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Concurrent measurements of the free cytosolic concentrations of H⁺ and Na⁺ ions with fluorescent indicators

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Abstract We report a method for the concurrent measurement of intracellular [Na⁺]_i ([Na⁺]_i) and pH (pH_i) in cells co-loaded with SBFI, a Na⁺-sensitive fluorophore, and either carboxy SNARF-1 or SNARF-5F, H⁺-sensitive fluorophores. With the optical filters specified, fluorescence emissions from SBFI and either SNARF derivative were sufficiently distinct to allow the accurate measurement of [Na⁺]_i and pH_i in rat hippocampal neurons. Neither the Na⁺ sensitivity of SBFI nor the pH sensitivities of carboxy SNARF-1 or SNARF-5F was affected by the presence of a SNARF derivative or SBFI, respectively. In addition, the calibration parameters obtained in neurons single-loaded with SBFI, carboxy SNARF-1 or SNARF-5F were not significantly influenced by the presence of a second fluorophore. In contrast to the established weak sensitivity of SBFI for protons, both SNARF derivatives appeared essentially insensitive to changes in [Na⁺]_i. The utility of the technique was demonstrated in neurons co-loaded with SBFI and SNARF-5F, which was found to have a lower pK_a in situ than carboxy SNARF-1. There were no significant differences in the changes in [Na⁺]_i and pH_i observed in response either to intracellular acid loads imposed by the NH₄⁺ prepulse technique or to transient periods of anoxia in neurons single-loaded with SBFI or SNARF-5F or co-loaded with both probes. The findings

support the feasibility of using SBFI in conjunction with either carboxy SNARF-1 or SNARF-5F to concurrently and accurately measure [Na⁺]_i and pH_i.

Keywords Intracellular pH · Intracellular sodium · SBFI · SNARF

Introduction

Quantitative fluorescence imaging microscopy with ion-sensitive probes has provided a wealth of information about cytosolic H⁺ and Na⁺ homeostasis in a large number of cell types. To date, however, fluorescent probe-based measurements of intracellular pH (pH_i) and free Na⁺ concentration ([Na⁺]_i) have been conducted separately in experiments performed in parallel, an approach that limits attempts to understand the interrelationships that exist between [H⁺]_i and [Na⁺]_i and the role of either ion in mediating specific cellular responses. Changes in pH_i and [Na⁺]_i may be linked directly through mechanisms as diverse as Na⁺/H⁺ exchange [15], Na⁺-dependent Cl⁻/HCO₃⁻ exchange [29], electrogenic Na⁺/HCO₃⁻ cotransport [9] and the activities of transmitter reuptake mechanisms [35], or indirectly via the coordinated activities of two or more transport mechanisms. In a number of cell types, for example, a rise in [Na⁺]_i promotes reverse-mode Na⁺/Ca²⁺ exchange and the subsequent rise in intracellular free Ca²⁺ concentration ([Ca²⁺]_i) can cause a fall in pH_i by activating the acid-loading Ca²⁺,H⁺-ATPase [16]. Furthermore, changes in pH_i and [Na⁺]_i can influence the activities of not only pH_i regulating transporters [13, 15] but also mechanisms that contribute to Na⁺ flux across biological membranes, including Na⁺/Ca²⁺ exchange and Na⁺/K⁺/Cl⁻ cotransport [5, 30].

The complex relationships that exist between changes in [H⁺]_i and [Na⁺]_i, and the recognized importance of both of these ions as determinants of cell function under a variety of physiological and pathophysiological con-

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ditions, underscores the need for concurrent quantitative measurements of both ions with good temporal and spatial resolution. In the present study, we have developed and characterized a technique for the near-simultaneous measurement of $[Na^+]_i$ and pH_i using, respectively, the dual excitation indicator sodium-binding benzofuran isophthalate (SBFI; [21]) and the dual emission seminaphthorhodafluor indicators carboxy SNARF-1 [38] or SNARF-5F [18]. Although SNARF-5F retains the spectral properties of other SNARF derivatives, it displays a lower pK_a value under cell-free in vitro conditions ($pK_a \sim 7.2$) that may make it more suitable than carboxy SNARF-1 ($pK_a \sim 7.5$) for measuring changes in pH_i below ~ 6.5 , as may occur, for example, in mammalian central neurons during anoxia or ischemia.

Materials and methods

Cell culture

Primary cultures of hippocampal neurons from 2- to 4-day-old postnatal Wistar rats (Animal Care Centre, University of British Columbia) were prepared as previously described [10]. Briefly, rat pups were anaesthetized, decapitated and the hippocampi removed. The hippocampi were enzymatically and mechanically dissociated and the resulting cell suspension was plated at a density of $5\text{--}7 \times 10^5$ neurons cm^{-2} onto glass coverslips coated with poly-D-lysine and laminin. The initial growth medium was DMEM/F-12 supplemented with 10% fetal bovine serum (Invitrogen Canada, Burlington, Ont., Canada). After 24 h, this medium was changed to serum-free Neurobasal Medium A (Invitrogen Canada). Cultures were then fed every 4–5 days by half-changing the existing medium with fresh Neurobasal Medium A. Glial proliferation was inhibited 48 h after initial plating by adding 5–10 μM cytosine arabinoside (Sigma-Aldrich Canada, Oakville, Ont., Canada). Neurons were used 7–14 days after plating.

Experimental media

The standard perfusion medium contained (in mM): 136.5 NaCl, 3 KCl, 1.5 NaH_2PO_4 , 1.5 $MgSO_4$, 10 D-glucose, 2 $CaCl_2$ and 10 HEPES (titrated to pH 7.35 with 10 M NaOH). In solutions containing 40 mM NH_4Cl , NH_4Cl isosmotically replaced NaCl. Anoxic media were prepared immediately prior to use by adding 1–2 mM sodium dithionite, an O_2 scavenger, to the standard perfusion medium and bubbling vigorously with 100% N_2 or Ar (see [10]). During anoxia, the atmosphere in the recording chamber was switched from room air to 100% N_2 or Ar.

Media used for full calibrations of SBFI ratio measurements contained (in mM): 0.6 $MgCl_2$, 0.5 $CaCl_2$, 10 HEPES, Na^+ and K^+ such that $[Na^+] + [K^+] = 130$,

100 gluconate and 30 Cl^- (titrated with 10 M KOH to the desired pH at 37°C), to which 4 μM gramicidin D (Sigma-Aldrich Canada) was added [11]. Media used for full calibrations of carboxy SNARF-1 and SNARF-5F ratio measurements were the same as the standard perfusion medium except for the substitution of NaCl and KCl with K gluconate (130.5 mM) and Na gluconate (9 mM) and the addition of 10 μM nigericin (Sigma-Aldrich Canada); pH was adjusted to a range of temperature-corrected values (pH 5.5–8.5 in 0.5 pH unit increments) with 10 M KOH [3]. To prevent cross-contamination by ionophores, perfusion lines were replaced and the perfusion chamber was decontaminated after each experiment (see [27]).

Recording techniques

The acetoxymethyl esters of SBFI (SBFI-AM), carboxy SNARF-1 (carboxy SNARF-1-AM) and SNARF-5F (SNARF-5F-AM) were obtained from Molecular Probes (Eugene, Ore., USA). To load SBFI, coverslips plated with neurons were placed in standard perfusion medium containing 10 μM SBFI-AM in the presence of 0.1% Pluronic F-127 and 5 $mg\ ml^{-1}$ bovine serum albumin, and incubated for 120–180 min at 32°C. To load SNARF derivatives, either individually or following SBFI-AM incubation, coverslips with neurons attached were placed in standard perfusion medium containing 0.1% Pluronic F-127 and either 10 μM carboxy SNARF-1-AM or SNARF-5F-AM for 30 min at 32°C. Following loading, coverslips were placed in standard medium for 20 min to ensure de-esterification of the fluorophore(s) and then mounted in a temperature-controlled perfusion chamber to form the base of the chamber. Neurons were superfused at a rate of 2 $ml\ min^{-1}$ for 15 min with the initial experimental solution at the appropriate temperature before the start of an experiment.

