

special communication

In situ calibration and $[H^+]$ sensitivity of the fluorescent Na^+ indicator SBFI

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Received 5 June 2000; accepted in final form 18 January 2001

Diarrar, Abdoullah, Claire Sheldon, and John Church. In situ calibration and $[H^+]$ sensitivity of the fluorescent Na^+ indicator SBFI. *Am J Physiol Cell Physiol* 280: C1623–C1633, 2001.—Despite the popularity of Na^+ -binding benzofuran isophthalate (SBFI) to measure intracellular free Na^+ concentrations ($[Na^+]_i$), the in situ calibration techniques described to date do not favor the straightforward determination of all of the constants required by the standard equation (Gryniewicz G, Poenie M, and Tsien RY. *J Biol Chem* 260: 3440–3450, 1985) to convert the ratiometric signal into $[Na^+]$. We describe a simple method in which SBFI ratio values obtained during a “full” in situ calibration are fit by a three-parameter hyperbolic equation; the apparent dissociation constant (K_d) of SBFI for Na^+ can then be resolved by means of a three-parameter hyperbolic decay equation. We also developed and tested a “one-point” technique for calibrating SBFI ratios in which the ratio value obtained in a neuron at the end of an experiment during exposure to gramicidin D and 10 mM Na^+ is used as a normalization factor for ratios obtained during the experiment; each normalized ratio is converted to $[Na^+]_i$ using a modification of the standard equation and parameters obtained from a full calibration. Finally, we extended the characterization of the pH dependence of SBFI in situ. Although the K_d of SBFI for Na^+ was relatively insensitive to changes in pH in the range 6.8–7.8, acidification resulted in an apparent decrease, and alkalization in an apparent increase, in $[Na^+]_i$ values. The magnitudes of the apparent changes in $[Na^+]$, varied with absolute $[Na^+]_i$, and a method was developed for correcting $[Na^+]_i$ values measured with SBFI for changes in intracellular pH.

sodium-binding benzofuran isophthalate; hippocampal neuron; intracellular sodium; intracellular pH

THE INTRACELLULAR FREE CONCENTRATION of Na^+ ions ($[Na^+]_i$) is an important determinant of cellular function. In neurons, the electrochemical gradient of Na^+ across the plasma membrane plays a central role in

determining excitability, and changes in $[Na^+]_i$ can modulate the activities of ion channels (40), Na^+ -coupled transporters and uptake mechanisms (4, 5, 20), and enzymes (12). Disturbances in neuronal intracellular Na^+ homeostasis also play a role in pathophysiological events, including excitotoxic/anoxic injury (7, 9, 11, 39).

Although a variety of methods have been employed to estimate $[Na^+]_i$, Na^+ -sensitive fluorescent dyes, especially Na^+ -binding benzofuran isophthalate (SBFI), are assuming an increased importance. Despite the many advantages of SBFI (see Refs. 26 and 32), a number of difficulties are associated with the calibration procedures required to convert experimentally derived SBFI ratio values into $[Na^+]_i$. Thus in vitro calibration fails to take into account the spectral shifts that are introduced when the dye is present in the cytosol (3, 8, 15, 23, 28). On the other hand, the in situ techniques described to date do not favor the straightforward determination of separate values for all of the constants necessary to convert the ratio of emitted SBFI fluorescence signals into $[Na^+]$ values according to the standard equation of Gryniewicz et al. (16)

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

where $\beta = S_{f2}/S_{b2}$ and is the ratio of the fluorescence of the free (unbound) dye (S_{f2}) to bound dye (S_{b2}) at the second excitation wavelength (λ_{2}^{ex}), K_d is the apparent dissociation constant of SBFI for Na^+ , R is the fluorescence ratio, R_{\min} is the fluorescence ratio at $[Na^+] = 0$ mM, and R_{\max} is the fluorescence ratio at saturation. The commonly employed “three-point” calibration technique (18), for example, provides only a composite value for βK_d ; the accurate determination of a separate value for β (and, thus the K_d of SBFI for Na^+) is precluded because a value for S_{b2} cannot be derived. In the present study, therefore, we developed a method to

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determine, from data derived from full in situ calibrations, all of the constant parameters required to resolve Eq. 1. We also describe a one-point technique for the in situ calibration of SBFI ratios and examine the utility of this procedure to calibrate the changes in SBFI ratios evoked in neurons by veratridine or anoxia.

Another difficulty associated with SBFI is that, in common with many fluorophores, SBFI fluorescence is sensitive to changes in $[H^+]$ (13, 18, 28, 29, 35, 36). Although the full pH sensitivity of SBFI has been described in a cell-free in vitro system (26), the effects of changes in intracellular H^+ concentration ($[H^+]_i$) on measurements of $[Na^+]_i$ made with SBFI in situ remain relatively poorly defined. This represents a potential limitation to the accurate estimation of $[Na^+]_i$ with SBFI, in part because changes in $[Na^+]_i$ may give rise to changes in intracellular pH (pH_i), either directly (e.g., via alterations in Na^+/H^+ exchange) or indirectly [e.g., via alterations in Na^+/Ca^{2+} exchange and subsequent changes in intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$)]. Therefore, we used the procedures developed in the first part of the study to assess the effect of changes in pH_i on the K_d of SBFI for Na^+ in situ. We also characterized the $[H^+]$ sensitivity of SBFI in situ at different values of $[Na^+]_i$ and developed a method for correcting, if necessary, $[Na^+]_i$ values measured with SBFI for changes in pH_i .

EXPERIMENTAL PROCEDURES

Cell Culture

Primary cultures of hippocampal neurons from 4-day postnatal Wistar rats were prepared as described (14). Briefly, rat pups were anesthetized with 3% halothane in air, rapidly decapitated, and the hippocampi removed. The hippocampi were enzymatically and mechanically dissociated, and the resulting cell suspension was underlain with fetal bovine serum and centrifuged at 150 g at 4°C for 10 min. Cells were then resuspended and plated onto glass coverslips coated with poly-D-lysine and laminin at a low density of 4×10^5 neurons/cm². The initial growth medium was Eagle's minimum essential medium supplemented with 5% horse serum and 5% fetal bovine serum (Life Technologies, Grand Island, NY). After 24 h, this medium was half-changed with serum-free N2-supplemented medium. Cultures were then fed every 4–5 days by half-changing the existing medium with serum-free N2-supplemented medium. Glial proliferation was inhibited 48 h after initial plating by adding 10 μ M cytosine arabinoside. Neurons were used 6–14 days after plating.

Solutions

The standard perfusion medium contained (in mM) 136.5 NaCl, 3 KCl, 1.5 NaH₂PO₄, 1.5 MgSO₄, 10 D-glucose, 2 CaCl₂, and 10 HEPES (titrated to the appropriate temperature-corrected pH with 10 M NaOH). Calibrating media contained (in mM) 0.6 MgCl₂, 0.5 CaCl₂, 10 HEPES, Na⁺ and K⁺ such that $[Na^+] + [K^+] = 130$, 100 gluconate, and 30 Cl⁻ (titrated with 10 M KOH to the desired temperature-corrected pH); gramicidin D, monensin, ouabain, and/or nigericin (Sigma-Aldrich Canada, Oakville, ON) were added, as indicated in the text. To limit cross-contamination by ionophores, perfusion lines were replaced, and the imaging chamber was decontaminated after each experiment (22). Anoxic media

were prepared immediately before use by adding 1–2 mM sodium dithionite to the standard perfusion medium and bubbling vigorously with 100% N₂ or Ar (14). During anoxia, the atmosphere in the recording chamber was switched from room air to 100% N₂ or Ar.

