the dynamic range of fluorescence ratio determinations. Finally, the probe is very susceptible to photobleaching, particularly when exposed to a focused beam in microscopic setups. Not only does this lead to progressive deterioration of the signal, but it can also cause damage of the biological preparation, likely associated with the generation of oxygen radicals.

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8-Hydroxypyrene-1,3,6-trisulfonic acid (pyranine) is a small, highly water-soluble fluorophore that has previously been shown to exhibit excellent pH-sensitive spectral properties (10). In particular, the excitation spectrum of pyranine undergoes a large shift when the molecule becomes protonated, displaying a well-defined isosbestic point. With the calculation of the ratio of intensity at two wavelengths on either side of the isosbestic point, pH1 can be measured with great sensitivity and accuracy. Unlike BCECF, however, pyranine is not available in membrane-permeant form. Consequently, delivery of pyranine has been restricted to the use of micropipettes, liposome fusion, or scrape-loading (11, 13). These techniques are all cumbersome, rather invasive, and in the case of microinjection or patch pipettes, restricted to small numbers of cells. In the current study, we used two procedures for loading pyranine into various cell types. For the first method, we took advantage of the observation that in several cell types, including the monotypic J774 line, extracellular addition of ATP 4+ induces the opening of a nonselective permeability pathway that allows the passage of molecules of molecular weight ~850 across the plasma membrane, without affecting endomembrane compartments (18). The formation of this pore is readily reversed upon addition of divalent metals, restoring the permeability properties of the cells. We reasoned that pyranine (mol wt 524) could be readily loaded into the cytosol with minimal damage to the cells. Because use of this method is restricted to cells expressing P2x7 receptors, we also implemented a second, more general loading procedure. A variety of cultured cells were subjected to a hypotonic shock that induced a transient increase in cytosolic permeability. We then proceeded to compare the properties of pyranine and BCECF as probes for the ratiometric determination of pH. Pyranine proved to have considerably better stability, greater dynamic range, and lower toxicity than BCECF.

**EXPERIMENTAL PROCEDURES**

Materials. The free acid and AM forms of BCECF, 8-hydroxypyrene-1,3,6-trisulfonic acid, and nigericin were from Molecular Probes (Eugene, OR). The potassium salt of ATP and MgCl2 were purchased from Sigma. BSA (fraction V) was from Boehringer Mannheim. Fetal calf serum was obtained from Cansera (Rexdale, Ontario, Canada). Lyophilized streptolysin O (SLO) was provided by Dr. S. Bhakdi (Johannes-Gutenberg Universität, Mainz, Germany) and dissolved in a phosphate-buffered saline (PBS) solution at 1 mg/ml and stored at −80°C. I immediately before use, SLO was diluted in a Na+-rich buffer that contained 1 mg/ml BSA and 2 mM dithiothreitol to a final concentration of 0.1 µM. All other chemicals and reagents were of the highest purity available. J774 cells, RAW cells, Chinese hamster ovary (CHO-WT5) cells, and COS cells were from the American Tissue Culture Collection. Cell culture media and antibiotics were from GIBCO.

Solutions. Na+-rich buffer contained (in mM) 140 NaCl, 3 KCl, 10 glucose, 1 CaCl2, 1 MgCl2, and 20 HEPES (pH 7.4). K+-rich buffer contained (in mM) 140 KCl, 10 HEPES, and 10 MES (pH ranged from 4.0 to 10.0, as specified; for pH >7.5, Tris was added instead of MES). Loading buffer consisted of (in mM) 70 KCl, 70 potassium glutamate, 5 ATP (potassium salt), 1 EGTA, 5 pyranine, 1 dithiothreitol, 10 HEPES, and 5% (wt/vol) BSA (pH 7.2). Recovery buffer contained (in mM) 70 KCl, 70 potassium glutamate, 1 MgCl2, 5 pyranine, 1 dithiothreitol, 10 HEPES (pH 7.2), and 5% (wt/vol) BSA. Ammonium-load solution consisted of (in mM) 110 NaCl, 25 Na+-HEPES, 10 NH4Cl, 5 KCl, 1.7 MgCl2, 1.4 CaCl2, and 5 glucose. Na+-free solution contained (in mM) 120 N-methyl-D-glucammonium (NMG) chloride, 25 NMG-HEPES, 5 KCl, 1.7 MgCl2, 1.4 CaCl2, and 5 glucose. The osmolality of the above media was adjusted to 290 ± 10 mosM with either water or the major salt. Hypotonic buffer contained 20 mM pyranine in PBS, diluted to a final osmolality of 150 mosM.

