Simultaneous Measurements of Intracellular pH in the Leech Giant Glial Cell Using 2′,7′-bis-(2-Carboxyethyl)-5,6-carboxyfluorescein and Ion-Sensitive Microelectrodes

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ABSTRACT We have employed two independent techniques to measure the intracellular pH (pH_i) in giant glial cells of the leech *Hirudo medicinalis*, using the fluorescent dye 2′,7′-bis-(2-carboxyethyl)-5,6-carboxyfluorescein (BCECF) and double-barreled neutral-carrier, pH-sensitive microelectrodes, which also record the membrane potential. We have compared two procedures for calibrating the ratio of the BCECF signal, excited at 440 nm and 495 nm: 1) the cell membrane was H^+-permeabilized with nigericin in high-K^+ saline at different external pH (pH_o) values, and 2) the pH_i of intact cells was perturbed in CO_2/HCO_3^--buffered saline of different pH, and the BCECF ratio was calibrated according to a simultaneous microelectrode pH reading. As indicated by the microelectrode measurements, the pH_i did not fully equilibrate to the pH_o values in nigericin-containing, high-K^+ saline, but deviated by -0.12 ± 0.02 (mean ± SEM, $n = 37$) pH units. In intact cells, the microelectrode readings yielded up to 0.15 pH unit lower values than the calibrated BCECF signal. In addition, larger dye injections into the cells (>100 μM) caused an irreversible membrane potential loss indicative of some damage to the cells. The amplitude and kinetics of slow pH_i changes were equally followed by both sensors, and the dye ratio recorded slightly higher amplitudes during faster pH_i shifts as induced by the addition and removal of NH_4^+.

INTRODUCTION

Intracellular pH (pH_i) plays an important role in a variety of cellular processes. Among these are the modulation of enzymatic activity, of receptors, and of ion channels (cf. Moody, 1984; Chesler, 1990; Deitmer and Rose, 1996). Several techniques have been used to monitor pH_i, including ion-sensitive microelectrodes (Thomas, 1974, 1984), the distribution of weak acids and/or bases, the phosphoric shift as measured with nuclear magnetic resonance (see Roos and Boron, 1981), and fluorescent dyes, such as 2′,7′-bis(2-carboxyethyl)-5,6-carboxyfluorescein (BCECF) (Rink et al., 1982).

The techniques most often applied to determine the pH in single cells in recent years have been the use of either pH-sensitive microelectrodes or the fluorescent dye BCECF. The principles of measurement of these two techniques, however, are quite different. Using BCECF, two fluorescent dye signals produced by excitation at two wavelengths are recorded to obtain a ratio signal, which provides a pH_i value that is assumed to be largely independent of the dye concentration. The BCECF ratio signal may provide spatial resolution of pH_i measurement by using video imaging techniques, whereas the microelectrode reading is a point-source measurement, providing a local cytosolic pH value. Although the fluorescent dye method can be mechanically noninvasive, if the dye is loaded as the membrane-permeable acetoxymethylester, microelectrode tips must impale the cell and always cause, at least transiently, some damage to the cell. Therefore, pH microelectrode measurements are usually limited to cells larger than 15 μm. In addition, the response time of BCECF is considerably shorter than that of ion-sensitive microelectrodes. Because of these considerable advantages over pH-sensitive microelectrodes, the importance of the BCECF technique has grown ever since it has been introduced.

On the other hand, several problems can occur with BCECF ratio measurements. First, the dye may enter cellular organelles and may hence give pH readings that are the means of cytosolic and organellar pH (Slayman et al., 1994). In contrast, the tip of a pH microelectrode, usually 1 μm or larger, is not expected to impale the small organelles. Bleaching of the dye during illumination of the cell may lead to accumulation of bleach products, which can harm the cell (Harris-Warrick and Flamm, 1987; Kemenes et al., 1991). Measurements of pH_i with microelectrodes, in contrast, may last for several hours without any apparent loss of cell viability. Finally, the calibration procedure for pH-sensitive microelectrodes, which are calibrated in salines of different pH before and after the experiment, is very easy, assuming a similar sensitivity and selectivity of the electrodes for H^+ in the cytosol and in bath salines (Thomas, 1984). In contrast, calibration procedures for BCECF and other fluorescent dyes are rather complex. Dye spectra are dependent on the composition of the solvent; therefore, intracellular dye spectra may differ from in vitro spectra, and hence in vitro calibration is often discarded. The commonly used in situ calibration of the BCECF ratio depends on making cell membranes permeable to H^+ by the K^+/H^+ ionophore nigericin, which should allow equilibration between intracellular and extracellular pH. This procedure

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allows the intracellular calibration of the dye and is assumed to provide accurate measurements of intracellular pH.

A comparison between pH measurements obtained with either dyes or pH-sensitive microelectrodes was first carried out by Chaillet and Boron (1985), using the nonfluorescent dye 4',5'-dimethyl-5 (and -6-) carboxyfluorescein (Me_2CF). It was found that the Me_2CF absorbance ratio, calibrated in situ with the nigericin/high-K^+ technique and pH-sensitive microelectrodes in salamander proximal tubules, indicated very similar pH_i changes, with the exception that the dye-derived pH_i value was alkaline shifted by about 0.1 pH units as compared with pH-sensitive microelectrode recordings.

We have now compared pH_i measurements using the fluorescent pH-sensitive dye BCECF and pH-sensitive, neutral carrier microelectrodes in the giant neuropile glial cell of the medicinal leech (Deitmer and Schlue, 1987; Munsch and Deitmer, 1994). The pH_i regulation in this cell has been studied in some detail (cf. Deitmer, 1995) by use of intracellular ion-sensitive microelectrodes (Deitmer and Schlue, 1987; Deitmer, 1991, 1992). A fluorescent dye technique has been established for Ca^{2+} measurements in these cells (Munsch and Deitmer, 1992; Munsch et al., 1994). Our results suggest that complete equilibration of intracellular with extracellular pH in nigericin-containing solutions is not established, and that excessive dye levels may markedly derogate the viability of the cells, while relative pH_i changes are recorded similarly by both techniques. A preliminary account of this study has been presented in abstract form (Nett and Deitmer, 1995).