Microspectrofluorometry

The acetoxymethyl esters of SBFI (SBFI-AM) and 2',7'-bis(2-carboxyethyl)-5(6)-carboxyfluorescein (BCECF-AM) were obtained, respectively, from Texas Fluorescence Labs (Austin, TX) and Molecular Probes (Eugene, OR). Coverslips plated with neurons were placed in standard perfusion medium containing either 10 μ M SBFI-AM (in the presence of 0.15% Pluronic F-127 and 5 mg/ml of bovine serum albumin) or 2 μ M BCECF-AM and incubated at room temperature for, respectively, 150 and 30 min. Coverslips were then placed in standard medium for 30 min to ensure deesterification of the fluorophore and mounted in a temperature-controlled perfusion chamber to form the base of the chamber. Neurons were superfused at a rate of 2 ml/min for 15 min with the initial experimental solution at the appropriate temperature before the start of an experiment. Experiments were performed at room temperature (20–22°C) and at 37°C, as indicated in the text.

Measurements of $[Na^+]_i$ and pH_i were performed using the dual excitation ratio method, employing an imaging system (Atto Instruments, Rockville, MD) in conjunction with an Axiovert 10 epifluorescence microscope (Carl Zeiss Canada, Don Mills, ON). Full details have been provided previously (2, 14, 38). In brief, SBFI- or BCECF-loaded neurons were excited via a $\times 40$ LD Achromplan objective with light provided by a 100-W Hg arc burner and band-pass filtered alternately at 334 and 380 nm (SBFI) or at 488 and 452 nm (BCECF). To reduce photobleaching of the fluorophores, the output of the ultraviolet (UV) light source was attenuated electronically, neutral density filters were placed in the light path, and a high-speed shutter was employed to limit UV exposure to the periods required for data acquisition. Fluorescence emissions, measured at 510 or 520 nm from neurons loaded with SBFI or BCECF, respectively, were detected by an intensified charge-coupled device camera (Atto Instruments) and collected from regions of interest placed on individual neuronal somata. Experiments were repeated on at least three (usually ≥ 5) different coverslips, each allowing collection of data from up to 99 individual neurons simultaneously. Raw emission intensity data at each excitation wavelength were corrected for background fluorescence before the calculation of a ratio; the intensity of background fluorescence was typically $<15\%$ of the total signal at any given excitation wavelength and remained constant during the course of a given experiment. Ratio pairs were acquired at 1- to 12-s intervals and analyzed off-line. The one-point high- $[K^+]$ /nigericin technique was employed to convert background-corrected BCECF emission intensity ratios (BI_{488}/BI_{452}) into pH_i values, as detailed previously (2, 6, 38). The procedures employed to calibrate the ratios of the emitted SBFI signals are detailed in RESULTS.

Data Analysis

Results are reported as means \pm SE, with the accompanying *n* value referring to the number of cell populations (i.e., number of coverslips) analyzed. For clarity, values obtained during the course of the study are presented to two significant decimal places, although all calculations were performed using values accurate to four decimal places. Statistical comparisons were carried out using Student's two-tailed *t*-test, paired or unpaired as appropriate, with a 95% confidence limit.

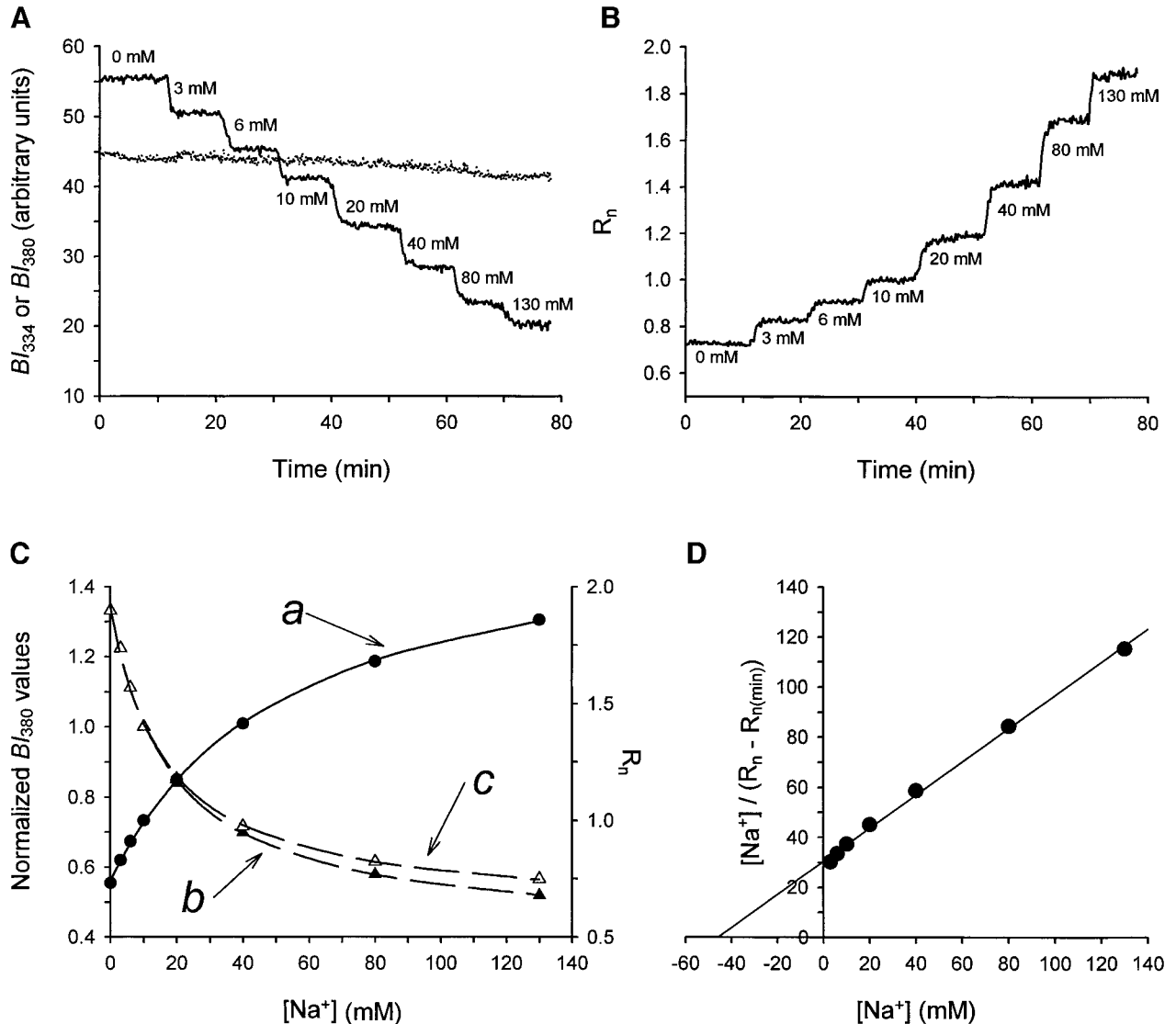


Fig. 1. In situ full calibration of Na^+ -binding benzofuran isophthalate (SBFI) at room temperature and extracellular pH (pH_o) 7.35. **A**: A full calibration experiment in which 10 neurons on a single coverslip were exposed to $5 \mu\text{M}$ gramicidin D-containing solutions at the $[\text{Na}^+]$ values shown above or below the traces. Displayed are changes in emitted fluorescence intensities during excitation at 334 nm (dotted line) and 380 nm (continuous line); the signals were corrected for background fluorescence. Whereas the BI_{380} value displayed stepwise changes in response to changes in $[\text{Na}^+]$, the BI_{334} value appeared insensitive. The trace is representative of 23 independent experiments. **B**: BI_{334}/BI_{380} ratio values were computed from the data shown in **A** and normalized to unity at intracellular free Na^+ concentration ($[\text{Na}^+]_i$) = 10 mM. The trace shows the stepwise changes in normalized BI_{334}/BI_{380} ratio values (R_n) evoked by successive increases in $[\text{Na}^+]$. **C**: curve *a* (solid line) is a plot of $[\text{Na}^+]$ vs. mean normalized BI_{334}/BI_{380} ratio values (R_n) obtained from 4 calibration experiments of the type shown in **B**, conducted on sister cultures on the same day. The curve is the result of a three-parameter hyperbolic fit (Eq. 3) to the data points (\bullet) indicated. Standard error bars are contained within each datum point. Curves *b* and *c* (dashed lines) are plots of $[\text{Na}^+]$ vs. mean normalized background-subtracted emission intensities during excitation at 380 nm. The curves are the result of three-parameter hyperbolic decay fits (Eq. 6) to the respective data points indicated. The data points employed for curve *b* (\blacktriangle) and curve *c* (\triangle) were not drift corrected and were drift corrected, respectively (see text). In each case, standard error bars are contained within each datum point. **D**: Hanes plot of the calibration data shown in **C** (plot *a*). The continuous line is a linear least-squares regression fit to the data points indicated ($r^2 = 0.99$). $R_{n(\text{max})}$ is drawn from the slope = $1/(R_{n(\text{max})} - R_{n(\text{min})})$, and $-\beta K_d$ is obtained from the intercept on the abscissa.