Cell culture. All cell lines were kept at 37°C in a humidified environment with 5% CO2. J774 and RAW cells were grown as monolayers in DME-M.H-21 medium supplemented with 10% fetal calf serum, 100 U/ml penicillin, and 100 µg/ml streptomycin. CHO-WT5, COS-7, and H-1421 cells were cultured in α-MEM supplemented with 10% serum, 100 U/ml penicillin, and 100 µg/ml streptomycin. Twenty-four hours before the experiments, cells were harvested and plated onto 25-mm glass coverslips in six-well plastic culture dishes, and experiments were performed when cells reached ~40–60% confluence.

Loading of BCECF. For BCECF loading, cells were washed three times with Na+-rich buffer to remove all serum components and subsequently incubated for 10 min at 37°C in Na+-rich buffer containing 1 µg/ml BCECF-AM. Cells were then rapidly washed three times with Na+-rich buffer and used for fluorescence acquisition.

Loading of pyranine with ATP 4+. J774 and RAW cells adherent to glass coverslips were washed once with PBS that contained 1 mM EGTA. Cells were next treated with the loading buffer for 5 min at 37°C which was then replaced with the recovery buffer for an additional 2 min at 37°C. Cells were then allowed to equilibrate for 20 min before starting the experiments. During this period, residual extracellular pyranine was eliminated by repeated washes with Na+-rich buffer. Hypotonic loading of pyranine by J774, CHO-WT5, COS, and H-1421 cells adherent to glass coverslips were treated with the 50% hypotonic buffer that contained 20 mM pyranine for 4 min at room temperature, after which the cells were returned for 5 min to an isosmolar PBS solution containing 20 mM pyranine. The cells were then washed with an isosmolar Na+-rich buffer (without pyranine) and allowed to recover for an additional 15 min at room temperature (7, 14). Cells were warmed to 37°C and used for fluorescence acquisition.

Measurement of intracellular concentrations of pyranine and BCECF. Cells grown to confluency in 10-cm plastic tissue culture dishes were loaded with either BCECF or pyranine according to their respective protocols, as described above. Cells were collected by scraping the plates. With the use of a Coulter Counter apparatus, cell number and volume were determined in an aliquot of the cell suspension. The concentration of intracellular dye was determined by lysing the cells in a Triton X-100 buffer (1% Triton X-100, 1 mM EDTA, 150 mM NaCl, and 50 mM Tris-HCl, pH 8.0) and measuring the fluorescence at the appropriate wavelengths in a fluorescence spectrometer (model 650–40, Perkin-Elmer). The concentration of the dye was determined by interpolating the measured fluorescence in a standard curve constructed using known amounts of pyranine or BCECF free acid dissolved in the Triton X-100 buffer.

Acquisition of fluorescence spectra. Pyranine and the free acid of BCECF were dissolved in the K+-rich buffer at a final concentration of 1 µM, and the pH was titrated between 6.0 and 10.0 using Tris or MES, as indicated. The excitation spectrum of the dyes was then determined in a Hitachi F-4000 fluorescence spectrophotometer with a bandwidth of 20 nm.
The fluorescence intensity decreases as the wavelength increases in the 6.0–9.0 range. Conversely, the fluorescence increases along with pH at wavelengths longer than the isosbestic point. As illustrated in Fig. 1, inset, the ratio of the fluorescence intensity measured with excitation at 440 nm over that at 380 nm provides a sensitive measure of the pH. The fluorescence ratio increases 50-fold between pH 6.0 and 9.0, with an apparent pKₐ of 7.82, consistent with that reported earlier (10). In contrast, the excitation maximum of BCECF remains near 500 nm throughout the pH range studied, showing only a modest blue shift at very acidic pH (Fig. 1B). Note that the isosbestic point for BCECF is ill defined and occurs at a wavelength (~445 nm) where the fluorescence intensity is comparatively low. As a result, ratiometric determinations of pH in the physiological range are expected to be more sensitive and accurate using pyranine than BCECF.

Loading of J774 cells with pyranine. Exposure of several cell types, including monocytes and macrophages, to the tetravalent form of ATP induces the formation of pores in the plasma membrane that allow the permeation of solutes of up to molecular weight 850 (18). We used this approach to load the cytosol of J774 monocytes with pyranine. As illustrated in Fig. 2, B and C, reversible permeabilization with ATP₄⁻ is a convenient method to trap pyranine in the cytosol of these cells. All the cells in the coverslips were loaded with the dye, although the extent of loading varied between cells. The source of this heterogeneity is not known but likely relates to variable density of P₂X₇ receptors.
resealing of the membrane by extracellular addition of divalent cations, the dye was retained intracellularly for extended periods of time. As illustrated in Fig. 2A, the relative fluorescence intensity decreased minimally after incubation for 1 h at 37°C.