MATERIALS AND METHODS

Preparation of glial cells

The experiments were performed on neuropile glial cells in isolated ganglia of the leech *Hirudo medicinalis* L. The dissection for exposing the two neuropile glial cells of each segmental ganglion in situ has been described previously (Munsch and Deitmer, 1992). In short, individual ganglia were pinned in a Sylgard-coated perspex chamber in a modified Leibovitz (L-15; see below) tissue culture medium. The ventral ganglionic capsule was removed mechanically with fine forceps. The ganglia were then incubated for 1 h in 2 mg ml^{-1} collagenase-dispase (Boehringer-Mannheim, Germany) containing L-15 medium at room temperature (20–25°C). After enzyme treatment the ganglia were thoroughly washed with enzyme-free medium. Neurons overlying the glial cells were removed by suction into a glass micropipette, thereby exposing the giant glial cells of the neuropile. For experimentation, ganglia were then transferred into standard leech saline.

Solutions

Original L-15 medium (Gibco, Eggenstein, Germany) was modified by dilution (1:3) with a salt solution of the following composition (in mmol l^{-1}): $CaCl_2$, 6.87; $MgCl_2$, 2.51; KCl, 3.32; Na-malate, 20.1; Na-pyruvate, 12.5; HEPES, 15; glucose, 15; gentamycin (10 mg ml^{-1}), 0.3%; adjusted to pH 7.4 with NaOH.

The standard, nominally CO_2/HCO_3^--free saline had the following composition (in mM): NaCl, 85; KCl, 4; $CaCl_2$, 2; $MgCl_2$, 1; HEPES, 10 (pH adjusted to 7.4 with 3–5 mM NaOH). In CO_2/HCO_3^--buffered saline, 24 mM NaCl was replaced by equimolar amounts of $NaHCO_3$, and the saline was bubbled with 5% CO_2/95% O_2. When the pH of the salines was adjusted to 7.8, 60 mM NaCl was replaced by 60 mM $NaHCO_3$. In CO_2/HCO_3^--buffered salines, which were adjusted to pH 7.0 or 6.6, HEPES was replaced by MOPS (3-(*N*-morpholino)ethanesulphonic acid) or PIPES (piperazine-*N*,*N*'-bis(2-ethanesulfonic acid)), and 10 mM or 3.8 mM NaCl was replaced by equimolar amounts of $NaHCO_3$, respectively.

Microfluorimetric pH measurement

Microfluorimetric measurement of the intracellular pH (pH_i) was performed using the fluorescent dye BCECF. BCECF (19 mM dissolved in 0.2 M K-acetate)-filled microelectrodes were used for iontophoretic dye injection into the giant glial cell with a negative constant current of 1–10 nA, first with a single injection pulse, and then continuously throughout the experiment (see Munsch and Deitmer, 1992, 1995). Attempts to load these cells with dye by incubation in the membrane-permeable BCECF-acetoxymethylester have been unsuccessful (Pfeiffer, 1992; Munsch and Deitmer, 1992).

Experiments were performed using a Deltascan dual-excitation spectrofluorimeter (PTI, Wedel, Germany) in which shutters, monochromator settings, and data acquisition were controlled by computer software and interfaces from PTI. The cells were excited alternately at 440 nm and 495 nm (bandwidth 4 nm) through the epifluorescence port of an upright Zeiss Axioskop (Zeiss, Oberkochem, Germany) with a water immersion Zeiss Achroplan 40×/W objective. Fluorescence intensity (at wavelength > 520 nm) was measured with a photon-counting photomultiplier tube. Measurements were limited to a field of view slightly larger than the cell body of the injected glial cell by a rectangular diaphragm.

The cellular background fluorescence was subtracted from the raw data before ratio calculation. The values of the background fluorescence for each excitation wavelength were obtained before the impalement of the cell on the dye-filled microelectrode after the field of view had been selected.

Because the isosbestic point of the BCECF excitation spectrum is around 440 nm, the fluorescence measured during excitation at this wavelength is a good indicator of the intracellular dye concentration. However, the absolute value cannot be determined directly in our preparation. We estimated the intracellular BCECF concentration by measuring the fluorescence intensity at 440 nm excitation (F440) of solutions in vitro, containing 80 mM KCl, 10 mM NaCl, 10 mM HEPES, and different concentrations of BCECF (0–300 μM). Ten microliters of diluted BCECF solutions was pipetted into a Neubauer counting chamber, and the field of view was set to approximately 3500 μm^2, equivalent to the mean dimension of neuropile glial cells (Pfeiffer, 1992). This procedure yielded a linear dependence of the fluorescence at 440 nm on the dye concentration over the range measured. According to this relationship, 4×10^4 and 8×10^4 photons/s corresponded to 75 and 150 μM BCECF, respectively.

The calibration of the BCECF ratio signal was performed by the method described by Thomas et al. (1979), using nigericin in high-K^+ salines at different bath pH values. Nominally CO_2/HCO_3^--free salines were used, in which 76 mM NaCl was replaced by equimolar KCl to a final K^+ concentration of 80 mM, which was measured to be the intracellular K^+ concentration (Brune, 1991). Nigericin (10 μM or 20 μM) (Molecular Probes, Eugene, OR) was added to permeabilize the glial cell membrane, and the pH was adjusted to 7.8, 7.4, 7.0, or 6.6. In salines adjusted to pH 7.0 or pH 6.6, HEPES was replaced with MOPS or PIPES, respectively.