RESULTS

Calibration of SBFI Ratio Values

Determination of R_{min} , R_{max} , and βK_d . Full calibrations were performed at room temperature in 23 neuronal populations by exposing neurons sequentially to

pH 7.35 media containing $5 \mu\text{M}$ gramicidin D and eight different $[\text{Na}^+]$ values (range, 0–130 mM). Typical changes in BI_{334} and BI_{380} values are illustrated in Fig. 1A. Fluorescence emitted during excitation at 334 nm was essentially unaffected by changes in $[\text{Na}^+]$, whereas during excitation at 380 nm, emitted fluores-

cence intensity decreased as $[Na^+]$ increased. The lack of an effect of changes in $[Na^+]$ on fluorescence emitted in situ during excitation at 334 nm in epifluorescence systems has been reported and discussed previously (3, 13, 15, 21, 23, 24, 28, 29, 34, 35).

To determine all the constants ($R_{n(\min)}$, $R_{n(\max)}$, β , and K_d) necessary to convert the ratio of emitted SBFI signals into $[Na^+]$ values, the standard equation (Eq. 1) was rearranged to give

$$[Na^+] = \beta K_d [(R_n - R_{n(\min)}) / (R_{n(\max)} - R_n)] \quad (2)$$

where R_n is the background-subtracted SBFI fluorescence ratio (BI_{334}/BI_{380}) normalized to $[Na^+] = 10$ mM, and $R_{n(\min)}$ and $R_{n(\max)}$ are, respectively, the minimum and maximum obtainable values for the normalized ratio. Background-subtracted SBFI fluorescence ratios were normalized to the BI_{334}/BI_{380} value at $[Na^+] = 10$ mM (Fig. 1B), and the data points relating $[Na^+]$ to R_n were then fit by a three-parameter hyperbolic equation having the form

$$R_n = R_{n(\min)} + [a([Na^+]) / (b + [Na^+])] \quad (3)$$

where a and b are constants. The data points relating $[Na^+]$ to R_n (Fig. 1C, curve a) were accurately fitted ($r^2 > 0.99$) by Eq. 3, and the resulting fitted parameters were $R_{n(\min)} = 0.74 \pm 0.01$, $a = 1.55 \pm 0.02$, and $b = 51.85 \pm 2.12$. The value for $R_{n(\min)}$ obtained in this manner was identical to the value of $R_{n(\min)}$ derived experimentally at $[Na^+] = 0$ mM. The hyperbolic form of curve a in Fig. 1C underscores the difficulty of experimentally determining accurate values for $R_{n(\max)}$ when using SBFI, especially in light of reports that the dye may not saturate in situ even when $[Na^+]_i$ is raised to 150 mM (3, 23). To estimate $R_{n(\max)}$ and a composite value for βK_d , Eq. 3 was rearranged to give

$$[Na^+] = b \{ (R_n - R_{n(\min)}) / [(a + R_{n(\min)}) - R_n] \} \quad (4)$$

It is apparent that Eq. 4 is identical to Eq. 2 when $b = \beta K_d$ and $[a + R_{n(\min)}] = R_{n(\max)}$. Thus the fitted parameters of Eq. 3 could be employed to determine those of the standard equation, Eq. 2. The calculated values of $R_{n(\max)}$ and βK_d were 2.30 ± 0.03 and 51.85 ± 2.12 mM, respectively.

Hanes plots and related methods have frequently been employed to derive parameters for the calibration of SBFI ratio values (e.g., Refs. 13, 15, 19, 33). Therefore, the values of $R_{n(\max)}$ and βK_d obtained via the three-parameter hyperbolic fit were compared with those resulting from a Hanes plot of the same data. To derive a Hanes plot, the standard equation (Eq. 2) was rearranged such that

$$[Na^+] / (R_n - R_{n(\min)}) = \{ [Na^+] / (R_{n(\max)} - R_{n(\min)}) \} + [\beta K_d / (R_{n(\max)} - R_{n(\min)})] \quad (5)$$

Accordingly, a plot of $[Na^+] / [R_n - R_{n(\min)}]$ vs. $[Na^+]$ gives a straight line, the slope $\{1 / [R_{n(\max)} - R_{n(\min)}]\}$ providing an estimate of $R_{n(\max)}$ and the intercept on the abscissa giving $-\beta K_d$ (Fig. 1D); $R_{n(\min)}$ is derived from experimental data. The values of $R_{n(\max)}$ and βK_d derived in this manner were 2.24 ± 0.03 and $46.75 \pm$

2.65 mM, respectively, and were not significantly different from those obtained via the three-parameter hyperbolic fit.

The values of the calibration parameters estimated from hyperbolic fits to data obtained in different full calibrations showed little interassay variability (Table 1). In addition, although it has been suggested that inhibition of the plasmalemmal Na^+K^+ -ATPase is required for optimum transmembrane Na^+ equilibration at $[Na^+] < 5$ mM in cardiac myocytes (Ref. 15, but see Ref. 23), in the present study, in neurons, the values of $R_{n(\min)}$, $R_{n(\max)}$, and βK_d obtained after the addition of 1 mM ouabain to gramicidin D-containing media (0.72 ± 0.05 , 2.30 ± 0.08 , and 47.83 ± 2.09 mM, respectively; $n = 3$) were not significantly different from those obtained in the absence of the pump inhibitor. Twelve full calibrations were also performed at 37°C and at extracellular pH (pH_o) 7.35; calculated values of $R_{n(\min)}$, $R_{n(\max)}$, and βK_d were 0.76 ± 0.02 , 2.26 ± 0.12 , and 50.66 ± 3.92 mM, respectively ($P > 0.05$ in each case for the difference to the respective value obtained at room temperature).

Determination of β and K_d . Neither Hanes plots of data derived from full calibrations nor the commonly employed three-point procedure (18) permits the straightforward determination of a separate value for β (and, thus a value for the K_d of SBFI for Na^+). To determine β , one needs to know S_{f2} and S_{b2} , the intensities of fluorescence emissions when exciting the fluorophore at the second wavelength ($\lambda_2^{ex} = 380$ nm) at $[Na^+] = 0$ mM and at saturating Na^+ , respectively. Although a value for S_{f2} can be obtained experimentally, the determination of S_{b2} requires very high $[Na^+]$ and is compromised by the sensitivity of SBFI to changes in ionic strength and other factors (3, 23, 26, 28). To derive S_{b2} , we employed the BI_{380} value at $[Na^+] = 10$ mM as a normalization factor. Normalized BI_{380} values (S_{n2}) at different $[Na^+]$ are shown in Fig. 1C (plot b). The data points were fitted ($r^2 > 0.99$) with a three-parameter hyperbolic decay equation having the form

$$S_{n2} = \delta + [\epsilon(\phi) / (\phi + [Na^+])] \quad (6)$$

Table 1. Values of $R_{n(\min)}$, $R_{n(\max)}$, and βK_d determined by three-parameter hyperbolic fits to data points obtained in full calibrations