Measurements of $[Na^+]_i$ and pH_i were performed using the dual-excitation and dual-emission ratio methods, respectively, employing an imaging system (Atto Bioscience, Rockville, Md., USA) in conjunction with an Axiovert 135 epifluorescence microscope (Carl Zeiss Canada, Don Mills, Ont., Canada). Fluorescence emissions from regions of interest placed on individual neuronal somata were detected with intensified charge-coupled device cameras (Atto Bioscience). A schematic diagram of the optical equipment used for measurements of $[Na^+]_i$ and pH_i in neurons loaded with SBFI and/or carboxy SNARF-1 or SNARF-5F is presented in Fig. 1. Filter selection was based upon the published in vitro spectra of SBFI, carboxy SNARF-1 and SNARF-5F [18, 19, 21] (see Results). In experiments in which SBFI alone was employed, neurons were excited alternately at 334 ± 5 and 380 ± 5 nm and fluorescence emissions at 550 ± 40 nm were detected sequentially by a single camera (camera 2 in Fig. 1). In experiments in which carboxy SNARF-1 or SNARF-5F alone were

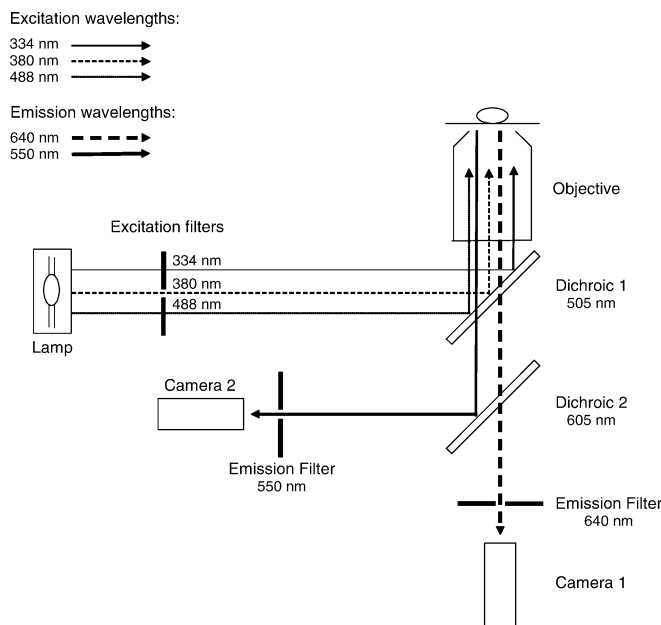


Fig. 1 A schematic representation of the optical equipment used to measure $[Na^+]_i$ and pH_i in hippocampal neurons loaded with sodium-binding benzofuran isophthalate (SBFI) and/or carboxy SNARF-1 or SNARF-5F (adapted from [7]). Neurons were excited with light provided by a 100-W mercury lamp and band-pass filtered at 488 ± 5 nm (for SNARF dyes) or alternately at 334 ± 5 and 380 ± 5 nm (for SBFI). Filtered excitation light was reflected off a 505 nm long-pass dichroic mirror (dichroic 1), passed through a $\times 40$ LD Achromplan objective (0.6 n.a.) and illuminated neurons loaded with fluorophore(s). SBFI fluorescence emissions passed through dichroic 1, reflected off a long-pass dichroic mirror centred at 605 nm (dichroic 2), passed through a 550 ± 40 nm band-pass emission filter and were detected by camera 2. Fluorescence emissions from carboxy SNARF-1 or SNARF-5F passed through dichroic 1, were split by dichroic 2 and passed through 640 ± 20 nm or 550 ± 40 nm band-pass emission filters before being detected by cameras 1 and 2, respectively. Optical filters, including dichroic mirrors 1 (505dxcru) and 2 (605drlpxr), were obtained from Chroma Technology (Rockingham, Vt., USA)

employed, neurons were excited at 488 ± 5 nm and fluorescence emissions at 550 ± 40 and 640 ± 20 nm were detected simultaneously by two cameras, the registration of which was confirmed prior to every experiment. In experiments in which neurons were co-loaded with SBFI and either carboxy SNARF-1 or SNARF-5F, ratio pairs were collected continuously by alternating between the excitation and emission modes; each automated cycle took ~ 1.5 s to complete, including a ~ 0.5 -s delay between collecting SBFI- and SNARF-derived ratio pairs, and was repeated every 2–15 s during the course of an experiment.

A one-point calibration technique was employed to convert background-corrected SBFI and carboxy SNARF-1 or SNARF-5F ratio values into $[Na^+]_i$ and pH_i values, respectively. At the end of an experiment, neurons loaded with SBFI were exposed to a pH 7.35 medium containing 10 mM Na^+ and 4 μ M gramicidin D [11] whereas neurons loaded with carboxy SNARF-1 or SNARF-5F were exposed to a pH 7.00 high- $[K^+]_i$

solution containing 10 μ M nigericin [3]; in neurons loaded with both SBFI and a SNARF derivative, the SBFI and SNARF one-point calibrating media were applied sequentially. The resulting background-corrected ratio values at $[Na^+]_i = 10$ mM (for SBFI) and at $pH_i = 7.00$ (for carboxy SNARF-1 or SNARF-5F) were used as normalization factors for experimentally-derived background-subtracted SBFI and SNARF ratio values, respectively. At the end of some experiments conducted at $37^\circ C$, we were unable to obtain stable normalizing ratio values for carboxy SNARF-1 or SNARF-5F during the one-point calibration at pH 7.00. In these cases, the one-point calibration was either repeated at pH 7.50 or the experimental data were normalized with ratio values obtained under identical experimental and optical conditions from a fresh sister culture loaded with the appropriate dye(s) and exposed to SNARF calibrating medium at pH 7.00. A similar instability of carboxy SNARF-1 and SNARF-5F ratio values, characterized by anomalous increases and decreases in background-subtracted 550 and 640 nm emission intensities, respectively, was also sometimes experienced during full calibration experiments conducted at $37^\circ C$ when pH values were ≤ 7.00 . The reasons for these atypical behaviours, which could occur in neurons loaded with carboxy SNARF-1 or SNARF-5F in the absence or presence of SBFI, remain unclear, although similar difficulties have been experienced by others [2, 4, 6, 19, 33, 34]. Nevertheless, we were able to obtain reproducible calibration parameters for carboxy SNARF-1 and SNARF-5F at $37^\circ C$ in situ that, in the case of carboxy SNARF-1, are consistent with those reported by others (see Results).

Normalized SBFI ratio values were converted into $[Na^+]_i$ values using the equation

$$[Na^+] = \beta K_d [(R_n - R_{n(\min)}) / (R_{n(\max)} - R_n)] \quad (1)$$

where R_n is the background-subtracted SBFI fluorescence intensity ratio (BI_{334}/BI_{380}) normalized to $[Na^+] = 10$ mM; $R_{n(\min)}$ and $R_{n(\max)}$ are, respectively, the minimum and maximum obtainable values for the normalized ratio; β is the ratio of the fluorescence of the free (unbound) dye to bound dye during excitation at 380 nm; and K_d is the apparent dissociation constant of SBFI for Na^+ [11].

Normalized carboxy SNARF-1 or SNARF-5F ratio values were converted into pH_i values using the equation

$$pH = pK_a + \log F_{640_{\min/\max}} - \log [(R_n - R_{n(\min)}) / (R_{n(\max)} - R_n)] \quad (2)$$

where R_n is the background-subtracted carboxy SNARF-1 or SNARF-5F fluorescence intensity ratio (BI_{550}/BI_{640}) normalized to pH 7.00 (or pH 7.50; see above); pK_a is the $-\log$ of the dissociation constant of the fluorophore; and $F_{640_{\min/\max}}$ is the ratio of fluorescence measured at 640 nm for low pH (pH 5.5) to that for high pH (pH 8.5) [7].