In addition, a nigericin-independent calibration method was used, in which the BCECF ratio was directly compared with the pH reading of a neutral-carrier, pH-sensitive microelectrode, which was used to impale intact neuropile glial cells simultaneously, while the steady-state pH_i was challenged in different CO_2/HCO_3^--buffered salines (see Results).

pH-sensitive microelectrodes

Microelectrodes made from theta capillaries (TST150; World Precision Instruments, New Haven, CT) were used for recording pH_i and the reference membrane potential. The prospective ion-sensitive barrel was silanized in principle as described by Borelli et al. (1985), using a drop of 5%

tri-*N*-butylchlorosilane (Fluka) mixed in 99.9% pure carbon tetrachloride, which was backfilled into the tip. The pipette was then baked on a hot plate at 470–475°C for 4.5–5 min. The H^+ cocktail (Fluka 95291) was backfilled into the tip of the silanized barrel, which was then filled up with 0.1 M Na-citrate, pH 6.0. The reference barrel was filled with 3 M KCl. The electrodes were beveled on a rotating disc; the reference barrel of the electrodes typically had a resistance of 50 MΩ in normal leech saline after beveling. Each channel of the theta-microelectrode was connected via chlorided silver wires to an input of a differential electrometer. The electrometer outputs were recorded on chart paper for direct display and on-line on a computer.

The electrodes were calibrated in nominally CO_2/HCO_3^--free as well as in CO_2/HCO_3^--containing leech saline buffered to 7.8 or 7.4 with HEPES and to 7.0 or 6.6 with MOPS or PIPES, respectively. The sensitivity to 5% CO_2 was tested for each electrode, and a response of up to 3 mV (0.05 pH units) was accepted, but taken into account for ratio calibration. This could not affect the microelectrode pH_i measurement during the nigericin-based calibration, because nominally CO_2/HCO_3^--free salines were used in those experiments.

RESULTS

The pH_i during dye injection

The background fluorescence intensity of the glial cells, measured before impalement of the cell on the dye electrode, was about 5×10^3 photon counts s^{-1} for both excitation wavelengths (Fig. 1, *inset*) and was subtracted before ratio calculation. After impalement of the cell on the dye-filled microelectrode, fluorescence intensity slowly increased because of BCECF-diffusion into the cell, while the fluorescence ratio strongly decreased (Fig. 1). When the iontophoretic injection current was turned on, the fluorescence intensity at both excitation wavelengths increased severalfold and reached typically $50–100 \times 10^3$ photons/s at 440 nm, and $200–500 \times 10^3$ photons/s at 495 nm, which corresponds to an intracellular dye concentration of 100–200 μM. The fluorescence ratio moderately decreased further, until a fluorescence intensity was reached, where repeated, moderate injection only slightly changed the ratio, indicating the resting pH_i level. The decrease in fluorescence ratio upon dye injection did not represent a true change in pH_i, because a pH-sensitive microelectrode, which a cell was simultaneously impaled with, showed no change in pH_i during normal, moderate dye injection (not shown here).

Fig. 2 shows the effect of different magnitudes of BCECF injection, indicated by the injection current (*lowest trace*), on the membrane potential of a glial cell, on the pH_i as recorded by the pH-sensitive microelectrode, on the BCECF ratio, and on the fluorescence intensity at 440 nm. The resting membrane potential at the beginning of the experiment was −71 mV, and the pH_i as measured by the pH-sensitive electrode was near 7.1. Turning on the injection current not only increased the fluorescence emission, but also decreased the BCECF ratio without any apparent change of pH_i as measured by the microelectrode (Fig. 2, see *). Turning off the injection current led to a slow increase of the BCECF ratio without a change in pH_i. When

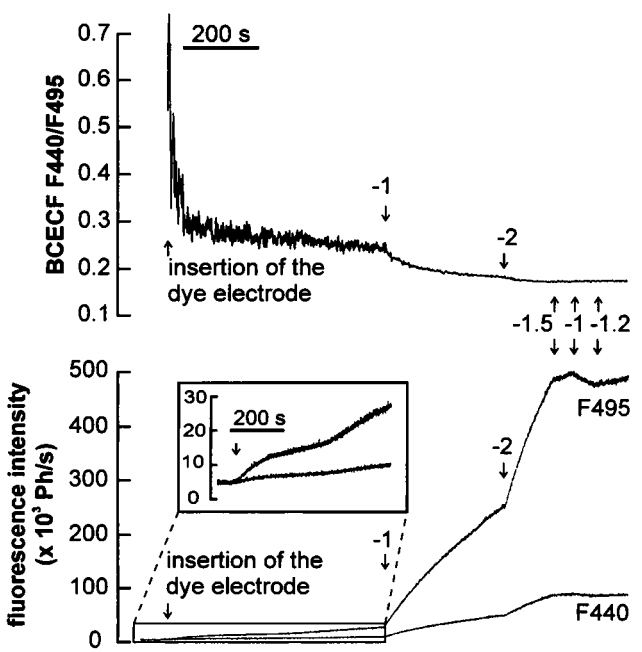

FIGURE 1 BCECF ratio (F440/F495, *upper trace*) and fluorescence intensity recorded at the excitation wavelengths 440 nm and 495 nm in photons/s (Ph/s, *lower traces*) before and during iontophoretic dye injection. The first 11 min are zoomed in the inset (above lower traces). The negative numbers indicate the iontophoretic current in nanoamperes, for dye injection.