Calibration Number	n	$R_{n(\min)}$	$R_{n(\max)}$	βK_d
1	4	0.74 ± 0.01	2.30 ± 0.03	51.85 ± 2.12
2	5	0.81 ± 0.01	1.87 ± 0.03	48.62 ± 2.25
3	4	0.74 ± 0.01	2.23 ± 0.05	50.14 ± 1.81
4	4	0.75 ± 0.01	2.14 ± 0.04	48.39 ± 1.42
5	3	0.72 ± 0.02	2.23 ± 0.08	50.24 ± 2.93
6	3	0.72 ± 0.01	2.36 ± 0.03	51.07 ± 2.50

Values are means \pm SE. The full in situ calibrations listed were performed over a 12-mo period on neurons from 6 different batches of cultures. n refers to the number of cell populations (i.e., no. of coverslips) from the same batch of cultures in which a full calibration experiment was repeated on the same day to provide the values for the calibration number indicated.

where δ , ϵ , and ϕ are constants. From Eq. 6 it can be seen that when $[\text{Na}^+] = 0$, $S_{n2} = S_{f2} = (\delta + \epsilon)$, and when $[\text{Na}^+] = \infty$, $S_{n2} = S_{b2} = \delta$. Consequently

$$\beta = S_{f2}/S_{b2} = (\delta + \epsilon)/\delta \quad (7)$$

The values of the parameters for Eq. 6, obtained from the fit to the data points shown in Fig. 1C (plot b), were δ (i.e., S_{b2}) = 0.41 ± 0.01 , $\epsilon = 0.94 \pm 0.01$, and $\phi = 17.96 \pm 0.97$; thus $S_{f2} = 1.34 \pm 0.03$ and β (S_{f2}/S_{b2}) = 3.31 ± 0.03 . As noted above, $\beta K_d = 51.85 \pm 2.12$ mM; thus the calculated K_d of SBFI for Na^+ = 15.69 ± 0.15 mM.

A source of error that can arise during in situ calibrations is a gradual decline ("drift") in emitted fluorescence intensity values (3, 15, 23, 24). In the present study, declines in BI_{334} and BI_{380} values were sometimes observed during the latter part of a full calibration (i.e., at $[\text{Na}^+] > 10$ mM; Fig. 2). Although this drift will not affect BI_{334}/BI_{380} ratio values (because BI_{334} and BI_{380} decline in parallel; see Fig. 2; also see Ref. 3), it will decrease S_{b2} and increase β , leading to an artifactually low value for K_d . We were able to correct for drift by normalizing the BI_{334} and BI_{380} values at $[\text{Na}^+] = 10$ mM to unity (Fig. 2). Because BI_{334} values are insensitive to changes in $[\text{Na}^+]$ under our experimental conditions, signal changes during excitation at 334 nm are necessarily due to Na^+ -independent factors such as dye loss (e.g., photobleaching) and/or other nonspecific artifacts. Furthermore, because BI_{334} and BI_{380} decline in parallel, the magnitude of any decline in BI_{334} values from unity at any given time point during a calibration can be employed to correct normalized BI_{380} values for drift. Normalized, drift-corrected BI_{380} values at different $[\text{Na}^+]$ are shown

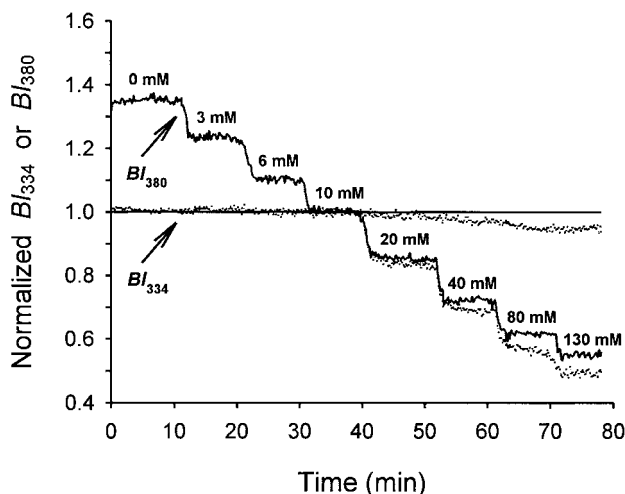


Fig. 2. Normalized and drift-corrected BI_{334} and BI_{380} values. Ten neurons on a single coverslip were exposed to $5 \mu\text{M}$ gramicidin D-containing solutions at the $[\text{Na}^+]$ values shown. Changes in BI_{334} and BI_{380} values were normalized to unity at $[\text{Na}^+] = 10$ mM (dotted lines). The magnitude of the decline in BI_{334} values from unity at any given time point was then employed to correct (solid line) the normalized BI_{380} values for the drift that occurred over the course of the experiment.

in Fig. 1C, where the data points have been fitted by Eq. 6 (curve c). The drift-corrected parameters for the fit were $S_{b2} = 0.47 \pm 0.01$ and $S_{f2} = 1.34 \pm 0.03$; thus β , corrected for drift, is 2.88 ± 0.05 . As noted above, $\beta K_d = 51.85 \pm 2.12$ mM; thus the K_d following correction is 17.99 ± 0.31 mM. The latter value is greater ($P < 0.05$) than the uncorrected value (15.69 ± 0.15 mM) and is similar to that reported in vitro in solutions with a combined $[\text{Na}^+] + [\text{K}^+]$, which approximates physiological strength ($K_d = 17\text{--}19$ mM) (26, 28).

One-point calibration. Full calibrations may be impractical at the end of an experiment, due to a loss of fluorescence signal with time. We therefore explored the possibility of applying a one-point procedure to calibrate SBFI ratios in situ. To illustrate the one-point technique, we measured the changes in SBFI ratios that occurred in hippocampal neurons at 37°C in response to $30 \mu\text{M}$ veratridine ($n = 5$, a total of 32 neurons) or anoxia ($n = 12$, a total of 91 neurons). At the end of an experiment, neurons were exposed to a single calibrating solution containing $5 \mu\text{M}$ gramicidin D and 10 mM Na^+ . The resulting BI_{334}/BI_{380} value at $\text{Na}^+ = 10$ mM for a given neuron in the sampled population was then used as a normalization factor for that neuron. After dividing experimentally derived BI_{334}/BI_{380} values from a given neuron by the normalization factor for that neuron, each R_n was converted to $[\text{Na}^+]_i$ using Eq. 2 and the parameters [$R_{n(\text{min})}$, $R_{n(\text{max})}$, β , and K_d] determined in a full calibration. Estimated in this manner, resting $[\text{Na}^+]_i$ before veratridine or anoxia was 9.8 ± 0.3 mM ($n = 17$), a value similar to that reported by others in hippocampal neurons (30, 34). During anoxia, $[\text{Na}^+]_i$ increased to 43.1 ± 2.8 mM ($n = 12$; Fig. 3A), a rise similar to that observed during energy deprivation or exposure to excitotoxins in a variety of mammalian central neurons (9, 10, 30). Veratridine ($30 \mu\text{M}$) evoked an increase in $[\text{Na}^+]_i$ that failed to recover toward resting values during the recording period (Fig. 3A).

In a separate series of experiments, we compared values for $[\text{Na}^+]_i$ derived via the one-point technique with those derived via the commonly employed three-point calibration procedure. Neurons were subjected to 5 min of anoxia and were then exposed sequentially to calibrating media containing $5 \mu\text{M}$ gramicidin D and 6, 10, or 40 mM Na^+ ($n = 8$; Fig. 3, B and C). The BI_{334}/BI_{380} values obtained from each selected cell in a given experiment were then calibrated separately, either by the three-point procedure [using the equations provided by Harootunian et al. (18)] or by the one-point procedure (using $\text{Na}^+ = 10$ mM as the normalization factor). Similar values for both resting $[\text{Na}^+]_i$ before anoxia and peak $[\text{Na}^+]_i$ evoked by anoxia were derived via the one- and three-point procedures (resting $[\text{Na}^+]_i = 9.9 \pm 0.9$ and 9.3 ± 1.0 mM, respectively; peak $[\text{Na}^+]_i = 45.2 \pm 3.4$ and 45.2 ± 3.9 mM, respectively). The results support the utility of a one-point procedure for the in situ calibration of SBFI ratio values.