The parameters fitting Eqs. 1 and 2 were derived from full in situ calibration experiments, as described in the Results.

Data analysis

Results are reported as mean \pm SEM. In experiments in which internal acid loads were imposed by the NH_4^+ prepulse technique or anoxia, experiments were performed on at least three coverslips obtained from at least two different batches of cultures and the accompanying n value refers to the number of neurons from which data were analyzed. For all other experiments, including full calibration experiments, the accompanying n value refers to the number of cell populations (i.e. number of coverslips) analysed. Statistical comparisons were carried out using Student's two-tailed t -test, paired or unpaired as appropriate, with a 95% confidence limit.

Results

Separation of SBF1 and SNARF fluorescence emissions in situ

With the optical filters specified in Materials and methods, the excitation and emission characteristics of SBF1, carboxy SNARF-1 and SNARF-5F in vitro appear sufficiently distinct to permit the discrimination of Na^+ - and pH_i -dependent signals from dual dye-loaded cells. However, the spectral properties of many fluorescent

probes, including SBF1 (see [11, 24]) and carboxy SNARF-1 (see [20, 34]), may differ in situ compared to in vitro. Initially, therefore, we examined whether the behaviours of SBF1, carboxy SNARF-1 and SNARF-5F in hippocampal neurons in situ would allow us to isolate signals coming from the respective dyes under our experimental conditions. To do so, we measured the intensities of emitted fluorescence relative to background fluorescence values under a variety of conditions.

In neurons single-loaded with SBF1, excitation at 334 and 380 nm (emissions collected at 550 nm) elicited fluorescence signals respectively ~ 20 and ~ 11 times greater than those originating from neurons single-loaded with carboxy SNARF-1, and ~ 16 and ~ 14 times greater than those originating from neurons single-loaded with SNARF-5F (Fig. 2a). In neurons single-loaded with carboxy SNARF-1 or SNARF-5F, excitation at 488 nm (emissions collected at 550 nm and 640 nm) elicited fluorescence signals respectively ~ 8 (at 550 nm) and ~ 7 (at 640 nm) times greater than those obtained from neurons single-loaded with SBF1 (Fig. 2b). When neurons were co-loaded with SBF1 and either carboxy SNARF-1 or SNARF-5F, fluorescence emissions originating from SBF1 and carboxy SNARF-1 or SNARF-5F continued to be adequately resolved (Fig. 2a, b). Nevertheless, emission intensities measured at 550 nm following excitation at 334 or 380 nm in neurons co-loaded with SBF1 and either carboxy SNARF-1 or SNARF-5F were significantly reduced, compared to those measured in neurons single-loaded with SBF1 (Fig. 2a). In addition, following excitation at 488 nm,

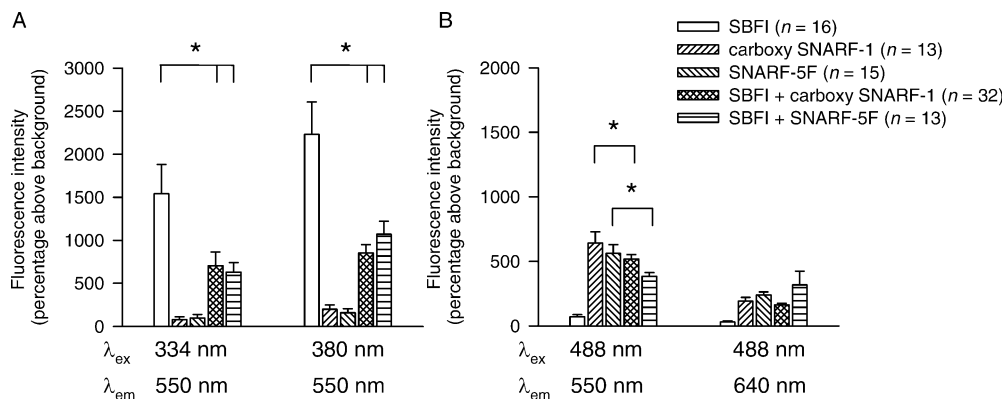


Fig. 2a, b Fluorescence emissions from single and dual dye-loaded hippocampal neurons. Neurons were single- or co-loaded with fluorophores, as shown on the figure, and illuminated at the indicated excitation wavelengths (λ_{ex}). The intensities of emitted fluorescence were measured at either 550 nm or 640 nm (λ_{em}) and are reported as percentages above background fluorescence values (\pm SEM). **a** In contrast to neurons single-loaded with either carboxy SNARF-1 or SNARF-5F, neurons single-loaded with SBF1 exhibited large fluorescence emissions (measured at 550 nm) during excitation at 334 and 380 nm. Emission intensities from neurons single-loaded with carboxy SNARF-1 were 5% (excitation at 334 nm) and 9% (excitation at 380 nm) of those observed in neurons single-loaded with SBF1 and excited at the same wavelengths. In neurons single-loaded with SNARF-5F and excited at 334 and 380 nm, emission intensities were, respectively, 6% and 7% of those observed in neurons single-loaded with SBF1. Also

shown are fluorescence emission intensities (measured at 550 nm) during 334 and 380 nm excitation of neurons co-loaded with SBF1 and either carboxy SNARF-1 or SNARF-5F (see text). **b** In neurons single-loaded with either carboxy SNARF-1 or SNARF-5F, excitation at 488 nm evoked fluorescence emissions at both 550 and 640 nm. In neurons single-loaded with SBF1 and also excited at 488 nm, fluorescence emissions measured at 550 and 640 nm were, respectively, 11% and 15% of those observed in neurons single-loaded with carboxy SNARF-1 and 13% and 13% of those observed in neurons single-loaded with SNARF-5F. Also shown are fluorescence emission intensities (measured at 550 and 640 nm) during 488 nm excitation of neurons co-loaded with SBF1 and either carboxy SNARF-1 or SNARF-5F (see text). * $P < 0.05$ between the indicated measurements in single- and dual dye-loaded neurons

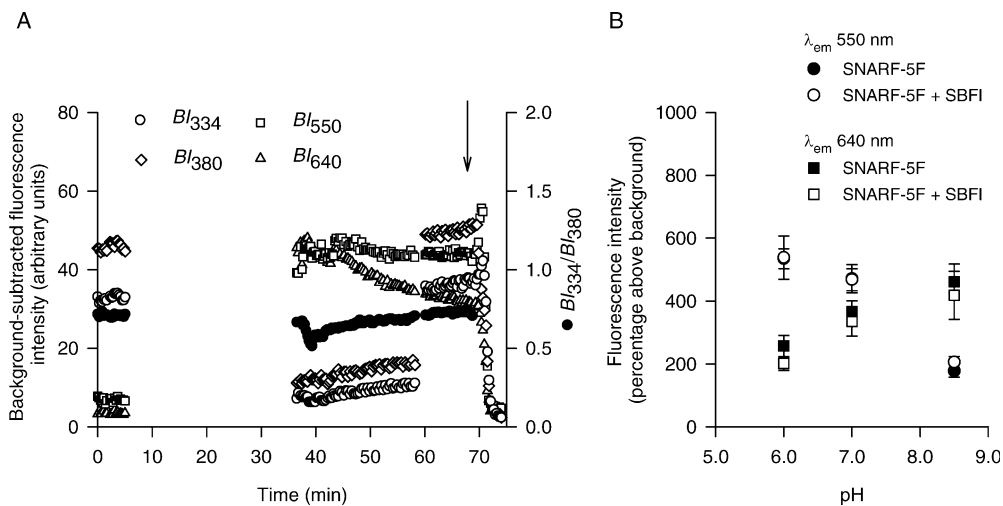


Fig. 3a, b Quenching effects between SBF1 and SNARF-based fluorophores. **a** In a population of 19 neurons single-loaded with SBF1, background-subtracted emission intensities were measured at 550 nm during excitation at 334 nm (BI_{334} , open circles) and 380 nm (BI_{380} , open diamonds). Perfusion and data collection were then interrupted and 10 μ M SNARF-5F-AM was added to the recording chamber. After loading with SNARF-5F, SBF1-derived BI_{334} and BI_{380} values were reduced to a proportionately similar extent, resulting in little change in SBF1 BI_{334}/BI_{380} ratio values (filled circles). Data collection was then interrupted again, during which time the gain of camera 2 was adjusted to increase emission intensities measured during excitation at 334 and 380 nm. Finally, the addition of 0.005% saponin (arrow) caused rapid decreases in BI_{334} and BI_{380} values. Also measured throughout the experiment were background-subtracted emission intensities at 550 nm (BI_{550} ,

