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Fluorescence Ratio Imaging of Cytosolic Free Na^+ in Individual Fibroblasts and Lymphocytes*

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New fluorescent Na^+ indicators, SBFI (short for sodium-binding benzofuran isophthalate) and SBFP (short for sodium-binding benzofuran phthalate) (Minta, A., and Tsien, R. Y. (1989) *J. Biol. Chem.* 264, 19449–19457), were tested in Jurkat tumor lymphocytes and in REF52 rat embryo fibroblasts. Both dyes could be introduced by direct microinjection. However, when cells were incubated with the tetra(acetoxymethyl) esters of the dyes, only SBFI gave intracellular loading that was reasonably responsive to $[Na^+]_i$. Because some compartmentation of the SBFI was visible and because the indicator properties are somewhat affected by cytoplasm, the relationship between intracellular free Na^+ ($[Na^+]_i$ and the 340/385 nm excitation ratio of the indicator was calibrated *in situ* using pore-forming antibiotics to equilibrate cytosolic $[Na^+]$ ($[Na^+]_i$) with extracellular $[Na^+]$. The excitation ratio was sufficiently sensitive to resolve small changes, ≤1 mM, in $[Na^+]_i$ in single cells. Basal $[Na^+]_i$ values in lymphocytes and serum-starved fibroblasts were 9.4 and 4.2 mM, respectively. As expected, large increases in $[Na^+]_i$ were elicited by blocking the Na^+ pump with ouabain or withdrawal of extracellular K^+. Mitogens such as phytohemagglutinin acting on the lymphocytes, or serum or vasopressin in fibroblasts, caused $[Na^+]_i$ to increase up to 2-fold. In fibroblasts, the rise in $[Na^+]_i$ was due at least partly to a stimulation of Na^+ influx, which was not wholly through the Na^+/H^+ exchanger. The mitogen-induced increases in $[Na^+]_i$ and the rate of Na^+ influx are consistent with earlier estimates based on measurements of total $[Na^+]$ or tracer fluxes. However, the absolute values for free $[Na^+]_i$ are much lower than previous values for total intracellular Na^+, suggesting that much of the latter is bound or sequestered.

The cytosolic activity of sodium, or concentration of free sodium, $[Na^+]_i$,[1] is of great significance in many biological processes including action potentials and synaptic potentials in excitable cells, secondary active transport processes such as Na^+/H^+ exchange, Na^+/Ca^{2+} exchange, Na^+-coupled uptake of nutrients, and regulation of cell volume. Speculative hypotheses have linked defects in Na^+ homeostasis with such varied pathologies as hypertension (Insel and Motulsky, 1984; Garay *et al.*, 1987), injury by pore-forming toxins (Bashford *et al.*, 1988), and malignant transformation (Liebling and Gupta, 1987; Jansson, 1986). Thus knowledge of $[Na^+]_i$ is of fundamental importance in assessing many physiological processes occurring within a cell. Many techniques for measuring cellular sodium such as flame photometry (Mendoza and Rozengurt, 1987), ^{22}Na tracer experiments (Mendoza and Rozengurt, 1987), or electron microprobe analysis (Abraham *et al.*, 1985) measure total Na^+ not free Na^+, are destructive assays, and require either careful removal of extracellular Na^+ without loss of intracellular Na^+ or prevention of Na^+ migration during sample preparation for electron microscopy. Nuclear magnetic resonance of ^{23}Na (Springer, 1987) is nondestructive but demands large quantities of tissue, long acquisition times, expensive instrumentation, and exposure of the tissue to lanthanide shift reagents. Na^+-sensitive microelectrodes (Thomas, 1978) are limited to relatively large cells that can withstand penetration with two electrode barrels; any leak at the site of puncture tends to let extracellular Na^+ enter and raise the observed $[Na^+]_i$. Fluorescent indicators combined with digital imaging have provided a nondestructive, fast responding method for measuring other cations such as Ca^{2+} or H^+ (Tsien and Poenie, 1986; Tsien, 1988, 1989); if a good indicator were available for Na^+, this powerful technology should be able to quantify $[Na^+]_i$ simultaneously in many individual cells or subcellular regions.