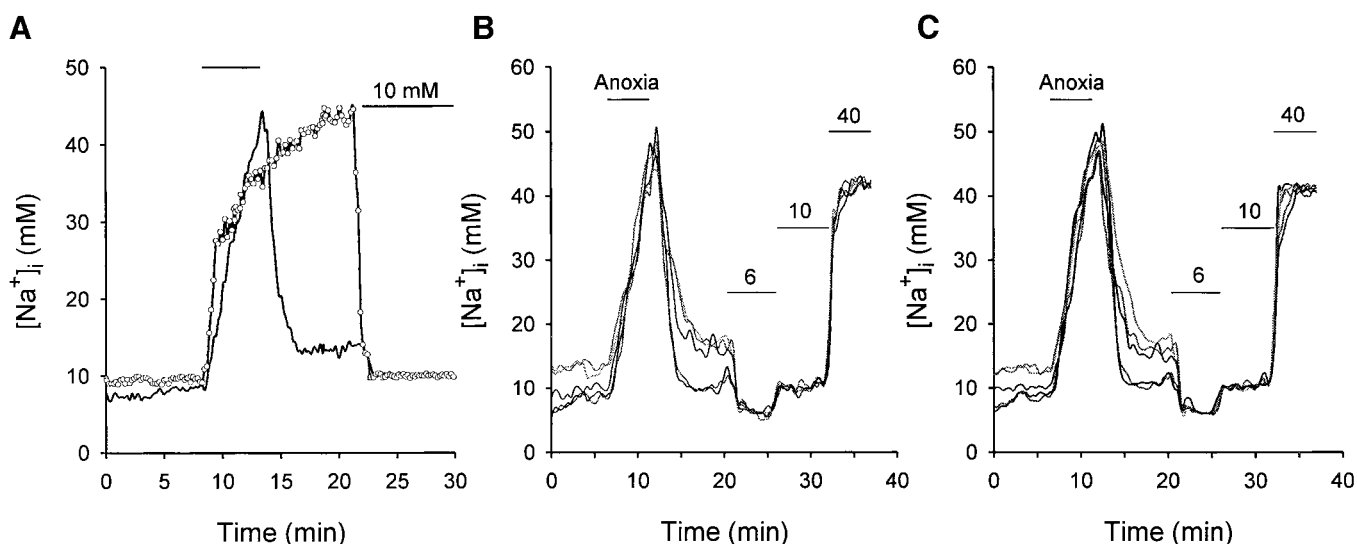


Fig. 3. Changes in $[Na^+]_i$ evoked by veratridine and/or anoxia. **A**: changes in $[Na^+]_i$ induced by a 5-min exposure, indicated by the first bar above the traces, to either $30 \mu M$ veratridine (\circ) or an anoxic medium (continuous line) in 2 populations of neurons on different coverslips. Each experiment was performed at $37^\circ C$ and pH_o 7.35. For each selected neuron in the sampled population, a one-point in situ calibration of the SBFI ratio signal, indicated by the second bar above the traces, was performed with a pH 7.35 medium containing $5 \mu M$ gramicidin D and $10 mM Na^+$. The responses to veratridine and anoxia are means of data obtained simultaneously from 9 and 12 neurons, respectively. **B** and **C**: after a 5-min period of anoxia, indicated by the first bar above the traces, 5 neurons on a single coverslip were exposed sequentially to calibrating media (pH 7.35) containing $5 \mu M$ gramicidin D and 6, 10, or $40 mM Na^+$. Ratios of the BI_{334} and BI_{380} signals obtained from each neuron during the course of the experiment were then calibrated separately by the three-point (**B**) and one-point (**C**) methods, the former using the equations provided by Harootunian et al. (18) and the latter using $Na^+ = 10 mM$ as the normalization factor and values for βK_d , $R_{n(min)}$, and $R_{n(max)}$ obtained from a full calibration (calibration 3, Table 1).

Effects of Changes in pH on $[Na^+]_i$ Measurements With SBFI In Situ

Effect of calibration media on pH_i . A change in pH_i during a calibration might affect the precision of the procedure. In addition, although SBFI ratios are often calibrated with a combination of gramicidin D, monensin, and ouabain (e.g., see Refs. 10, 13, 29, 34), it was originally suggested that gramicidin D alone might provide a more accurate calibration for estimating cytosolic $[Na^+]_i$ (18). Therefore, we assessed the effects on pH_i of exposing neurons to $5 \mu M$ gramicidin D alone or in combination with $10 \mu M$ monensin and $1 mM$ ouabain; in all cases, pH_o was 7.35 at $37^\circ C$.

Exposure to a solution containing gramicidin D alone evoked a change in pH_i , the direction and magnitude of which depended on the resting pH_i (Fig. 4, A and B); overall, the effect of medium containing $5 \mu M$ gramicidin D and $10 mM Na^+$ was to bring pH_i to 7.33 ± 0.01 ($n = 5$; a total of 46 neurons). Similar results were obtained with medium containing $130 mM Na^+$ ($n = 3$; not shown). As illustrated in Fig. 4A, the addition of nigericin to gramicidin D-containing medium did not further alter pH_i ($n = 5$). We also measured the effects on pH_i of altering the pH of media containing $5 \mu M$ gramicidin D. As illustrated in Fig. 4C, at pH_o 6.80, steady-state pH_i in the presence of gramicidin D was 6.85 ± 0.02 , whereas at pH_o 7.80, pH_i was 7.83 ± 0.03 ($n = 5$ in each case; a total of 51 neurons in each case). Similar results were obtained when neurons were exposed to media containing $10 mM Na^+$ and $5 \mu M$

gramicidin D, $10 \mu M$ monensin, and $1 mM$ ouabain. Examined in six populations of neurons (a total of 61 cells) at pH_o 7.35, pH_i was 7.37 ± 0.04 (a value that was not significantly different compared with the pH_i measured in the presence of gramicidin D alone), and the addition of $10 \mu M$ nigericin failed to further influence pH_i . At pH_o 6.80 and 7.80, pH_i measured in the presence of $5 \mu M$ gramicidin D, $10 \mu M$ monensin, and $1 mM$ ouabain was 6.87 ± 0.03 ($n = 3$) and 7.83 ± 0.04 ($n = 3$), respectively ($P > 0.05$ in each case for the difference to the pH_i measured at the respective pH_o in the presence of gramicidin D alone).

Effects of changes in pH_i on the K_d of SBFI for Na^+ . To assess whether the K_d of SBFI for Na^+ in situ is sensitive to changes in pH , full calibrations were performed at pH_o 6.80 and pH_o 7.80 ($n = 8$ in each case). The resulting normalized BI_{334}/BI_{380} ratios and normalized drift-corrected fluorescence intensities (at $\lambda_{2ex} = 380 nm$) were then fitted by the appropriate equations (Eqs. 3 and 6, respectively). BI_{334} values were essentially unaffected as pH_o increased from 6.80 to 7.80 (data not shown), as was the value for $R_{n(min)}$ (0.75 ± 0.01 and 0.74 ± 0.03 at pH_o 6.80 and 7.80, respectively), whereas $R_{n(max)}$ increased slightly from 2.24 ± 0.02 to 2.36 ± 0.05 . Values for βK_d also increased slightly, from $50.04 \pm 1.88 mM$ to $54.67 \pm 3.26 mM$, as pH_o increased from 6.80 to 7.80; this change reflected an increase in β , and K_d values obtained at pH_o 6.80 and 7.80 (17.95 ± 0.03 and $17.55 \pm 0.20 mM$, respectively) were, in both cases, not significantly dif-