The pH sensitivity of intracellular pyranine was compared with that of BCECF, using the microscopic imaging apparatus detailed in Ref. 4. The pH of the cytosol was manipulated by means of ionophores, and the resulting changes in the fluorescence ratio of the dyes are shown in Fig. 1, C and D. Note that the dynamic range of the fluorescence ratio is smaller for both dyes when measured within the cells than when determined in vitro. This is attributable to the wider bandpass of the filters in the imaging system compared with the monochromator used for the macrofluorometry determinations in Fig. 1, A and B. More importantly, the dynamic range is much greater for cytosolic pyranine (a 30-fold change in fluorescence ratio between pH 5.5 and 8.5) than for BCECF (4-fold change in the same pH range).

Photobleaching and retention of pyranine and BCECF in cells. Experiments were performed to measure the relative intracellular concentration of pyranine and BCECF after their respective loading protocols. Intracellular pyranine was found to reach 47.1 ± 7.8 (SE) µM (n = 6) after the P2X7 receptor stimulation loading protocol. In comparison, the intracellular concentration of BCECF was 330 ± 39 (SE) µM (n = 6) in J774 cells exposed to 1.6 µM BCECF-AM for 10 min. The stability and retention of pyranine and BCECF were next compared: J774 cells loaded with the dyes were exposed to repetitive excitation at the appropriate wavelengths, and the decay in emission intensity followed (Fig. 2, A and D). The cells were irradiated for 250 ms with sufficient light to provide a signal that was 5- to 10-fold higher than the background, at either 5- or 30-s intervals for up to 1 h. Excitation of pyranine every 30 s resulted in negligible decrease in fluorescence, whereas excitation at 5-s intervals induced a loss of 25–30% of the initial fluorescence after 1 h. In fact, the fluorescence of pyranine remaining after 3 h was adequate to...
measure pH$_i$ (data not shown). In contrast, nearly 50% of the fluorescence of BCECF was lost when irradiating every 30 s, and the fluorescence was almost undetectable when irradiating every 5 s. It therefore appears that BCECF undergoes extensive photolysis and/or leakage during the course of imaging, whereas pyranine appears more stable.

Comparison of the morphology of the cells after repeated excitation for 1 h revealed a second advantageous feature of pyranine. The morphology of the cells loaded with this dye was indistinguishable at the beginning and at the end of the 1-h irradiation regime. In contrast, cells loaded with BCECF displayed extensive blebbing of the plasma membrane after repeated illumination (cf. Fig. 2, B and E vs. C and F). This toxic effect was likely caused by photoactivation products of BCECF, since cells loaded with the dye remained healthy for even longer periods of time if not irradiated (data not shown).

Subcellular localization of pyranine and BCECF. The means used to load pyranine and BCECF into J774 cells are expected to deliver the dyes preferentially to the cytosol. This is further suggested by the diffuse fluorescence pattern observed microscopically (Figs. 2 and 3). To verify the subcellular localization of the dyes, loaded cells were subjected to permeabilization with SLO. At the concentration used, this toxin permeabilizes the plasmalemma, allowing the loss of cytosolic components, while endomembrane compartments remain intact. As shown in Fig. 3, E and J, pyranine loaded into J774 cells using ATP$_4^-$ and BCECF loaded as its AM are readily lost when the cells are treated with SLO, consistent with a cytosolic localization of both dyes. A small fraction of the cells (~10%) retained both dyes even after prolonged exposure to SLO, possibly as a result of heterogeneous sensitivity to the toxin (Fig. 3, E and J). Interestingly, when measuring actual fluorescence intensity, we found a higher residual pyranine fluorescence in cells permeabilized with SLO (Fig. 3A). Close examination of these cells revealed that the remaining fluorescence was not due to trapping of pyranine in a particular organelle but rather to diffuse binding of the dye by the permeabilized cells.

Assessment of the responsiveness of pyranine and BCECF to changes in pH$_i$. To evaluate the ability of pyranine to monitor pH in the cytosol, J774 cells were acid-loaded by a gentle ammonium prepulse (10 mM), and the ensuing physiological recovery was recorded. The initial alkalosis induced by influx of NH$_3$, the pH$_i$ recovery due to entry of NH$_4$+, and the large acidification noted upon removal of external NH$_3$/NH$_4$+ was readily and similarly detected with both pyranine and
with BCECF (Fig. 4). Addition of extracellular Na\(^+\) to the acid-loaded cells induced a rapid recovery of pH\(_i\), most likely mediated by Na\(^+\)/H\(^+\) exchange. It is noteworthy that the rates of Na\(^+\)-induced recovery determined with both dyes were similar (0.27 ± 0.02 pH/min for BCECF and 0.24 ± 0.01 pH/min for pyranine; values are means ± SE from 4 experiments).