In this article we report the use of newly developed fluorescent Na^+ indicators, SBFP and SBFI, in fibroblast and lymphocyte cell lines. The indicators respond appropriately to changes in $[Na^+]_i$ brought about by gross manipulations such as blockage of the plasma membrane Na^+/K^+ pump. They are also sensitive enough to detect changes on $[Na^+]_i$ and Na^+ flux which occur upon mitogenic activation of cells. Thus serum or vasopressin but not phorbol esters increase $[Na^+]_i$ of fibroblasts of the Fischer rat embryo cell line REF52. This increase is due to a stimulation of Na^+ influx but is not entirely attributable to Na^+/H^+ exchange. Similarly, stimulation of Jurkat T lymphoma cells with mitogenic lectins also elicits increases in $[Na^+]_i$.

MATERIALS AND METHODS

Fischer rat embryo fibroblasts of the cell line REF52 (Logan *et al.*, 1981; a gift of J. Feramisco, Cancer Center, University of California, San Diego) were grown in Dulbecco's Modified Eagle Medium (DME) supplemented with 10% (v/v) calf serum, 50 units/ml penicillin, and 50 μg/ml streptomycin. Cells were detached by exposure to trypsin-EDTA solution, seeded onto 25-mm glass coverslips, and cultured for 3–5 days before use. Jurkat lymphocytes (Goldsmith and Weiss, 1988;

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[1] The abbreviations used are: $[Na^+]_i$, cytosolic free Na^+ concentration; $[Ca^{2+}]_i$, cytosolic free Ca^{2+} concentration; pH_i, cytosolic pH; DME, Dulbecco's Modified Eagle Medium; HBS, Hanks' Balanced Salt Solution; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; PHA-P, phytohemagglutinin; PDGF, platelet-derived growth factor; EGTA, [ethylenebis(oxyethylenenitrilo)]tetraacetic acid; SBFP, sodium-binding benzofuran phthalate; SBFI, sodium-binding benzofuran isophthalate; SBFO, sodium-binding benzofuran oxazole; AM, acetoxymethyl ester; BCECF, 2′,7′-bis(carboxyethyl)-5(6)-carboxyfluorescein.

a gift of Dr. A. Weiss, University of California, San Francisco) were grown in RPMI 1640 medium supplemented with 10% (v/v) fetal calf serum, 50 units/ml penicillin, and 50 μg/ml streptomycin. The lymphocytes were grown to densities of 10^6 cells/ml before use.

Cells were loaded with the sodium indicator either by microinjection of the free acid (fibroblasts only) or by hydrolysis of the membrane-permeant acetoxymethyl esters. Injection solutions contained either 27 mM K_2HPO_4, 8 mM NaH_2PO_4, 26 mM KH_2PO_4, and 2–10 mM dye (SBFP or SBFI), or 10 mM SBFI potassium salt and 10 mM K-Hepes. Both types of solution were titrated to pH 7.3 and injected according to previously described methods (Harootunian *et al.*, 1988); the hypotonic medium without phosphate gave better results and became preferred. For ester loading, REF52 cells were incubated for 1–3 h with 2–10 μM tetra(acetoxymethyl) ester of the dye in Hepes-buffered DME media at room temperature (25 °C), whereas the Jurkat cells were incubated with similar ester concentrations for 40–60 min in an incubator containing 5% CO_2 at 37 °C. The ester was dissolved in dimethyl sulfoxide at a concentration of 5 mM and mixed with an equal volume of 25% w/v Pluronic F-127 (BASF Corp., Wyandotte, MI): dimethyl sulfoxide just before dilution into the culture medium containing the cells. Pluronic F-127 is a nonionic surfactant that has proven valuable in delivering many hydrophobic dyes including fura-2/AM to cells (Poenie *et al.*, 1986; Cohen *et al.*, 1974); premixture with Pluronic was essential for successful loading of the SBFI. The cells were washed twice in Hanks' balanced salt solution (HBS) and then transferred to Petri dishes with No. 1 glass coverslip windows for microscopic observation or quartz cuvettes for spectroscopic analysis. As the acetoxymethyl groups hydrolyze, the 340/385 nm excitation ratio gradually rises. Cells were usually kept in ester-free saline for an hour to allow completion of hydrolysis, judged by attainment of a stable 340/385 nm ratio and by Na^+ titration of indicator recovered from lysed cells as described under "Results."