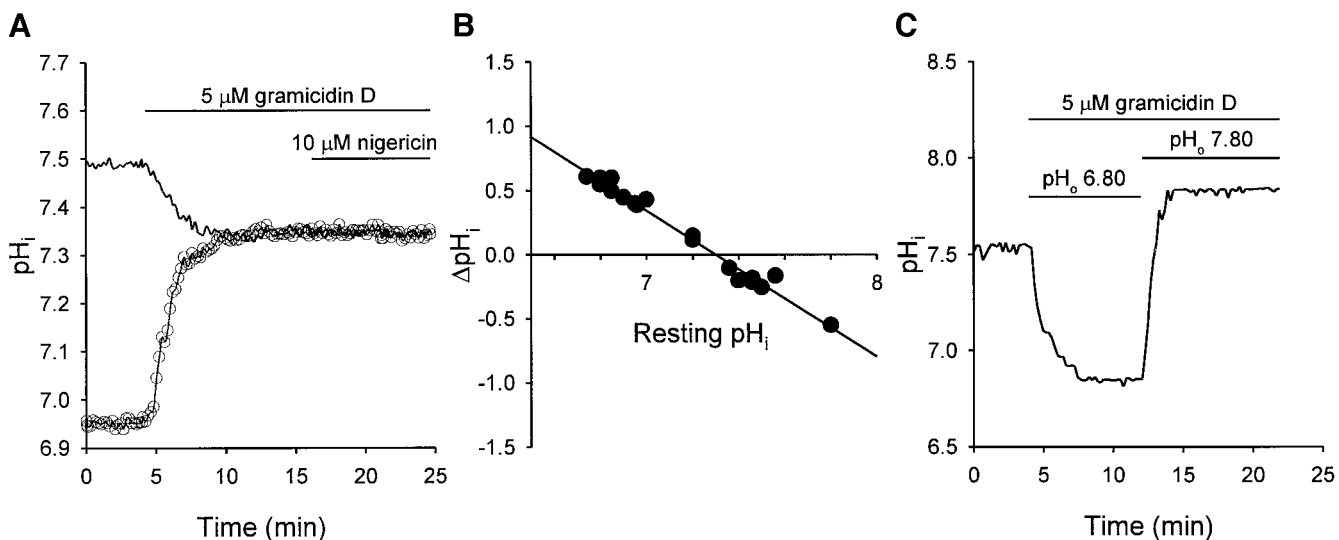


Fig. 4. Effect of calibrating media on intracellular pH (pH_i) in neurons loaded with 2',7'-bis(2-carboxyethyl)-5(6)-carboxyfluorescein (BCECF). **A**: 2 different neurons on a single coverslip, one with resting $pH_i \sim 7.48$ and the other with resting $pH_i \sim 6.95$, during perfusion with standard pH 7.35 medium at 37°C , were exposed to a pH 7.35 medium containing 10 mM Na^+ and 5 μM gramicidin D for the period indicated by the bar above the traces. The calibrating medium decreased the pH_i of the neuron with a high initial resting pH_i (continuous line) and increased the pH_i of the neuron with a low initial resting pH_i (\circ). The subsequent addition of 10 μM nigericin failed to evoke an additional change in pH_i . **B**: the changes in steady-state pH_i (ΔpH_i) evoked in 22 different neurons by exposure to a pH 7.35, 10 mM Na^+ medium containing 5 μM gramicidin D are plotted against the pH_i values measured before application of the calibrating medium. The line shown is a linear least-squares regression fit to the data points indicated ($r^2 = 0.96$). **C**: a neuron perfused with a standard pH 7.35 medium at 37°C was exposed to a pH 6.80 calibration medium containing 10 mM Na^+ and 5 μM gramicidin D; pH_i fell to a new steady-state value. The pH_o was subsequently increased to 7.80, which caused pH_i to increase to a new steady-state level. A one-point BCECF calibration with 10 μM nigericin was performed immediately after the end of the record shown. The trace is representative of responses observed in 51 neurons in 5 independent experiments.

ferent from the K_d value established at pH_o 7.35 (17.99 ± 0.31 mM; see above).

Effects of changes in pH_i on SBFI ratio values and $[\text{Na}^+]_i$. Initially, we examined the effects on SBFI ratios of a series of calibrating media containing 5 μM gramicidin D and 0, 10, 40, or 130 mM Na^+ , at three different pH values (6.80, 7.35, and 7.80). Experiments were performed at room temperature ($n = 6$) and 37°C ($n = 3$); no differences were observed between results at the two temperatures, and the data were, therefore, pooled. As illustrated in Fig. 5A, R_n values at a given $[\text{Na}^+]$ were reduced at pH_o 6.80 and increased at pH_o 7.80, compared with pH_o 7.35, both effects increasing in magnitude as $[\text{Na}^+]$ was increased from 0 to 130 mM. Normalized ratios were converted into $[\text{Na}^+]_i$ using values for $R_{n(\text{min})}$, $R_{n(\text{max})}$, β , and K_d determined at pH_o 7.35 in full calibrations, and the resulting traces of the effects of changes in pH on $[\text{Na}^+]_i$ at the different Na^+ concentrations are shown in Fig. 5B. Acidification resulted in an apparent decrease and alkalinization in an apparent increase in $[\text{Na}^+]_i$ when $[\text{Na}^+] \geq 10$ mM.

To quantify the effects of changes in pH on $[\text{Na}^+]_i$ values measured with SBFI, we calculated the magnitude of the apparent pH_i -induced changes in $[\text{Na}^+]_i$ by taking the measured $[\text{Na}^+]_i$ at pH_i 7.33 (i.e., the pH_i measured in the presence of gramicidin D at pH_o 7.35) as a reference point and calculating the apparent change in $[\text{Na}^+]$ ($\Delta[\text{Na}^+]$) at $pH_i = 6.85$ and 7.83 (i.e.,

the pH_i values measured in the presence of gramicidin D at pH_o 6.80 and 7.80, respectively) according to

$$\Delta[\text{Na}^+] = [\text{Na}^+]_{pH_i(x)} - [\text{Na}^+]_{pH_i,7.33} \quad (8)$$

where $[\text{Na}^+] = 10, 40, \text{ or } 130$ mM and $x = 6.85, 7.33, \text{ or } 7.83$ (pH_i -evoked changes in $\Delta[\text{Na}^+]$ were very small at $[\text{Na}^+] = 0$ mM; these data were excluded). The results, which are presented in Fig. 5C, indicate that the effects of changes in pH on $\Delta[\text{Na}^+]_i$ increase as $[\text{Na}^+]$ increases and that a linear relationship exists between the effects of changes in pH on $\Delta[\text{Na}^+]$ for each $[\text{Na}^+]$ examined. The linear nature of the relationship enabled us to construct an empirical equation by means of which a correction factor could be calculated and then added to or subtracted from the apparent $[\text{Na}^+]_i$ to yield a pH-corrected $[\text{Na}^+]_i$. The form of this equation was

$$[\text{Na}^+]_{(\text{CF})} = [\text{Na}^+](\Delta pH_i/\sigma) \quad (9)$$

where $[\text{Na}^+]_{(\text{CF})}$ is the correction factor, $\Delta pH_i = 7.33 - pH_{i(x)}$ [where $pH_{i(x)}$ corresponds to any pH_i within the tested range of 6.85 to 7.83], and $1/\sigma$ is a proportionality factor. Figure 5D shows linear least-squares regression fits to data points obtained experimentally at pH_i 6.85 and 7.83 and plots obtained using Eq. 9 at four different values of pH_i (6.85, 7.10, 7.60, and 7.83) for $\sigma = 3.5$. The results suggest that Eq. 9 provides a reasonable estimate of $[\text{Na}^+]_{(\text{CF})}$ at any given pH_i . Fur-