Loading of pyranine by hypotonic shock. The results above indicate that pyranine is a superior dye for the measurement of pH by fluorescence imaging. Unfortunately, not all cell types are endowed with P\(_{2x7}\) receptors, limiting the use of the loading technique described earlier. In an attempt to extend the use of pyranine to cells that do not express the P\(_{2x7}\) receptors, we loaded pyranine into their cytosol using a hypotonic shock. Brief exposure to hypotonic media has been used in the past to render the membrane permeable to small hydrophilic substrates (7, 14). Figure 5 illustrates the application of this procedure to four different cell types: J 774, Chinese hamster ovary, COS-7, and H-1421 cells. Thirty minutes after the hypotonic treatment, all cells exhibited normal morphology, assessed by differential interference contrast (Nomarski) microscopy. Such cells trapped pyranine and retained the dye for up to 2 h after loading (data not shown).

Under the conditions used (see EXPERIMENTAL PROCEDURES), the amount of pyranine trapped by hypotonic shock was sufficient to measure pH\(_i\) by ratio imaging. Indeed, the concentration of trapped pyranine in J 774 cells after loading by hypotonic shock was determined to be 46.4 ± 10.3 µM, which is similar to that attained by the ATP\(^4-\) loading protocol. The alkalosis induced by
an NH₄⁺ prepulse, the acid-load induced by its removal, and the recovery mediated by Na⁺/H⁺ exchange were readily detected in all cell types, although their rates of recovery of pHᵢ differed. For example, the initial recovery rate in COS-7 cells subjected to an acid load was 0.17 ± 0.03 pH/min (Fig. 6A; other cell types are not illustrated). This rate is significantly slower than that of J774 cells acidified to a comparable extent (see illustrated). This rate is significantly slower than that of J774 cells acidified to a comparable extent (see above).

Activation of Na⁺/H⁺ exchange can also be elicited without resorting to pronounced acid loading by shrinking the cells in hypertonic solutions (5). This response was readily detectable in cells loaded with pyranine but not in cells loaded with BCECF (Fig. 6B). We believe that the impaired responsiveness of BCECF-loaded cells results from the toxic products released upon repeated irradiation of BCECF with the intense excitation light required for digital imaging (see above). Indeed, BCECF-loaded cells that fail to respond in the imaging setup display normal osmotic responses when excited with the much lower intensities required for photon counting or macrofluorescence determinations (data not shown).

**DISCUSSION**

Fluorescence imaging using pH-sensitive dyes is a highly sensitive and accurate method to investigate the mechanisms underlying the homeostasis of pHᵢ. It allows for real-time measurements of pH in defined intracellular compartments of living cells (12). Moreover, when measuring the ratio of fluorescence at two different wavelengths, variations in dye concentration, photobleaching, and other systematic errors can be eliminated (8, 11). On a theoretical basis, the optimal dye to measure cytoplasmic pH would possess the following characteristics: 1) a pKᵢ in the physiological range and extreme H⁺ selectivity, i.e., a low reactivity with other intracellular components; 2) high absorption coefficient and quantum yield; 3) a large dynamic range, allowing for measurement of minute changes in pH; 4) exclusive distribution in the cytoplasm, with little or no sequestration in other intracellular compartments; 5) a long intracellular retention time; 6) minimal decomposition upon irradiation, avoiding cellular damage by toxic photoproducts; and 7) a universal and simple mechanism of loading, allowing for the use in a variety of cell types.

Several of the criteria described above are met by the fluorescein derivative BCECF which has, consequently, become the most frequently used dye for measuring pHᵢ. Despite its popularity, BCECF is an imperfect dye. It is generally loaded into cells by permeation and hydrolysis of the precursor AM. Products of the deesterification reaction, namely, acetate, formaldehyde, and protons, accumulate in the cells and can alter their pH and their metabolism in general (1). Second, because the isosbestic point of the BCECF excitation spectrum occurs at a wavelength where the quantum yield is low, the signal-to-noise relationship and dynamic range of the fluorescence ratio are not optimal. Third, like most fluorescein derivatives, BCECF appears to be rather susceptible to photobleaching. This not only reduces the effective signal but, more importantly, is associated with the generation of toxic photoproducts. At best, such toxic products can inhibit physiological responses, such as the osmotic activation of Na⁺/H⁺ exchange. At worst, they will induce extensive cell damage, as indicated by pronounced blebbing of the plasmalemma (Fig. 2).