open squares) and 640 nm (BI_{640} , open triangles) during excitation at 488 nm; BI_{550} and BI_{640} values increased after neurons were loaded with SNARF-5F and fell rapidly upon the subsequent addition of saponin. Temperature was 37°C throughout. **b** Neurons single-loaded with SNARF-5F (closed symbols) or co-loaded with SBF1 and SNARF-5F (open symbols) were exposed to SNARF calibration media at pH 6.0, 7.0 or 8.5. The intensities of emitted fluorescence (excitation at 488 nm) were measured at 550 nm (circles) and 640 nm (squares) and are reported as percentages above background fluorescence values ($n \geq 12$ for each data point). Compared with measurements made in neurons single-loaded with SNARF-5F, emission intensities at 550 and 640 nm were not significantly different ($P > 0.05$) in the presence of SBF1 at any of the pH values tested. Error bars are SEM

fluorescence emissions at 550 nm, but not 640 nm, were reduced by $\sim 25\%$ in neurons co-loaded with SBF1 and either carboxy SNARF-1 or SNARF-5F compared to those measured in neurons single-loaded with carboxy SNARF-1 or SNARF-5F (Fig. 2b).

These observations are consistent with the possibility that SNARF derivatives may quench SBF1 fluorescence, and vice versa. When neurons were single-loaded with SBF1 and subsequently loaded with carboxy SNARF-1 or SNARF-5F ($n = 3$ in each case), BI_{334} and BI_{380} values were reduced by $61 \pm 2\%$ and $63 \pm 1\%$ (carboxy SNARF-1; not shown) and by $60 \pm 13\%$ and $60 \pm 10\%$ (SNARF-5F; Fig. 3a), respectively. However, because BI_{334} and BI_{380} values were reduced to a proportionately similar extent, SBF1-derived BI_{334}/BI_{380} ratio values were minimally affected by the presence of either carboxy SNARF-1 (not shown) or SNARF-5F (Fig. 3a). Due to the length of time required for SBF1 loading, we were unable to further examine the apparent effect of SBF1 to quench carboxy SNARF-1 and SNARF-5F 550 nm emissions in a manner similar to that shown in Fig. 3a. Therefore, we measured fluorescence emission intensities at 550 and 640 nm (excitation at 488 nm) in neurons single-loaded with either carboxy SNARF-1 or SNARF-5F at pH 6.0, 7.0 and 8.5, and compared these intensities to those obtained in neurons from sister cultures co-loaded with SBF1 and carboxy SNARF-1 or SNARF-5F. Neither carboxy SNARF-1 (not shown)

nor SNARF-5F (Fig. 3b) emission intensities at 550 and 640 nm were significantly influenced by the addition of SBF1, indicating that SBF1 does not significantly reduce fluorescence emissions from SNARF-based dyes at any of the pH values examined.

Quenching between fluorophores requires intracellular co-localization. Exposure of neurons co-loaded with SBF1 and carboxy SNARF-1 or SNARF-5F to 0.005–0.01% saponin ($n = 3$ in each case) reduced emission intensities measured at 550 nm (following excitation at 334, 380 or 488 nm) and at 640 nm (following excitation at 488 nm) by $> 85\%$ (Fig. 3a; also see [4, 29]). Similar findings, which also indicate that SBF1- and SNARF-derived fluorescence emissions originate largely from the cytosolic compartment, were made when co-loaded neurons were exposed to 20 μ M digitonin ($n = 3$ in each case; not shown).

Full calibrations of SBF1, carboxy SNARF-1 and SNARF-5F ratio values in situ

Next, we performed full in situ calibrations of SBF1, carboxy SNARF-1 and SNARF-5F in single-loaded neurons and in neurons co-loaded with SBF1 and carboxy SNARF-1 or SNARF-5F.

As illustrated in Fig. 4a, full SBF1 calibrations were performed at 37°C by exposing neurons to pH 7.35

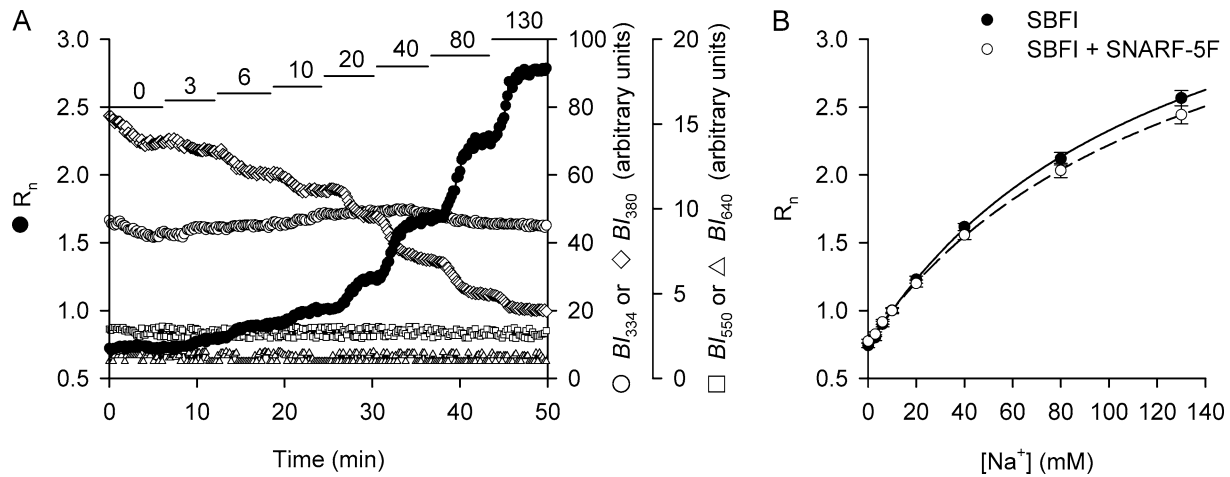


Fig. 4a, b In situ calibration of SBFI at 37°C, pH_o 7.35. **a** A full calibration experiment in which 20 neurons single-loaded with SBFI were exposed to 4 μM gramicidin D-containing solutions at the [Na⁺] values (in mM) indicated above the traces. Shown are the mean changes in emitted background-subtracted fluorescence intensities measured at 550 nm during excitation at 334 nm (BI₃₃₄, open circles) and 380 nm (BI₃₈₀, open diamonds) and the mean normalized BI₃₃₄/BI₃₈₀ ratio values (R_n , filled circles), which increased as [Na⁺] increased. Also shown are the background-subtracted fluorescence emission intensities measured at 550 nm (BI₅₅₀, open squares) and 640 nm (BI₆₄₀, open triangles) during excitation at 488 nm (i.e. the excitation and emission wavelengths

used for SNARF measurements); note the change of scale. **b** Plots of [Na⁺] versus R_n obtained from experiments of the type shown in **a**. Data points fitted by the continuous line were obtained from four experiments conducted on sister cultures in which neurons were loaded with SBFI alone. Data points fitted by the dashed line were obtained from four experiments conducted on sister cultures in which neurons were co-loaded with SBFI and SNARF-5F. In both cases, error bars are SEM. As detailed in Diarra et al. [11], the curves are the result of three-parameter hyperbolic fits to the respective data points indicated and were used to determine the values of the SBFI calibration parameters (i.e. K_d , β , $R_{n(\min)}$ and $R_{n(\max)}$) under the different dye loading conditions (see Table 1)

media containing 4 μM gramicidin D at eight different [Na⁺] values (range 0–130 mM). The resulting plot of the data points relating [Na⁺] to R_n in neurons single-loaded with SBFI is shown in Fig. 4b and the resulting fitted SBFI calibration parameters are presented in Table 1. Importantly, when neurons single-loaded with SBFI were illuminated at 488 nm (i.e. the excitation wavelength employed for carboxy SNARF-1 and SNARF-5F), fluorescence emissions measured at 550 and 640 nm (i.e. the emission wavelengths of the

SNARF dyes) remained small and stable as [Na⁺] was altered from 0 to 130 mM (Fig. 4a). Finally, when full SBFI calibrations were performed in neurons co-loaded with SBFI and either carboxy SNARF-1 or SNARF-5F, there were no significant differences between the resulting SBFI calibration parameters and those computed from neurons loaded with SBFI alone (Fig. 4b; Table 1). Thus, carboxy SNARF-1 and SNARF-5F do not influence the in situ sensitivity of SBFI to changes in [Na⁺]_i.