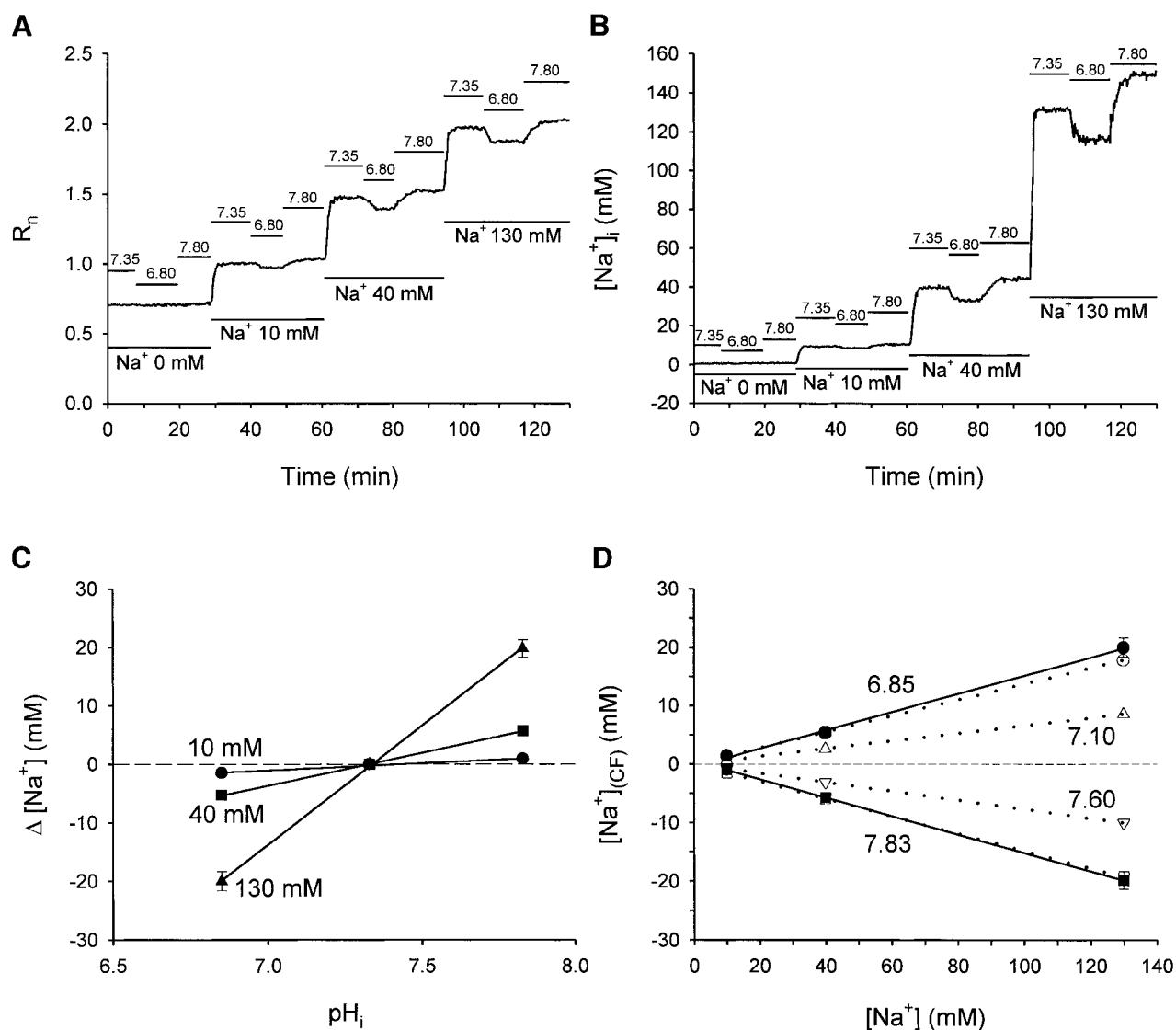


Fig. 5. $[H^+]$ sensitivity of SBFI in situ. **A**: neurons were superfused with a calibration medium containing 5 μ M gramicidin D, and $[Na^+]$ was increased sequentially from 0 to 10 to 40 to 130 mM, as indicated by the bars beneath the trace. At each $[Na^+]$, pH_o was changed from 7.35 to 6.80 to 7.80. BI_{334}/BI_{380} ratio values for each cell in the sampled population were normalized to unity at $[Na^+] = 10$ mM and $pH_o = 7.35$. The trace is a mean of data obtained simultaneously from 18 neurons on a single coverslip. **B**: the normalized BI_{334}/BI_{380} ratio values shown in **A** were transformed into $[Na^+]_i$ using the one-point procedure described in the text. **C**: apparent changes in $[Na^+]_i$ ($\Delta[Na^+]_i$) at different absolute values of $[Na^+]_i$ (10 mM, \bullet ; 40 mM, \blacksquare ; and 130 mM, \blacktriangle) are shown as a function of pH_i . For each absolute $[Na^+]_i$, values of $\Delta[Na^+]_i$ were calculated according to Eq. 8 and were obtained from 9 experiments (3 conducted at 37°C and 6 conducted at room temperature) of the type illustrated in **A**; where not shown, standard error bars are contained within the datum point. The lines shown are linear least-squares regression fits to the data points indicated for each absolute value of $[Na^+]_i$ ($r^2 > 0.99$ in each case). **D**: $[Na^+]_{(CF)}$ as a function of $[Na^+]_i$ at different pH_i values. $[Na^+]_{(CF)}$ is the value of $[Na^+]_i$ (in mM) that is required to correct the apparent $[Na^+]_i$ obtained at the pH_i values indicated for the pH sensitivity of SBFI in situ. The continuous lines are linear least-squares regression fits to data points obtained experimentally at pH_i 6.85 (\bullet) and 7.83 (\blacksquare) for $[Na^+]_i = 10, 40,$ and 130 mM in each case ($r^2 > 0.99$ in each case). Each datum point represents the mean value obtained from 6 to 9 similar experiments; where missing, standard error bars lie within the point. Dotted lines show the values for $[Na^+]_{(CF)}$ that were calculated for $pH_i = 6.85$ (\circ), 7.10 (\triangle), 7.60 (∇), and 7.83 (\square) by means of Eq. 9 and plotted as a function of absolute $[Na^+]_i$.

thermore, the linear form of Eq. 9 indicates that the percent error introduced in the estimation of $[Na^+]_i$ by a fixed change in pH_i will be constant at all values of $[Na^+]_i$. Thus, for example, if $[Na^+]_i$ has been estimated at 10 mM under conditions where pH_i is reduced by 0.2

pH units, the corrected value will be 10.57 mM {i.e., $[Na^+]_{(CF)} = 2/3.5 = 0.57$ mM}. On the other hand, at an estimated $[Na^+]_i$ of 80 mM, the same reduction in pH_i will give a $[Na^+]_{(CF)} = 4.57$ mM and the corrected $[Na^+]_i$ will be 84.57 mM.

DISCUSSION

The accurate in situ calibration of SBFI is important, not only because the spectral properties of the dye in situ differ markedly from those in vitro, but also because physiologically important changes in neuronal $[\text{Na}^+]_i$ may be small with respect to resting levels (4, 28). Although full SBFI calibrations have been fitted by a variety of different means (e.g., see Refs. 3, 13, 15, 19, 24, 29, 37), in this study we employed a three-parameter hyperbolic equation (Eq. 3) that not only provided a simple method for estimating R_{\min} and βK_d , but could also be transformed easily into the standard equation of Grynkiewicz et al. (16) to determine R_{\max} . This is advantageous, given that the high ionic strength media required for the experimental determination of R_{\max} may affect the characteristics of the fluorophore in situ (3, 23). Because 140 mM Na^+ is not the saturation point for SBFI, the equation employed in the present study also obviates the approximation made when the ratio of fluorescence intensities emitted during excitation at 380 nm for $[\text{Na}^+] = 0$ mM and ~ 140 mM are employed to derive values for β and thus the K_d of SBFI for Na^+ . We also took advantage of the insensitivity of the SBFI emission signal during excitation at 334 nm to changes in $[\text{Na}^+]$ in situ to correct the Na^+ -sensitive 380 nm signal for the drift that often occurs during the course of a full calibration experiment. The normalized drift-corrected BI_{380} signal was fitted with a three-parameter hyperbolic decay equation (Eq. 6), a procedure that facilitated the determination of β and thus the K_d of SBFI for Na^+ . Although values for R_{\max} and βK_d obtained by the aforementioned methods were similar to those derived from Hanes plots of full calibration data, the equations employed in the present study offer the advantage over previously described methods (including Hanes plots and the three-point procedure) of allowing the straightforward in situ determination of separate values for β and K_d as well as R_{\min} and R_{\max} (i.e., individual values for all the constant parameters of the standard equation). In this way, potential errors introduced by the use of K_d values obtained in vitro for the calibration of signals from experiments in intact cells can be avoided, and the effects of experimental maneuvers, such as changes in pH, on K_d values can easily be determined.