Most of the shortcomings inherent to BCECF are overcome by the improved optical properties of pyranine. Because of the sizable spectral shift it undergoes in response to changes in pH (Fig. 1A), the dynamic range of the fluorescence ratio of pyranine is much greater than that of BCECF (cf. Fig. 1, C and D). This increased dynamic range allows for a more accurate determination of minute changes in pH. Second, under the conditions required for adequate acquisition of images with a cooled charge-coupled device camera, pyranine proved to have much greater photostability than BCECF. This not only extended the period of
experimentation but also protected the cells from photodynamic damage (cf. Fig. 2, B and E).

The sulfonate groups of pyranine do not become protonated at or near the physiological pH, ensuring that the dye will remain in the polyanionic, impermeant form throughout. This property ensures retention of the cytosolic dye, but at the same time complicates its delivery into the cells. We have circumvented this problem by two different methods. The first one made use of the fact that some cell types, including monocytes and macrophages, express P2X receptors coupled to channels that, when activated, allow the passage of molecules of up to 850 (including pyranine, mol wt 524) into the cytosol (18). Loading of the cells by this procedure yielded reproducible and satisfactory determinations of pH1, with excellent recovery of pH1 after a mild ammonium prepulse acid load. We have noted that the fluorescence of pyranine is more intense in ~20% of the cells. We believe that this may be because of differences in loading efficiency, possibly caused by heterogeneity in the expression of purinergic receptors.

In cells not expressing P2X receptors, an alternate approach was used for loading pyranine, which consisted of a hypotonic treatment of the cells. When compared in the same cell type (J 774), the ATP"-method proved more effective in loading pyranine, considering that a fourfold higher concentration of pyranine was used during the hypotonic loading. Nevertheless, the amount of dye trapped using the latter method yielded satisfactory recordings (e.g., Fig. 6). As in the case of ATP-loaded cells, we noted some heterogeneity in the uptake of dye, with ~20% of cells exhibiting a significantly higher fluorescence intensity. When further examining COS-7 cells, we did notice a discrepancy between pH1 as measured with BCECF and pyranine. Cytosolic pH measured with pyranine at the end of the equilibration periods was somewhat more alkaline than those obtained with BCECF. This discrepancy is not likely because of the calibration procedure, since the same calibration methods were employed for both dyes and a comparable difference was noted when using nigericin or the null-point titration method (data not shown) (9). Instead, the more elevated pH of pyranine-loaded cells may have been due, at least in part, to activation of H+ extruding pathways during hypotonic pyranine loading. This was concluded because the pH1 was also elevated in cells that were first subjected to a hypotonic stress mimicking the loading conditions and then stained with BCECF (7.18 ± 0.12, n = 4 vs. 7.01 ± 0.05, n = 4). Alternatively, it is possible that the products of hydrolysis of BCECF-AM may systematically reduce pH1. In any event, the differential pH1 reported by both dyes is not universally observed, since the more alkaline pH1 detected by pyranine in COS cells was not found in J 774 cells.

One disadvantage of pyranine relates to the fact that inadequate washing of the cells will result in a contamination of the signal by the fluorescence of extracellular pyranine. This is not problematic in the case of BCECF, since the unhydrolyzed extracellular BCECF-AM exhibits very little fluorescence. The other apparent disadvantage of pyranine is that it requires excitation in the near-ultraviolet spectrum. In our experience, however, this did not result in increased autofluorescence interfering with the accurate measurement of cytosolic pH.

Whether loaded by hypotonic stress or using ATP, pyranine yielded recordings which suggested that the cells were better preserved and more responsive than those treated with BCECF-AM. This was concluded from the preservation of the osmotically induced activation of the antiporter in pyranine-containing cells, but not in those with BCECF (Fig. 6B). This effect could potentially be accounted for by depletion of intracellular ATP, which is needed for osmotic activation of the Na"+/H" antiporter (5). The blebbing noted after repeated irradiation of BCECF is also compatible with metabolic depletion of the cells induced by photolysis products.

In summary, we have demonstrated that pyranine is a more sensitive and stable indicator of pH1 than BCECF. Use of this dye was heretofore limited by the difficulties inherent in its delivery to the cytosol of intact cells. The two methods described in this report overcome this limitation by providing simple and reliable means of loading pyranine into a variety of mammalian cell types.

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