The sodium indicators used were originally synthesized in this laboratory by Dr. Akwasi Minta (see Minta and Tsien (1989), accompanying paper) and subsequently were obtained from Molecular Probes, Inc. (Eugene, OR). BCECF/AM was also obtained through Molecular Probes, Inc. All media, sera, and antibiotics used in cell culture were purchased from GIBCO. [Arg^8]Vasopressin and amphotericin B were obtained from Sigma. Nigericin, monensin, and ionomycin were produced by Calbiochem. Gramicidin D was purchased from Boehringer Mannheim. The porcine PDGF used was a product of R&D Systems, Inc. (Minneapolis, MN).

Fluorescence Instrumentation—A Spex Industries (Edison, NJ) Fluorolog system, modified to permit excitation ratioing (Tsien *et al.*, 1985), was used for spectroscopic measurements of both calibration solutions and cell suspensions. Digital imaging of Na^+ indicator fluorescence in individual cells at excitation wavelengths of 340 and 385 nm was performed on a ratio imaging system previously described (Poenie *et al.*, 1986; Ambler *et al.*, 1988). In order to standardize ratios against interlaboratory or day-to-day differences in the relative illumination intensities at 340 and 385 nm, all 340/385 nm excitation ratios were normalized with respect to that obtained the same day from a thin sample of 1 mM fura-2 in an essentially Ca^{2+}-free solution containing 100 mM EDTA and 20 mM Hepes at pH 7.3, sandwiched between two glass coverslips. Excitation ratios were averaged over 8-μm diameter circles in the center of the cell, avoiding regions of the cell which appeared punctate or compartmentalized. Analysis of the latter regions of the cells will be discussed below.

Calibration of $[Na^+]_i$—Calibration of the excitation ratio in terms of $[Na^+]_i$ was accomplished *in situ* by application of gramicidin D, amphotericin B, or a combination of monensin and nigericin. Gramicidin or amphotericin produce transmembrane pores that are highly conductive and selective for monovalent cations but that do not distinguish markedly between Na^+ and K^+. Prior evidence (Rink *et al.*, 1980; Medoff, 1983) suggests that when applied to intact cells, both these very hydrophobic antibiotics mainly affect the plasma membrane, not internal organelles. Therefore gramicidin rapidly equilibrates internal $[Na^+]$ and $[K^+]$ with external concentrations. Monensin and nigericin mediate Na^+/H^+ and K^+/H^+ exchange, respectively, and together should also abolish Na^+ and K^+ concentration gradients across all membranes. Exposure of the cells to various mixtures of sodium gluconate and potassium gluconate in the presence of these ionophores permits calibration of 340/385 nm excitation ratios in terms of $[Na^+]_i$ using techniques analogous to those used to calibrate fluorescent pH indicators *in situ* (Paradiso *et al.*, 1987). Observed values of the 340 nm/385 nm excitation ratio R at three different $[Na^+]_i$ values were needed to solve for the three unknown parameters, R_0, R_∞, and (K_dS_{f2}/S_{b2}) in the standard equation (Grynkiewicz *et al.*, 1985):

$$[Na^+]_i = (K_dS_{f2}/S_{b2})\cdot(R - R_0)/(R_\infty - R)$$

for indicators of 1:1 stoichiometry. In the above equation, R represents the ratio of fluorescence intensities at 340 and 385 nm excitation, R_0 is the ratio at 0 Na^+, R_∞ is the ratio at saturating Na^+, K_d is the Na^+ dissociation constant, and S_{f2}/S_{b2} is the ratio of the excitation efficiencies of free indicator to Na^+-bound indicator at 385 nm. If R_1, R_2, and R_3 are the observed ratios at $[Na^+]_i = C_1$, C_2, and C_3, respectively, then algebra shows that:

$$R_0 = [R_1R_2C_3(C_1 - C_2) + R_2R_3C_1(C_2 - C_3) + R_3R_1C_2(C_3 - C_1)]/[R_1C_1(C_3 - C_2) + R_2C_2(C_1 - C_3) + R_3C_3(C_2 - C_1)]$$

$$R_\infty = [R_1R_2(C_2 - C_1) + R_2R_3(C_3 - C_2) + R_3R_1(C_1 - C_3)]/[R_1(C_2 - C_3) + R_2(C_3 - C_1) + R_3(C_1 - C_2)]$$

$$(K_dS_{f2}/S_{b2}) = C_i(R_\infty - R_i)/(R_i - R_0) \text{ for } i = 1, 2, \text{ or } 3.$$

It should be noted that $[Na^+]_i$ calibration is much more difficult in free-floating than in adherent cells, because the process requires several complete changes of solutions with varying Na^+:K^+ ratios. Whereas extracellular Ca^{2+} or H^+ can be removed from a cuvette by small additions of concentrated EGTA or base solutions, removal of Na^+ or K^+ requires exchange of solutions.