Table 1 Calibration parameters for sodium-binding benzofuran isophthalate (SBFI), and the dual-emission seminaphthorhodafluor indicators carboxy SNARF-1 and SNARF-5F in single dye- and dual dye-loaded hippocampal neurons. Values are mean ± SEM [β ratio of the fluorescence of the free (unbound) dye to bound dye

during excitation at 380 nm; K_d dissociation constant; $R_{n(\min)}$, $R_{n(\max)}$ minimum and maximum obtainable values for the normalized ratio, respectively; pK_a is the $-\log$ of the dissociation constant; $F_{640_{\min/\max}}$ is the ratio of fluorescence measured at 640 nm for low pH (pH 5.5) to that for high pH (pH 8.5)]

SBFI calibration						
Neurons loaded with:	Temp (°C)	K_d	β	$R_{n(\min)}$	$R_{n(\max)}$	n
SBFI	37	21.7 ± 2.1	4.5 ± 0.7	0.77 ± 0.01	3.2 ± 0.4	21
SBFI + carboxy SNARF-1	37	17.6 ± 3.5	5.4 ± 1.3	0.77 ± 0.03	3.2 ± 0.4	5
SBFI + SNARF-5F	37	22.7 ± 4.8	4.5 ± 1.3	0.78 ± 0.03	3.3 ± 0.5	8
Carboxy SNARF-1 and SNARF-5F calibrations						
Neurons loaded with:	Temp (°C)	pK_a	$F_{640_{\min/\max}}$	$R_{n(\min)}$	$R_{n(\max)}$	n
Carboxy SNARF-1	22	7.40 ± 0.06	0.83 ± 0.06	0.34 ± 0.05	1.3 ± 0.04	7
Carboxy SNARF-1	37	7.59 ± 0.13	0.62 ± 0.05*	0.18 ± 0.04*	1.4 ± 0.07	19
Carboxy SNARF-1 + SBFI	37	7.62 ± 0.09	0.46 ± 0.07	0.19 ± 0.05	1.3 ± 0.06	9
SNARF-5F	37	7.25 ± 0.12	0.45 ± 0.05	0.29 ± 0.06	1.7 ± 0.19	12
SNARF-5F + SBFI	37	7.30 ± 0.09	0.58 ± 0.11	0.27 ± 0.06	1.8 ± 0.17	12

* $P < 0.05$ compared to the corresponding value at 22°C

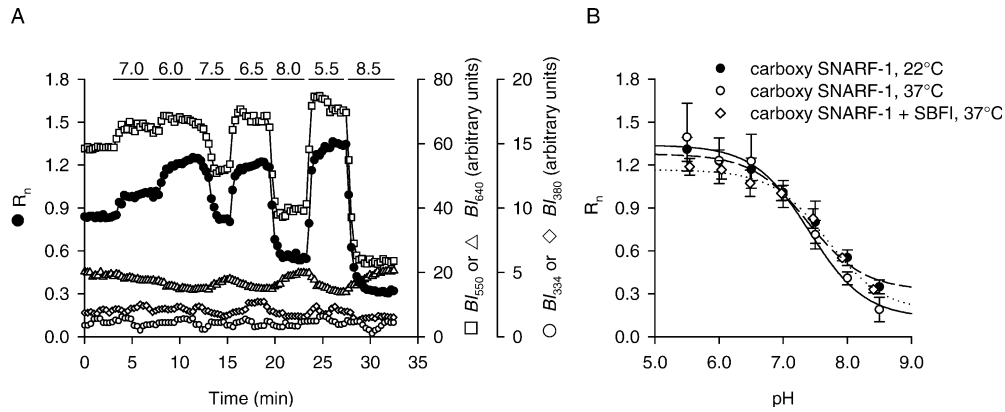


Fig. 5a, b In situ calibration of carboxy SNARF-1. **a** A full calibration experiment performed at 22°C in which 18 neurons single-loaded with carboxy SNARF-1 were exposed to high-[K⁺], 10 μ M nigericin-containing solutions at the pH values shown above the records. Background-subtracted fluorescence emissions measured at 550 nm (BI₅₅₀, open squares) increased upon protonation of the dye while background-subtracted emissions measured at 640 nm (BI₆₄₀, open triangles) decreased; thus, the resulting background-subtracted BI₅₅₀/BI₆₄₀ ratio values normalized to 1.00 at pH 7.00 (R_n , filled circles) increased as pH_i fell. Also shown are the background-subtracted fluorescence emission intensities measured at 550 nm during excitation at 334 nm (BI₃₃₄, open circles) and 380 nm (BI₃₈₀, open diamonds), i.e. the excitation and emission wavelengths used for SBFI measurements; BI₃₃₄ and BI₃₈₀

emissions from carboxy SNARF-1 (and SNARF-5F; not shown) remained small as pH was altered (note the change of scale). **b** Plots of pH versus R_n obtained from experiments of the type shown in **a**. In neurons single-loaded with carboxy SNARF-1, data points obtained from experiments performed at 22°C and 37°C ($n=3$ experiments conducted on sister cultures in each case) were fitted using non-linear least squares regression (dashed and continuous lines, respectively). Also shown are data points from three experiments performed on sister cultures in which neurons were co-loaded with carboxy SNARF-1 and SBFI; these are fitted by the dotted line. In all cases, error bars are SEM. The curves were used to determine the values of the carboxy SNARF-1 calibration parameters (i.e. pK_a , $\log F_{640_{\min/\max}}$, $R_{n(\min)}$ and $R_{n(\max)}$) under the different dye loading conditions (see Table 1)

Full in situ calibrations of carboxy SNARF-1 are presented in Fig. 5. As described by others [4, 7, 19], exposing neurons single-loaded with carboxy SNARF-1 to 10 μ M nigericin-containing high-K⁺ solutions at a variety of pH values influenced both BI₅₅₀ and BI₆₄₀ emission intensities (Fig. 5a). The resulting plots of the data points relating R_n and pH, at both 22°C and 37°C, are shown in Fig. 5b and the fitted carboxy SNARF-1 calibration parameters are presented in Table 1. Interestingly, the carboxy SNARF-1 $F_{640_{\min/\max}}$ value was significantly reduced at 37°C compared to 22°C (Table 1) and, in agreement with Ch'en et al. [8], the dynamic range of the dye's fluorescence ratio increased with increasing temperature (Fig. 5b; Table 1). As noted in the Introduction, SNARF-5F displays a lower pK_a value in vitro than carboxy SNARF-1. Therefore, we performed full in situ calibrations (at 37°C) of SNARF-5F single-loaded into hippocampal neurons. The fitted calibration parameters (Table 1) confirmed that SNARF-5F also possesses a lower pK_a value than carboxy SNARF-1 in situ. Carboxy SNARF-1 and SNARF-5F calibration parameters were then determined at 37°C in neurons also loaded with SBFI (see Fig. 5b). There were no significant differences between the values of the calibration parameters obtained from neurons loaded with carboxy SNARF-1 or SNARF-5F alone and those obtained from neurons co-loaded with SBFI and carboxy SNARF-1 or SNARF-5F (Table 1). Together, the results indicate that SBFI does not influence the in situ sensitivities of carboxy SNARF-1 or SNARF-5F to changes in pH_i.