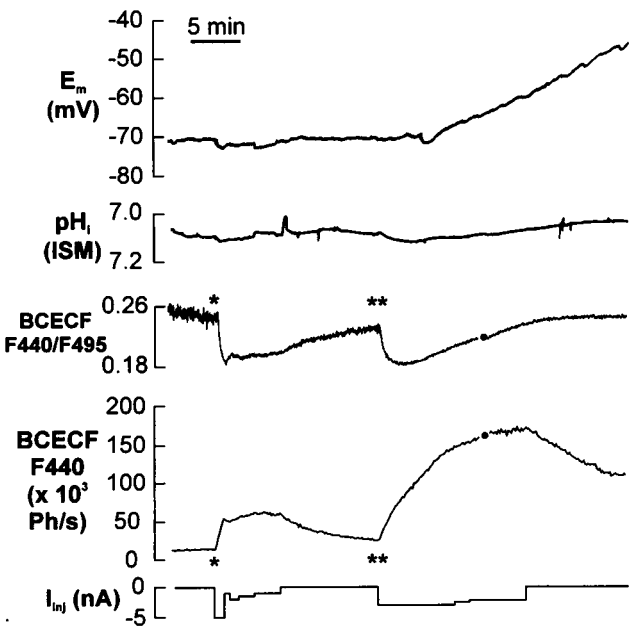

FIGURE 2 Membrane potential (E_m), intracellular pH as recorded with the microelectrode (pH_i (ion-sensitive microelectrode); BCECF ratio (BCECF F440/F495), and fluorescence intensity at 440 nm excitation wavelength (BCECF F440) during different magnitudes of iontophoretic BCECF injection (I_{inj}). Moderate (*) and extensive (**) dye injection affected the BCECF ratio at least transiently, but had only small effects on the ion-sensitive microelectrode reading. Very large dye injections (F440 > 100×10^3 photons/s) caused an irreversible membrane depolarization within about 10–15 min.

a large injection current was again applied (Fig. 2, see **), leading to an additional fluorescence increase to well above 100×10^3 photons/s at F440 nm, the BCECF ratio decreased markedly, and the pH microelectrode indicated a small transient pH_i increase (by ~0.03 pH units). Within a few minutes of this large dye injection, the glial cell membrane began to depolarize irreversibly, indicating a loss of cell viability.

This depolarization depended on illumination of the dye-filled cells and was not an effect of BCECF itself or of simultaneously injected acetate. When cells were loaded with a large amount of BCECF without continuous illumination, no depolarization occurred for 1 to 1.5 h (not shown). Only when illumination was turned on did the irreversible depolarization occur as described above. In 25 of 27 experiments, where F440 augmented 5×10^4 photons/s, and where the cells were continuously illuminated, an irreversible decay of the membrane potential was recorded within 10 to 30 min (see also Fig. 6). We therefore limited the dye injection to a F440 level below 5×10^4, typically to between 2×10^4 and 4×10^4 photons/s (corresponding to 40–80 μM BCECF), which allowed us experiments of at least 60 min before a decay of membrane potential eventually occurred.

In situ calibration of the BCECF ratio in H^+-permeabilized cells

In the calibration procedure for the BCECF described by Thomas et al. (1979), the cell membrane is H^+ permeabilized by the addition of the K^+/H^+ ionophor nigericin, and the external K^+ concentration is raised to cytosolic levels to abolish the transmembrane K^+ gradient. This should lead to equilibration between cytosolic and external pH, a presumption for the accuracy of the absolute pH_i value as determined with the BCECF ratio.

In salines containing 80 mM K^+ and 10 μM nigericin, the BCECF ratio reached a steady-state value in salines adjusted to 6.6, 7.0, 7.4, and 7.8, respectively, within about 20 min (Fig. 3 *A*). Excitation spectra at the different external pH values are shown in the inset, indicating that the fluorescence amplitude decreased by about 20% during the experiment, whereas the course of the spectra remained virtually the same. The steady-state fluorescence ratio values from nine experiments were plotted as a function of the external pH (Fig. 3 *B*). The relationship between BCECF ratio and external pH appeared nonlinear, and the values could be fitted to a modified Henderson-Hasselbalch equation described by James-Kracke (1992):

$$pH_i = pK_a - \log((R - R_{min})/(R_{max} - R)), \qquad (1)$$

where pK_a is the negative logarithm of the acid dissociation constant, R is the fluorescence ratio F440/F495, R_{min} is the ratio under saturating alkaline conditions, and R_{max} is the ratio under saturating acid conditions. This procedure

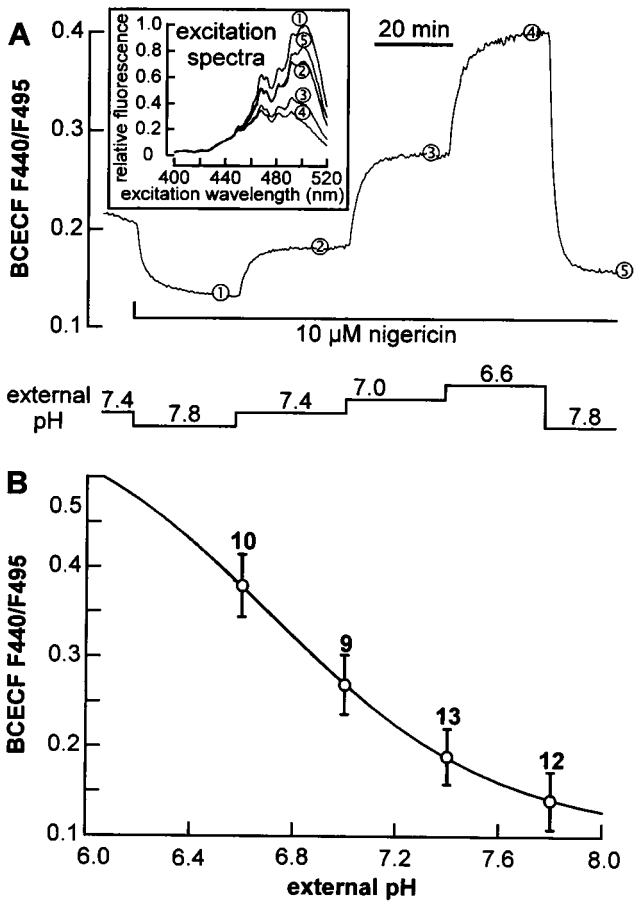

FIGURE 3 (*A*) BCECF ratio in nigericin-containing, high-K^+ saline at different external pH values between 6.6 and 7.8. Excitation spectra (*inset*) were recorded during steady-state ratio values indicated by 7–5, and normalized to the fluorescence intensity at the isosbestic point (440 nm). Note that the second excitation spectrum at external pH 7.8 5 exhibited about 20% smaller fluorescence intensities at wavelengths above 440 nm excitation. (*B*) Relationship between the BCECF ratio of H^+-permeabilized cells and the external pH. The symbols are the means, and the bars represent ±SD. The figures above the symbols indicate the number of experiments, and the solid line is a nonlinear regression according to Eq. 1 (see text).

yielded a pK_a of 6.72, an R_{min} of 0.104, and an R_{max} of 0.588.