Values for R_{\min} , R_{\max} , β , and K_d estimated from three-parameter hyperbolic fits to the data points obtained in full calibrations were highly reproducible. However, a full calibration employing ≥ 8 concentrations of Na^+ at the end of an experiment may be impractical (e.g., see Ref. 13). A frequently employed alternative is the three-point method introduced by Harootunian et al. (18). Nevertheless, marked loss of signal may occur even during this less protracted procedure under some circumstances (e.g., following anoxia in mammalian neurons; unpublished observations). Given these limitations, we examined the possibility of applying a one-point technique to calibrate SBFI ratio values in situ. In this procedure, a full calibration curve is constructed (Fig. 1C, curve *a*), and

the curve is constrained to pass through the points $BI_{334}/BI_{380} = 1.0$, $[\text{Na}^+] = 10$ mM. The advantage of this normalization step is that it permits a one-point calibration for each cell studied. At the end of every experiment, cell(s) are exposed to a medium containing 10 mM Na^+ and ionophore(s), and BI_{334}/BI_{380} values from the entire experiment for a given cell are divided by the BI_{334}/BI_{380} value at $[\text{Na}^+] = 10$ mM for that cell; the normalized BI_{334}/BI_{380} values are then used to calculate $[\text{Na}^+]_i$, utilizing Eq. 2 and the appropriate fitted calibration parameters. The latter are derived from full in situ calibration experiments, which are required only when the cell type under study or optical equipment is changed. Not only does a one-point calibration offer the advantages of being simpler and faster to perform at the end of an experiment than a full or a three-point calibration, but also the accuracy of the method appears at least equivalent to that of a three-point procedure. Thus changes in $[\text{Na}^+]_i$ evoked by anoxia in rat hippocampal neurons could be estimated as precisely by the one-point technique as by the three-point procedure (Fig. 3, *B* and *C*). The utility of the one-point calibration procedure is also illustrated by the data presented in Fig. 5B, where the solution employed for the one-point calibration contained 10 mM Na^+ at pH 7.35. From this figure, it is apparent that the calculated values of $[\text{Na}^+]_i$ at pH_o 7.35 closely approximate the values of $[\text{Na}^+]_o$ employed during the course of the experiment. Thus although only a single calibration point was employed, the normalized BI_{334}/BI_{380} values obtained at values of $[\text{Na}^+]_o$ other than 10 mM were accurately transformed into appropriate values of $[\text{Na}^+]_i$. These points having been noted, it is nevertheless important to state that absolute values for $[\text{Na}^+]_i$ derived via any calibration procedure should be held with caution.

The changes in pH_i that may occur not only in response to changes in $[\text{Na}^+]_i$ but also to the ionophores employed in calibration procedures represent a potential confound to estimates of $[\text{Na}^+]_i$ made with SBFI. In the present study, the application of gramicidin D alone was found to equilibrate pH_i and pH_o , suggesting that neither monensin nor nigericin are required in calibrating media to abolish transmembrane H^+ gradients. On the other hand, given the sensitivity of SBFI ratio measurements to $[\text{H}^+]_i$ in situ (see below), the fact that the ionophore(s) employed in SBFI calibration procedures cause pH_i to equal pH_o reinforce the suggestion (23, 35) that media employed to calibrate SBFI ratio signals should be titrated to the normal mean resting pH_i of the cell type under study.

Consistent with the reported apparent negative logarithm of the acidic dissociation constant (6.09) of SBFI in vitro (26; also see Ref. 17), changes in pH in the range 6.8–7.8 exerted minimal effects on the K_d of SBFI for Na^+ measured in situ. With regard to the $[\text{H}^+]$ sensitivity of SBFI ratio measurements at a constant $[\text{Na}^+]$, acidification resulted in an apparent decrease and alkalinization in an apparent increase in $[\text{Na}^+]_i$ when $[\text{Na}^+] \geq 10$ mM, results that are in broad

agreement with those presented previously (13, 28, 29, 33, 34, 36). Although Harootunian et al. (18) failed to observe significant changes in SBFI ratios when the intracellular compartment of REF52 cells was alkalinized by 0.4 pH units, Rose and Ransom (34, 35), for example, found that at $[Na^+] = 20$ mM, 0.4-pH unit changes induced apparent changes in $[Na^+]_i$ of 3.0 ± 0.6 mM and 6.1 ± 1.5 mM in hippocampal neurons and astrocytes, respectively (acidification and alkalinization evoking decreases and increases, respectively, in apparent $[Na^+]_i$). Similarly, Nett and Deitmer (29) observed an apparent 0.77 mM decrease in $[Na^+]_i$ for a 0.3-pH unit reduction in the pH of the calibrating solution at $[Na^+] = 10$ mM in leech giant glial cells. The greater magnitude of the apparent $[Na^+]_i$ changes evoked by alterations in pH reported by Rose and Ransom (34, 35), compared with Nett and Deitmer (29), may in part reflect the observation made in the present study that the magnitude of the effect of a change in pH on apparent $[Na^+]_i$ measured with SBFI is dependent upon the absolute value of $[Na^+]_i$. However, for a given $[Na^+]_i$, the relationship between the change in pH_i and the change in apparent $[Na^+]_i$ is reasonably linear (Fig. 5C), and a simple procedure was developed to correct for pH-induced changes in apparent $[Na^+]_i$ measured with SBFI. The correction procedure described may be of use in experiments in which $[Na^+]_i$ and pH_i are measured concurrently, under which conditions $[Na^+]_i$ could be corrected for pH_i on a region-by-region or pixel-by-pixel basis (as described for simultaneous measurements of $[Ca^{2+}]_i$ and pH_i ; see Refs. 25 and 27) or in studies where SBFI fluorescence measurements are made at a known pH_i (e.g., following the application of an H^+ ionophore or during combined patch-clamp and fluorescence ratio imaging, in which the patch pipette solution contains a high concentration of H^+ buffer). Nevertheless, together, the results of the present study indicate that the effects of changes in pH_i on neuronal $[Na^+]_i$ values estimated with SBFI are relatively small and are unlikely to affect the interpretation of results under most experimental conditions.

In summary, we developed and tested simplified procedures for calibrating SBFI ratio measurements in situ. Values for all of the SBFI calibration constants are drawn from in situ measurements by means of three-parameter hyperbolic equations, and, once the parameters of the standard equation have been determined, SBFI ratios measured during the course of an experiment are transformed into $[Na^+]_i$ values by a one-point procedure applied at the end of the experiment. The results also demonstrate that the in situ pH sensitivity of SBFI ratio measurements is related linearly to $[Na^+]_i$. Although SBFI is only weakly sensitive to changes in pH in the range pH 6.8–7.8 (compared, for example, with fura 2 or indo 1; see Refs. 1, 25, and 31), correction factors can be employed, if required, to correct SBFI-derived measurements of apparent $[Na^+]_i$ for pH_i .

We thank S. Atmadja for preparation of the neuronal cultures and Dr. E. D. W. Moore for helpful comments on an early version of the manuscript.

Financial support was provided by a Grant-in-Aid from the Heart and Stroke Foundation of British Columbia and Yukon.

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