Effects of changes in [Na⁺]_i on pH_i measurements with carboxy SNARF-1 and SNARF-5F in situ

The effects of changes in pH_i on [Na⁺]_i estimated with SBFI in situ have been well characterized [11, 24, 29]. To examine the effects of changes in [Na⁺]_i on SNARF-based pH_i measurements, neurons were single-loaded with carboxy SNARF-1 or SNARF-5F and [Na⁺]_i was varied from 0 to 10 to 130 mM at four different pH values (6.00, 6.50, 7.00 and 7.50) in the presence of 4 μ M gramicidin D at 37°C (see [11]). As illustrated in Fig. 6a, increasing [Na⁺]_i from 0 to 10 to 130 mM at a constant pH had minimal effects on carboxy SNARF-1 ratio measurements. Moreover, plots of R_n as a function of pH at each [Na⁺]_i (Fig. 6b) indicated that changes in [Na⁺]_i did not alter the computed $pK_a + \log F_{640_{\min/\max}}$, $R_{n(\min)}$ or $R_{n(\max)}$ values for carboxy SNARF-1. Thus, neither carboxy SNARF-1-derived R_n nor pH_i measurements were influenced significantly by changes in [Na⁺]_i in the range 0–130 mM ($P > 0.05$ at all pH values). Similar findings were made in neurons single-loaded with SNARF-5F ($n \geq 3$ at each pH value; not shown). These results indicate that the effects of changes in [Na⁺]_i on SNARF-based pH_i measurements are unlikely to affect the interpretation of results under most experimental conditions.

Concurrent measurements of [Na⁺]_i and pH_i in rat hippocampal neurons

To demonstrate the utility of concurrent measurements of [Na⁺]_i and pH_i in cells co-loaded with SBFI and a

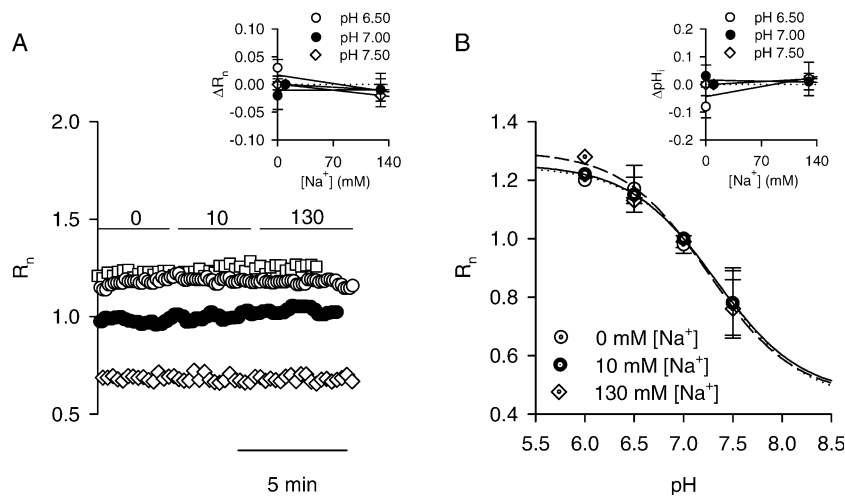


Fig. 6a, b Sodium sensitivity of carboxy SNARF-1 in situ. **a** Neurons loaded with carboxy SNARF-1 were exposed at 37°C to calibration media containing 4 μ M gramicidin D at pH 6.00 (open squares), 6.50 (open circles), 7.00 (filled circles) or 7.50 (open diamonds). At each pH, $[Na^+]_i$ was changed from 0 to 10 to 130 mM as indicated above the records. BI_{550}/BI_{640} ratio values (R_n) were normalized to unity at pH = 7.00 and $[Na^+]_i = 10$ mM. *Inset* To quantify the effects of changes in $[Na^+]_i$ on R_n values measured with carboxy SNARF-1, apparent changes in R_n (ΔR_n) were calculated as $R_{n(x)} - R_{n(10)}$ (where $x = 0, 10$ or 130) for each pH value indicated and plotted as a function of $[Na^+]_i$. **b** R_n values at a given $[Na^+]_i$ were plotted as a function of pH ($n = 3$ at pH 6.50, 7.00 and 7.50; $n = 1$ at pH 6.00) and data points were fitted using non-linear least squares regression (continuous, dotted and dashed

curves for data obtained at 0, 10 and 130 mM $[Na^+]_i$, respectively). The $pK_a + \log F_{640_{min/max}}$, $R_{n(min)}$ and $R_{n(max)}$ values for carboxy SNARF-1 did not change significantly as $[Na^+]_i$ was increased from 0 to 10 to 130 mM ($pK_a + \log F_{640_{min/max}}$, $R_{n(min)}$ and $R_{n(max)}$ values were, respectively, 7.35, 0.44 and 1.25 at 0 mM $[Na^+]_i$; 7.32, 0.47 and 1.26 at 10 mM $[Na^+]_i$; and 7.28, 0.46 and 1.30 at 130 mM $[Na^+]_i$). *Inset* R_n values were converted into pH_i and apparent changes in pH_i (ΔpH_i) were then calculated as $pH_{i(x)} - pH_{i(10)}$ (where $x = 0, 10$ or 130) for each pH value indicated and plotted as a function of $[Na^+]_i$. In the insets in **a** and **b**, each datum point represents measurements made in 3 separate experiments; error bars are SEM and the continuous lines represent linear regression fits to the data points indicated for each pH value

SNARF derivative, we subjected rat hippocampal neurons to internal acid loads imposed by the NH_4^+ pre-pulse technique or transient periods of anoxia. In light of its lower pK_a value, SNARF-5F was employed in these experiments in preference to carboxy SNARF-1. Consistent with previous measurements in hippocampal neurons [3, 11, 29], resting pH_i and $[Na^+]_i$ values in cells single-loaded with SNARF-5F or SBF1 were 7.38 ± 0.03 ($n = 57$) and 12 ± 1 mM ($n = 44$), respectively. These values were not significantly different to those obtained in 22 neurons co-loaded with SNARF-5F and SBF1 (resting pH_i 7.40 ± 0.04 ; resting $[Na^+]_i$ 10 ± 1 mM).

Under the nominally HCO_3^- -free, HEPES-buffered conditions employed in the present experiments, the recovery of pH_i from NH_4^+ -induced internal acid loads in rat hippocampal neurons is mediated in large part by Na^+/H^+ exchange [3, 26]. As expected, in neurons loaded with SNARF-5F alone, pH_i increased during NH_4^+ application, fell to below resting levels upon NH_4^+ washout and then recovered (Fig. 7a; Table 2). In neurons loaded with SBF1 alone, the washout of NH_4^+ was associated with increases in $[Na^+]_i$; that subsequently recovered towards resting levels (Fig. 7b; Table 2). When cells were co-loaded with SNARF-5F and SBF1, the magnitudes of the decreases in pH_i and increases in $[Na^+]_i$ observed upon NH_4^+ washout were not significantly different from those seen in neurons loaded with SBF1 or SNARF-5F alone (Fig. 7c; Table 2) and were comparable to the changes measured

concurrently with ion-sensitive microelectrode(s) [ISM(s)] in crayfish neurons (see Fig. 4 in [22]). Further, as shown in Fig. 7d, rates of pH_i recovery from NH_4^+ -induced internal acid loads were similar in neurons loaded with SBF1 and SNARF-5F or SNARF-5F alone. Finally, measurements in neurons co-loaded with SNARF-5F and SBF1 revealed a positive relationship between the magnitude of the recovery of pH_i and the increase in $[Na^+]_i$ seen after NH_4^+ washout (Fig. 7e).