The BCECF ratio was plotted in the same way as recordings of the pH- sensitive microelectrode, i.e., with acidifications going up (F440/F495) and alkalinizations going down, to follow the kinetics of increase and decrease of the H^+ concentration, respectively.

The steady-state pH_i was thus determined to be 7.32 ± 0.22 (mean ± SD, n = 19) for the neuropile glial cells in standard saline (pH_o = 7.4). This value differs significantly (paired t-test; $p < 0.001$) from the value measured with pH-sensitive microelectrodes (7.09 ± 0.13) in the same experiments.

For further examination of these discrepancies between microelectrode- and of dye-derived pH_i values, we have routinely measured pH_i during the nigericin calibration pro-

cedure with a simultaneously impaled pH-sensitive microelectrode (Fig. 4). The neuropile glial cells depolarized in salines containing 80 mM K^+ and 10 or 20 μM nigericin to a steady-state value of -6.3 ± 1.5 mV (mean $\pm$ SEM, $n = 12$). The steady-state membrane potential remained negative in 4 out of 4 experiments, even when the K^+ concentration was further increased to 94 mM (the K^+ concentration could not be increased beyond 94 nM without increasing the tonicity of the leech saline).

The pH microelectrode, which was calibrated in the bath before and after each experiment in salines adjusted to different pH values, both in high-K^+ and nigericin saline, as well as in standard saline, showed no sensitivity to nigericin itself (Fig. 4 *A*). The electrode indicated a pH_i of 7.25 in the nigericin-containing, high-K^+ saline at an external pH of 7.4; on average a pH_i of 7.29 $\pm$ 0.06 ($n = 11$) was measured. Even when the reference electrode recorded zero mV in this calibration solution, the pH_i measured by the microelectrode was always lower than the bath pH of 7.4. The BCECF ratio leveled at 0.15 in this saline, and increased stepwise with lowering of external pH, reaching a new steady state within about 15 min. The relative shifts in pH_i, as measured with the pH microelectrode, were of constant size, but with a somewhat slower time course than the change in BCECF ratio.

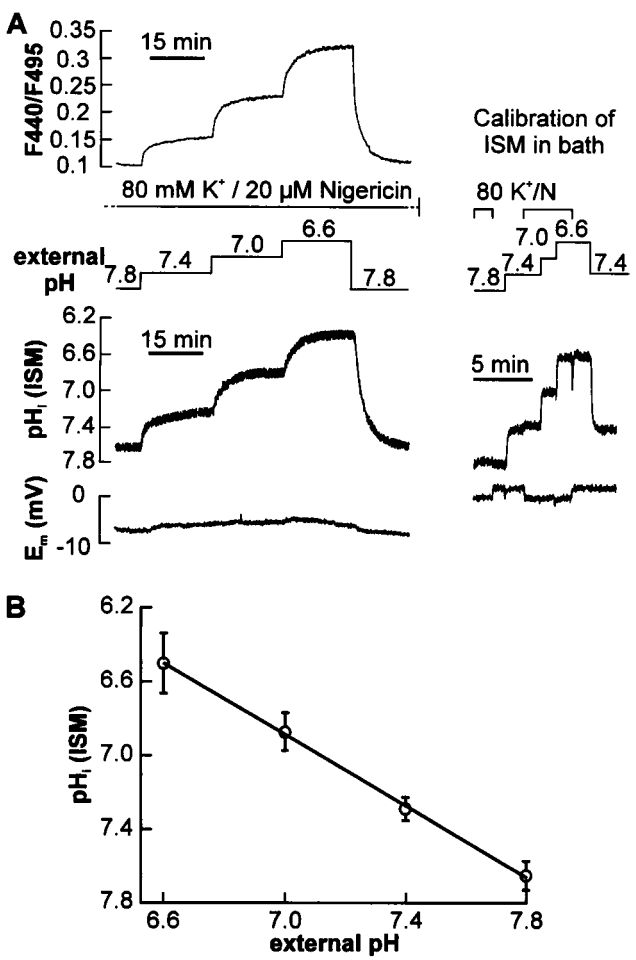

FIGURE 4 (*A*) BCECF ratio (*upper trace*), pH_i as recorded by the pH-sensitive microelectrode (pH_i (ISM), *middle trace*), and membrane potential (E_m, *lower trace*) during the calibration procedure in a glial cell, H^+-permeabilized by 20 μM nigericin at a high K^+ concentration. The calibration of the pH-sensitive microelectrode was performed before (not shown) and after the experiment, both in the presence and absence of nigericin (N) (*right*). (*B*) Relationship between the intracellular pH (pH_i) as recorded with the pH-sensitive microelectrode and the external pH. The line was drawn according to a linear regression fit to all points.

The relationship between the pH_i, as measured with the pH-sensitive microelectrode, and the external pH is plotted in Fig. 4 *B*. The absolute pH_i values as measured by the microelectrode were not identical to the pH_o values, as would be expected if the cytosolic pH had fully equilibrated with the bath pH values. The mean pH_i values measured were 0.12 ± 0.02 (mean $\pm$ SEM; $n = 37$) pH units more acid than the external (bath) pH values, indicating incomplete "clamp" of pH_i during this nigericin-based calibration procedure. Neither change of external K^+ to 94 mM nor raising the nigericin concentration to 40 μM had any effect on this discrepancy between intracellular and external pH.

In situ calibration in intact glial cells

To circumvent the problems of the nigericin-based calibration, the BCECF ratio was calibrated according to the reading of an intracellular pH microelectrode without the use of nigericin. Shifts of pH_i were evoked in salines with different pH values buffered with 5% CO_2 and various HCO_3^- concentrations (Fig. 5 *A*). In these glial cells pH_i largely follows the equilibrium of the reversible Na^+-HCO_3^- cotransporter and hence the external pH (Deitmer, 1991; Deitmer and Schneider, 1995). Decreasing pH_o reversibly depolarized the glial membrane because of the electrogenicity of the cotransporter (Deitmer and Schlue, 1989; Deitmer and Szatkowski, 1990). The BCECF ratio and the microelectrode reading changed with similar time courses, when pH_o was altered from 7.4 to 7.0, 7.8, and 6.6, respectively.

One hundred five steady-state values of the BCECF ratio obtained from 17 experiments were plotted against the steady-state pH_i as measured by the microelectrode (Fig. 5 *B*) and fitted to Eq. 1. This yielded a pK_a of 6.6, R_{min} was 0.091, and R_{max} was 0.54. The dotted curve in Fig. 5 *B* shows the fitted calibration curve from the nigericin-based calibration (Fig. 3 *B*). There appeared a significant deviation in absolute values between the two types of calibration curves; the BCECF ratio values calibrated by the pH microelectrode reading in intact cells indicated a pH value up to 0.15 pH units lower than the BCECF ratio values calibrated in H^+-permeabilized cells.

In 19 experiments where pH_i was measured with electrode and BCECF simultaneously, the mean resting pH_i obtained from the microelectrode reading in standard CO_2/HCO_3^--free saline was 7.09 ± 0.13 (mean $\pm$ SD), and the mean BCECF ratio was 0.207 ± 0.04. Converting this ratio value to a pH_i value yielded a pH_i of 7.32 ± 0.22 according

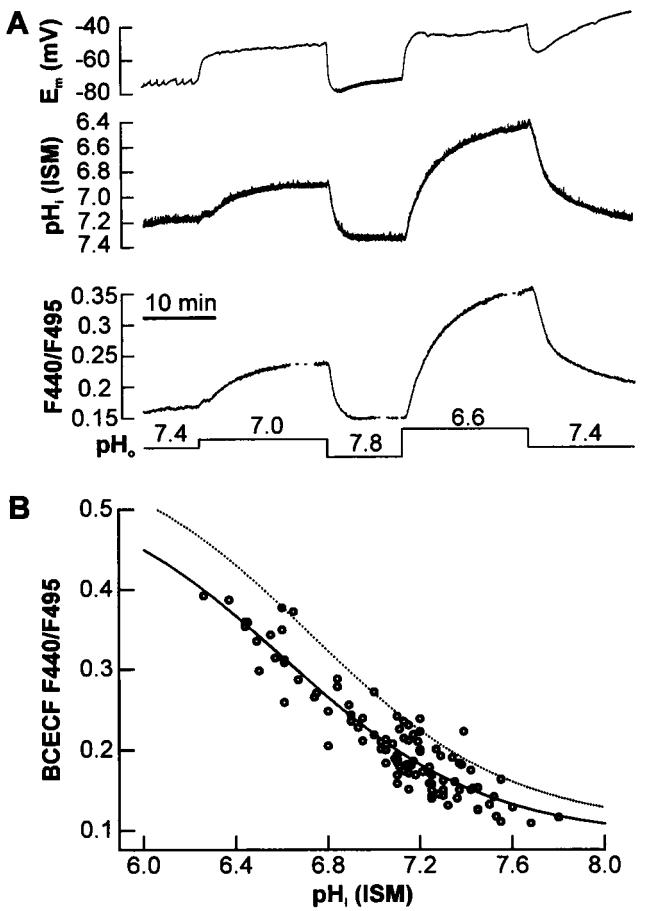

FIGURE 5 (*A*) Membrane potential (E_m, *upper trace*), intracellular pH as recorded with the microelectrode (pH_i (ISM), *middle trace*), and BCECF ratio, F440/F495 (*lower trace*), simultaneously recorded in an intact neuropile glial cell during superfusion with CO_2/HCO_3^--buffered salines adjusted to different pH (pH_o) between 6.6 and 7.8. (*B*) Relationship between BCECF ratio and pH_i as recorded with the microelectrode in 17 experiments of the kind shown in *A*. The solid line is a nonlinear regression to Eq. 1 described in the text, and the dotted line represents the fitted curve from Fig. 3 *B*.