In the final series of experiments we measured the changes in pH_i and $[Na^+]_i$ evoked in rat hippocampal neurons by anoxia. Consistent with previous reports (see [10, 11] and references therein), in hippocampal neurons loaded only with SNARF-5F (Fig. 8a; Table 2) or SBF1 (Fig. 8b; Table 2), 5 min anoxia produced falls in pH_i and increases in $[Na^+]_i$, respectively, with recoveries toward resting values upon the return to normoxia. Comparable changes in $[Na^+]_i$ and pH_i were observed in neurons co-loaded with SNARF-5F and SBF1 (Fig. 8c; Table 2). In addition, measurements in dual dye-loaded cells revealed that pH_i consistently declined before $[Na^+]_i$ started to increase (Fig. 8c).

Discussion

The dual excitation ratiometric indicator SBF1 has been used to measure $[Na^+]_i$ in a large number of cell types; the ion selectivity of the probe has been assessed, meth-

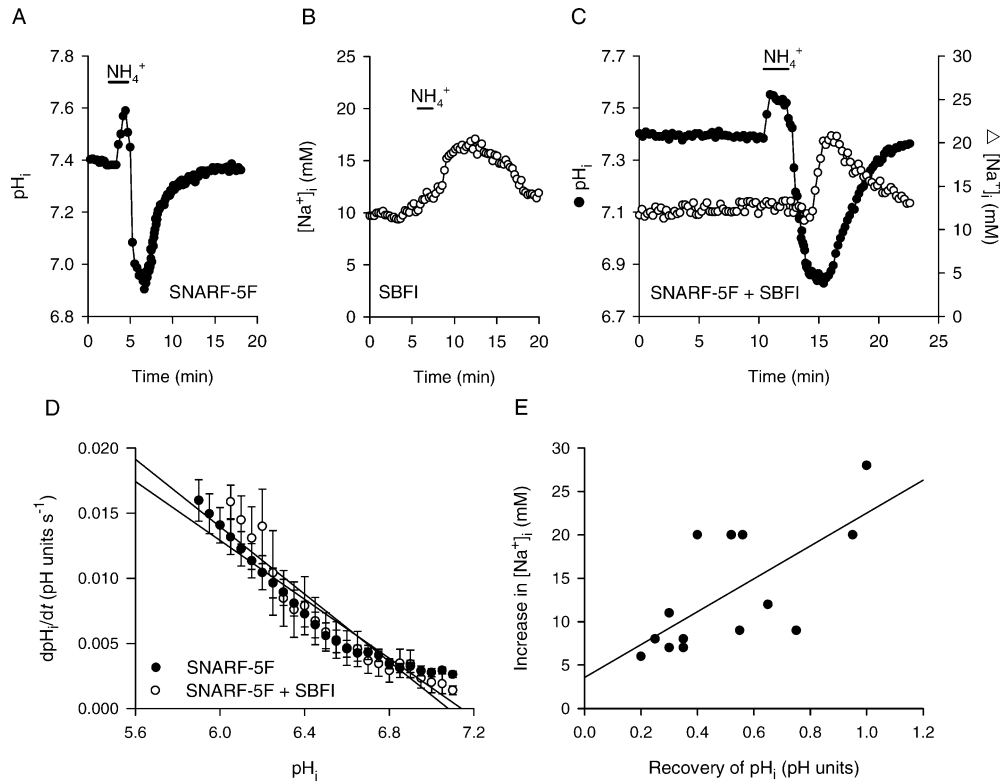


Fig. 7a–e Changes in pH_i and $[\text{Na}^+]_i$ observed in rat hippocampal neurons in response to intracellular acid loads imposed by the NH_4^+ prepulse technique. **a** In a neuron single-loaded with SNARF-5F, washout of NH_4^+ evoked a fall in pH_i which gradually returned to the resting level. **b** In a neuron single-loaded with SBFI, an increase in $[\text{Na}^+]_i$ occurred upon the washout of NH_4^+ . **c** In a neuron co-loaded with SNARF-5F and SBFI, the changes in pH_i (filled circles) observed on NH_4^+ washout were temporally associated with a transient increase in $[\text{Na}^+]_i$ (open circles). **d** Rates of pH_i recovery from NH_4^+ -induced internal acid loads measured in neurons either single-loaded with SNARF-5F (solid circles; $n=39$) or co-loaded with SNARF-5F and SBFI (open circles; $n=14$). Error bars are SEM and continuous lines represent

weighted nonlinear regression fits to the data points indicated for each experimental condition (see [3] for methodological details). Rates of pH_i recovery in neurons single-loaded with SNARF-5F were not significantly different from those in neurons co-loaded with SNARF-5F and SBFI ($P > 0.05$ at each absolute pH_i value). **e** Scatter plot demonstrating the relationship between the magnitude of the recovery of pH_i (measured as the difference between the minimum pH_i value attained after NH_4^+ washout and the steady-state pH_i value reached after recovery) and the increase in $[\text{Na}^+]_i$ observed after NH_4^+ washout in 14 neurons co-loaded with SNARF-5F and SBFI. The continuous line is a linear regression fit to the data points shown ($r=0.68$)

ods for its in situ calibration have been developed and a one-point technique for the conversion of SBFI-derived ratio measurements into $[\text{Na}^+]_i$ has been validated (see [11] and references therein). Similarly, the dual emission ratiometric dye, carboxy SNARF-1, has gained wide acceptance as a pH_i indicator, in part because of its wide dynamic range and good signal-to-noise ratio (see [7]). Nevertheless, its relatively high $\text{p}K_a$ may limit the accuracy of low pH_i measurements, a potential disadvantage that prompted us to assess the utility of SNARF-5F as a pH_i indicator in hippocampal neurons. We confirmed that the $\text{p}K_a$ of SNARF-5F in situ is lower than that of carboxy SNARF-1 but that it behaves in an otherwise similar manner to carboxy SNARF-1.

To date, concurrent single cell measurements of $[\text{Na}^+]_i$ (or intracellular Na^+ activity) and pH_i have been obtained with ISMs, sometimes in conjunction with an ion-sensitive fluorescent probe [9, 15, 17, 22, 23, 36]. Although ISMs offer some advantages over fluorescent probes (see [25, 37]), they cannot easily be applied to

small cells (e.g. mammalian central neurons) or cellular processes. In contrast, SBFI and SNARF derivatives offer means for measuring $[\text{Na}^+]_i$ and pH_i , respectively, in small cells that are not amenable to stable impalements with ISMs, but heretofore they have only been employed separately¹, precluding a clear understanding of the temporal and other relationships between cytosolic Na^+ and H^+ homeostasis and the role of either ion in the regulation of specific physiological responses. As noted in the Introduction, $[\text{H}^+]_i$ and $[\text{Na}^+]_i$, like $[\text{H}^+]_i$ and $[\text{Ca}^{2+}]_i$ (and also $[\text{Na}^+]_i$ and $[\text{Ca}^{2+}]_i$), are related by a variety of mechanisms; indeed, an appreciation of the latter relationships has led to the development of techniques for the concurrent measurement of pH_i and $[\text{Ca}^{2+}]_i$ with carboxy SNARF-1 and fura-2 [19, 20] or

¹Jung et al. [14] employed SBFI and carboxy SNARF-1 simultaneously in isolated heart mitochondria but few details were given and the behaviours of the fluorophores when co-loaded were not systematically examined.