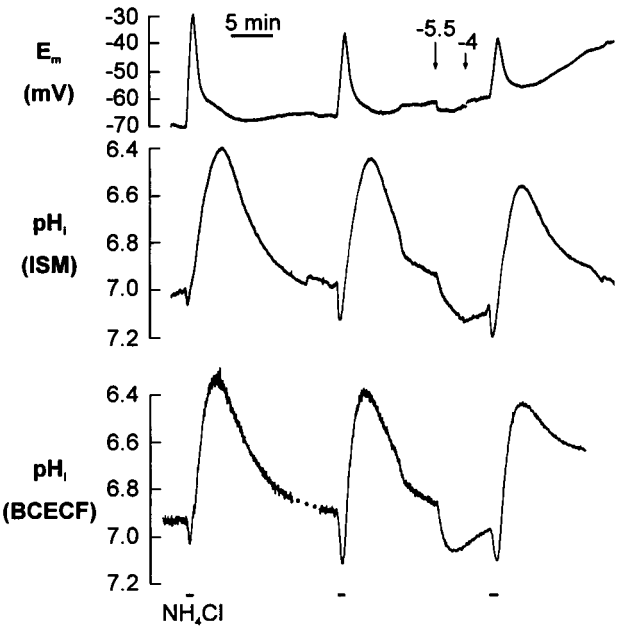

FIGURE 6 Membrane potential (E_m), intracellular pH as recorded with the microelectrode (pH_i (ISM)), and pH_i as recorded with BCECF (pH_i (BCECF)), calibrated according to the microelectrode, as shown in Fig. 5 during successive NH_4Cl (20 mM) applications. After the second NH_4^+ pulse, the dye injection current was increased from −1 nA to −5.5 and −4 nA, respectively, as indicated by the arrows, which resulted in a slurring of the BCECF ratio signal and a loss of membrane potential.

to the nigericin-based calibration (see above). A pH_i of 7.08 ± 0.21 was obtained when the dye calibration according to the microelectrode reading in CO_2/HCO_3^--containing solution was taken. Although the mean pH_i values measured with the pH electrode and BCECF (microelectrode-based calibration) were thus very similar, the pH_i values measured with both techniques in an individual experiment could differ by up to 0.2 pH units (see also Fig. 6).

Simultaneous pH_i recording during NH_4^+ pulses

Alkaline-acid shifts can be induced in living cells by the application and subsequent removal of NH_4^+ (Boron and De Weer, 1976). We applied 20 mM NH_4Cl for 1 min and recorded the pH_i changes with both the (microelectrode-based) calibrated BCECF ratio and the intracellular pH-sensitive microelectrode (Fig. 6). In the presence of NH_4^+, a fast intracellular alkalinization was followed by a slower acidification. After the removal of NH_4^+, the cell acidified nearly to pH_i 6.4 because of NH_3 leaving the cell. The cell recovered from this acid load by active pH_i regulation, which, in the nominal absence of CO_2/HCO_3^-, is achieved mainly by an amiloride-sensitive Na^+/H^+ exchange (Deitmer and Schlue, 1987).

The two pH_i recording techniques indicated a less than 0.1-unit-different, absolute, resting pH_i value. The fast alkalinization after the addition of NH_4 and the acidification upon removal of NH_4^+ was faster and larger, as monitored by the dye as compared to the microelectrode. Otherwise the pH_i recordings with the two techniques were very similar.

After the second NH_4^+ pulse, the injection current was increased from −1 nA to −5.5 and −4 nA, respectively, which caused an apparent rise in pH_i, as recorded by both the BCECF ratio and the microelectrode. The pH_i increase as measured with the pH microelectrode was again slower than the change in the BCECF ratio signal. The following (third) NH_4^+ pulse produced similar alkaline and acid shifts, as measured by both techniques; however, the recovery from the acid load was poorly recorded by the BCECF ratio as compared to the microelectrode recording. In addition, the membrane depolarization evoked by the NH_4^+ pulse was smaller than those elicited by the previous NH_4^+ pulses, and the membrane began to depolarize irreversibly (see also Fig. 2).

This type of experiment shows that the pH_i measurements were similar with the two techniques, with the following

differences: 1) the absolute values obtained with the two techniques may be different in individual experiments (see above) when pooled calibration data are used; 2) as expected, the BCECF ratio signal could follow pH_i changes faster than pH-sensitive microelectrodes; 3) the recordings as obtained with the two methods increasingly deviated with the amount of dye injected into the cell above a level of approximately 5×10^4 photons/s F440 for the used instrumentation. In addition, the injection of larger amounts of dye apparently caused a conductance change in the cell membrane, which led to an irreversible depolarization.

DISCUSSION

The present study is the first that directly compares the BCECF technique and ion-sensitive microelectrodes to measure pH_i in the same cell simultaneously. Because the two techniques rely on different principles of measurement and calibration, the results obtained with each technique have been difficult to evaluate with respect to the other. In the great majority of cells in which pH_i has been measured, only one technique has been used: either the BCECF ratio method (mostly in smaller vertebrate cells) or pH-sensitive microelectrodes (mostly in larger invertebrate and vertebrate cells). The direct comparison of the techniques in the leech giant glial cell was therefore intended to reveal some general features of the compatibility of the data obtained with these two methods. Our results indicate that the two techniques measure very similar relative pH_i changes, while the absolute pH_i values may deviate considerably, presumably because of incomplete pH equilibration across the cell membrane during the nigericin-based calibration of the BCECF ratio.

Effects of dye injection and dye bleaching

Continuous BCECF injection during the experiment was necessary because the fluorescence rapidly decayed, partly because of bleaching, and partly because of dye loss from the cell body by diffusion into other parts of the cell and across gap junctions into neighboring glial cells (Pfeiffer, 1992) or out of the cell. A similar dye loss has been described for this cell with the Ca^{2+}-sensitive dye fura-2, which could be counteracted by continuous dye injection (Munsch and Deitmer, 1995). Because the input resistance of the cells is around 0.5 MΩ or less (Munsch and Deitmer, 1994), injection of 1–3 nA offsets the membrane potential by not more than 1–2 mV to more negative values.

Our results indicate that the determination of pH_i by the BCECF ratio appears to be independent of the amount of intracellular dye only over a relatively small concentration range. The spectral properties of the dye may possibly change with rising dye concentrations, perhaps because of binding of the dye to intracellular structures. A dependence of absorption and excitation spectra on the dye concentration has previously been described for carboxyfluorescein (Babcock, 1983). Large injections of the dye, however, may change pH_i because of some increase in buffering power. Similarly, large changes in the concentration of the Ca^{2+}-sensitive dye fura-2 affected the amplitude and time course of the fura-2 ratio signals in this cell, believed to be partly due to some increase in Ca^{2+}-buffering power (Munsch and Deitmer, 1995). Because the intrinsic H^+ buffering power of these cells is about 25 mM (Deitmer and Schlue, 1987), the dye concentration has to be in the millimolar range to exert a significant contribution on the buffering. The alkalinization upon larger dye injections could also be due to the simultaneous injection of acetate, which was in a concentration of 200 mM in the dye-injecting electrode.

During prolonged illumination of dye-filled neuropile glial cells, an irreversible loss of membrane potential occurred. This was not observed with injections of fura-2 to concentrations well beyond those necessary for Ca^{2+} measurement with a good signal-to-noise ratio (Munsch and Deitmer, 1995). In our study the depolarization was not a direct effect of BCECF itself (e.g., due to inhibition of K^+ channels, described for other fluorescein derivatives; de Weille et al., 1992), but dependent on continuous illumination of the cells, suggesting that the toxicity originated from the bleach products of BCECF. 5(6)-Carboxy-fluorescein, another fluorescein derivative with a structure very similar to that of BCECF, has indeed been used for photoinactivation of cells (Harris-Warrick and Flamm, 1987; Kemenes et al., 1991). Because in most fluorescent dye studies measuring pH_i, the membrane potential has not been recorded (as, e.g., in cell cultures where a high final dye concentration is often used to improve the signal-to-noise ratio), a loss of cell viability may easily remain undetected.

Calibration of the BCECF ratio

Calibration of the BCECF ratio with the nigericin method (Thomas et al., 1979) yielded steady-state pH_i values that were alkaline shifted in comparison with values measured with pH-sensitive microelectrodes. Our results indicate that an incompleted "clamp" of the pH_i to pH_o during the calibration procedure may account for the different absolute pH_i measurements.

The transport reaction of this carboxylic ionophore is electroneutral and the equilibrium is achieved at $[H^+]_i$:$[H^+]_o = [K^+]_i$:$[K^+]_o$ (cf. Pressman, 1976), assuming high selectivity for K^+ over other cations. The external K^+ concentration was elevated to the mean intracellular K^+ concentration of 80 mM, as determined with K^+-sensitive microelectrodes (Brune, 1991) or with the reversal potential of K^+-dependent membrane responses (Munsch and Deitmer, unpublished observations). This should eliminate the K^+ gradient across the cell membrane, resulting in equilibration of cytosolic to external pH. We have tested this assumption with a pH-sensitive microelectrode, which the cell was impaled with during the calibration procedure. In our calibration experiments, however, the cytosol remained about 0.12 pH units more acid than the external saline.

This is unlikely to be due to a too low external K^+ concentration, because elevation of the external K^+ concentration to 89 or 94 mM, respectively, had no effect on pH_i, as measured with the pH-sensitive microelectrode. An error due to the slight affinity of nigericin for Na^+ can be excluded, because the external Na^+ concentration in the nigericin/high-K^+ salines was close to the intracellular Na^+ concentration as measured in normal saline (Deitmer, 1992).

The calibration of the BCECF ratio in intact cells, according to the simultaneous reading by an intracellular pH-sensitive microelectrode, provided a calibration curve, which was offset by up to 0.15 pH unit as compared to the calibration in H^+-permeabilized cells. It is concluded, therefore, that the absolute pH_i determined with the two techniques may deviate and that the calibration curve obtained in H^+-permeabilized cells must be corrected to align with the calibration obtained with the pH-sensitive microelectrodes. The pK_a values that have been measured for intracellular BCECF vary between 6.7 (James-Kracke, 1992) and 7.3 (Boyarsky et al., 1993); hence the pK_a values measured in our experiments were at the lower part of this range.

Even when the microelectrode-based calibration of the BCECF ratio is used, there might be a difference between the microelectrode-derived pH_i value and the BCECF-derived pH_i value in individual experiments that we cannot explain. However, these differences canceled out when mean values were calculated and hence were not likely to be due to a systematic error. For accuracy of the BCECF measurements it would be necessary to perform calibration curves for each experiment.

In a study of another, nonfluorescent pH-sensitive dye, 4′,5′-dimethyl-5 (and -6-) carboxyfluorescein, Chaillet and Boron (1985) also found very little relative difference between microelectrode and dye-based pH_i measurements carried out in salamander proximal tubules. They also measured pH_i simultaneously with the two techniques, but not during the calibration procedure. Again, for absolute pH_i determination, Chaillet and Boron (1985) found a similar deviation of ~0.1 unit as obtained with the two techniques. The treatment of the calibration procedure assumes that the pH-sensitive microelectrode measures the pH with no interference in the cytosol, giving the same absolute pH values as the pH values adjusted in the bath salines. If the deviation of the cytosolic pH in the H^+-permeabilized cells from the bath saline pH, as measured by the microelectrodes, were due to an interference from other ions on the electrode, the difference in calibration would result from some inaccuracy of the ion-sensitive microelectrodes. However, the selectivity of the H^+ cocktail for H^+ over physiologically relevant inorganic ions is very high, and no interference of other cellular components (e.g., proteins) has yet been reported.

It might be possible to circumvent the necessity of complete equilibration of pH_i and pH_o by using a full-range calibration method described by James-Kracke (1992), where R_{min} and R_{max} can be accurately determined by choosing very alkaline and acid external pH values, respectively. However, the determination of the acid dissociation constant, K_a, in situ still requires absolute intracellular pH values.

In conclusion, our study shows that the differences between the now commonly used pH-sensitive dye BCECF and recordings with pH-sensitive microelectrodes are similar to those reported for the nonfluorescent dye Me_2CF and microelectrodes (Chaillet and Boron, 1985). Our results suggested that these differences could be due to incomplete equilibration of pH_i to pH_o during the nigericin-based BCECF calibration. The simultaneous recording of the membrane potential indicated that large BCECF injections and continuous illumination may lead to loss of cell viability.

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