Table 2 NH_4^+ prepulse- and anoxia-evoked changes in pH_i and $[\text{Na}^+]_i$ in hippocampal neurons loaded with SNARF-5F and/or SBFI. Values are mean \pm SEM. The changes in pH_i and $[\text{Na}^+]_i$ shown are with respect to pre-stimulus resting values

NH_4^+ prepulse			
Neurons loaded with:	Decrease in pH_i on NH_4^+ washout (pH units)	Increase in $[\text{Na}^+]_i$ on NH_4^+ washout (mM)	<i>n</i>
SNARF-5F	0.81 ± 0.08		39
SBFI		12 ± 1	25
SBFI + SNARF-5F	0.87 ± 0.12	12 ± 2	14
Anoxia			
Neurons loaded with:	Decrease in pH_i (pH units)	Increase in $[\text{Na}^+]_i$ (mM)	<i>n</i>
SNARF-5F	0.40 ± 0.02		18
SBFI		18 ± 1	19
SBFI + SNARF-5F	0.44 ± 0.03	18 ± 3	8

indo-1 [1, 39], and $[\text{Na}^+]_i$ and $[\text{Ca}^{2+}]_i$ with SBFI and fluo-3 [31, 32] or fluo-4 [12].

A number of conditions must be satisfied for the accurate measurement of the concentrations of two ions simultaneously with fluorescent probes (see [20, 39]): (1) a lack of spectral overlap between the probes; (2) minimal interactions between the probes; (3) a lack of differential compartmentalization between the probes; (4) binding affinities in the physiological range; (5) distinct ion selectivities; and (6) lack of toxicity. In the present study, the combination of SBFI and carboxy SNARF-1 or SNARF-5F fulfilled each of these criteria. First, using the optical equipment specified, the fluorescence signals originating from the fluorophores in situ could be adequately resolved (Fig. 2). Second, although carboxy SNARF-1 and SNARF-5F quenched the fluorescence of SBFI (Figs. 2 and 3), SBFI-derived ratio values and the Na^+ -sensitivity of SBFI were minimally affected (Figs. 3, 4). Third, in agreement with previous reports [20, 29], SBFI- and SNARF-derived fluorescence emissions emanated largely from the same (i.e. cytosolic) compartment (Fig. 3). Fourth, the calibration parameters obtained in neurons single-loaded with SBFI or carboxy SNARF-1 were consistent with previous in situ estimates [4, 7, 8, 11] and were not significantly influ-

enced by the presence of a second fluorophore (Table 1; Figs. 4, 5); similarly, the $\text{p}K_a$ of SNARF-5F was not affected by the presence of SBFI (Table 1). Fifth, we found that carboxy SNARF-1 and SNARF-5F in situ are effectively insensitive to changes in $[\text{Na}^+]_i$ (Fig. 6); previously, SBFI in situ has been found to be weakly sensitive to changes in pH [11, 24, 29]. Sixth, although it is well established that fluorescent probes employed for ratiometric $[\text{ion}]_i$ determinations may, under some conditions, exert toxic effects (see [25, 37] and references therein), resting pH_i and $[\text{Na}^+]_i$ values (and rates of pH_i recovery from NH_4^+ -induced internal acid loads; Fig. 7d) obtained in neurons co-loaded with SBFI and SNARF-5F were not significantly different from the respective values obtained in neurons single-loaded with either dye and are consistent with previous measurements in hippocampal neurons [3, 11, 29]. In addition, in perforated patch electrophysiological recordings, we have observed that hippocampal neurons co-loaded with SBFI and SNARF-5F have normal resting membrane potentials, exhibit a stable input resistance over time and generate trains of overshooting action potentials in response to membrane depolarization (T. Kelly, C. Sheldon and J. Church, unpublished observations). These findings suggest that co-loading neurons with SBFI and

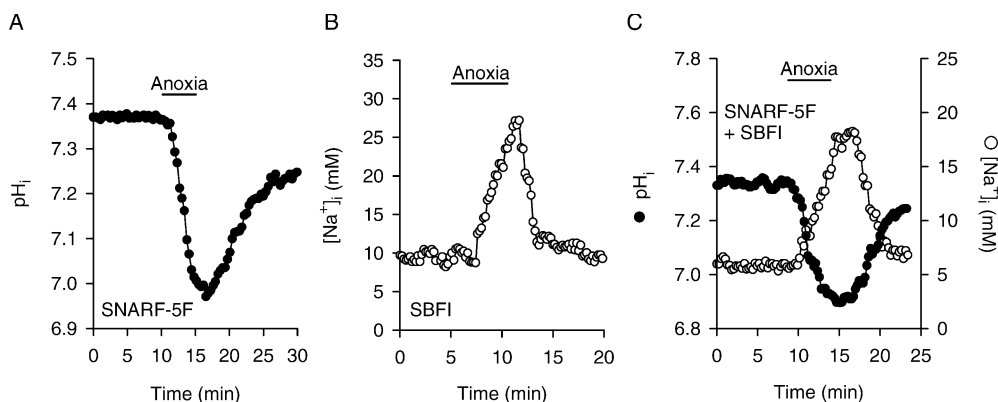


Fig. 8a–c Changes in pH_i and $[\text{Na}^+]_i$ induced by anoxia in rat hippocampal neurons. **a** In a neuron single-loaded with SNARF-5F, 5 min anoxia evoked a fall in pH_i that recovered towards the resting value upon the return to normoxia. **b** In a neuron single-loaded with SBFI, 5 min anoxia induced an increase in $[\text{Na}^+]_i$ that

recovered to the resting value upon the return to normoxia. **c** In a neuron co-loaded with SNARF-5F and SBFI, 5 min anoxia induced a fall in pH_i (solid circles) and an increase in $[\text{Na}^+]_i$ (open circles). Upon the return to normoxia, both pH_i and $[\text{Na}^+]_i$ recovered toward pre-anoxia values

SNARF-5F does not, at least in the short-term, adversely affect neuronal viability.

Together, therefore, our findings support the feasibility of using SBFI in conjunction with either carboxy SNARF-1 or SNARF-5F to concurrently and accurately measure $[Na^+]_i$ and pH_i . Indeed, no significant differences were observed in the changes in $[Na^+]_i$ and pH_i evoked by NH_4^+ -induced internal acid loads (Table 2; Fig. 7) or transient periods of anoxia (Table 2; Fig. 8) in neurons single-loaded with SBFI or SNARF-5F or co-loaded with both probes. The experiments illustrated in Figs. 7 and 8 also demonstrate the utility of concurrent measurements to resolve the interplay between changes in $[Na^+]_i$ and pH_i under various experimental conditions. Thus, the records shown in Fig. 7c and e demonstrate that the recovery of pH_i from NH_4^+ -induced internal acid loads in rat hippocampal neurons is accompanied by a transient rise in $[Na^+]_i$, a finding that provides further evidence for Na^+/H^+ exchange [15, 22, 23, 36] in a cell type in which the transport mechanism is insensitive to known pharmacological inhibitors [3, 26]. And the records shown in Fig. 8c illustrate the temporal relationship between the changes in $[Na^+]_i$ and pH_i evoked by anoxia; in contrast to excitotoxin-induced changes in $[Na^+]_i$ and pH_i (measured simultaneously with ISMs in leech neurons [17]), a fall in pH_i consistently preceded any rise in $[Na^+]_i$. Concurrent measurements of $[Na^+]_i$ and pH_i also provide the data required to correct, on a region-by-region basis, $[Na^+]_i$ values estimated with SBFI for changes in pH_i . Using the methods detailed in Diarra et al. [11], the magnitudes of the increases in $[Na^+]_i$ observed in response to NH_4^+ washout or anoxia increased from 12 ± 2 mM (uncorrected) to 16 ± 3 mM (corrected) and from 18 ± 4 mM (uncorrected) to 21 ± 4 mM (corrected), respectively ($P < 0.05$ in each case by paired Student's *t*-tests). Because small changes in $[Na^+]_i$ can exert important physiological effects [28], the ability to correct apparent $[Na^+]_i$ values measured with SBFI for the prevailing pH_i is another advantage offered by the simultaneous use of SBFI and carboxy SNARF-1 or SNARF-5F.

In summary, we have developed and characterized a method for the near-simultaneous measurement of $[Na^+]_i$ and pH_i in cells co-loaded with SBFI and carboxy SNARF-1 or SNARF-5F. The technique offers an alternative to the use of ISMs in experiments in which information is required on both $[Na^+]_i$ and pH_i .

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