Na^+ calibration solutions were made from appropriate mixtures of high Na^+ and high K^+ solutions. The former contained 110 mM sodium gluconate, 30 mM NaCl, 1.2 mM $CaCl_2$, 0.6 mM $MgCl_2$, 10 mM Na-Hepes; the high K^+ solution was identical except for complete replacement of Na^+ by K^+. The low chloride contents were chosen to prevent cell swelling, which was observed under transmitted light microscopy when cells were calibrated with solutions containing 144 mM Cl^- and no gluconate. The choice of 30 mM $[Cl^-]$ was intended to approximate the intracellular chloride levels of the cells (Iversen, 1976). If the concentrations of semipermeant anions (*e.g.* Cl^-) and impermeant anions (proteins *versus* gluconate) are balanced across the plasma membrane, any Donnan effect should disappear, so that the equilibrium achieved by the ionophores should represent true equalization of intracellular and extracellular $[Na^+]$.

RESULTS

Preliminary Tests of SBFP and SBFO—We started by preparing the acetoxymethyl esters of SBFP and SBFO (Minta and Tsien (1989), accompanying paper) and trying to use them to load the indicators into intact rabbit gastric glands (in collaboration with A. M. Paradiso and T. E. Machen), murine EL4 lymphoma cells, and REF52 fibroblasts. All three cell types readily took up fluorescence, which unfortunately was not properly Na^+-sensitive. Even when the cells were lysed and the lysate titrated with NaCl, the spectral shift due to Na^+ was much smaller than for true indicator and required much higher Na^+ concentrations, 0.1–1 M instead of 10–50 mM Na^+. Prolonged digestion of the lysate with tetramethylammonium hydroxide largely restored the correct spectral behavior, suggesting that the terminal carboxyl groups were incompletely de-esterified or otherwise covalently linked. Although these carboxyls do not themselves participate in binding the Na^+ in the cavity of the crown ether, ester or amide formation would exert some electron-withdrawing effect through the conjugated system and weaken cation binding. Thin layer chromatography of SBFP/AM-loaded cell pellets showed considerable fluorescent material that remained immobile at the base line under elution conditions that caused the ester, free acid, and all intermediates to migrate. We therefore suspected that the SBFP was becoming covalently bound to cell macromolecules, perhaps through amide linkages resulting from transient formation of phthalic anhydride intermediates. Microscopic examination of SBFP/AM-loaded REF52 fibroblasts supported this interpretation, since much of the fluorescence resisted digitonin lysis, although it could eventually be removed by high concentrations of the detergent Triton X-100. In contrast, direct microinjec-

tion of SBFP salt into REF52 cells showed that the dye could behave sensibly once properly introduced. Some preferential staining of the nucleus and some perinuclear reticulum was still observed; since the nuclear and diffuse cytoplasmic dye but not the reticular staining rapidly diffused away from the cell after membrane permeabilization with digitonin, the easiest part of the cell to monitor was the nucleus. Fig. 1 depicts $[Na^+]_i$ levels in a cell injected with SBFP. The initial level of $[Na^+]_i$ was high due to damage from the injection, performed in normal extracellular $[Na^+]$, but was soon reduced to a level of less than 10 mM as the cell recovered from the injection damage. It should be noted that injections of indicator with much quicker recovery from injection could be attained with practice. Upon removal of external K^+, $[Na^+]_i$ increased markedly due to blockage of the Na^+/K^+-ATPase. The pump blockage was then reversed by returning external K^+ to the solution. $[Na^+]_i$ increased again when external K^+ was removed a second time. Thus, the effect of simple manipulation of the sodium pump upon $[Na^+]_i$ can be observed using microinjected indicator SBFP; however, microinjection is much more injurious and laborious than ester loading.

SBFI was designed on the hypothesis that the problems encountered in loading of SBFP/AM ester and the labeling of perinuclear compartments might be due to the ability of the adjacent carboxyls of SBFP to form a cyclic anhydride, which would make the molecule more permeable through membranes and enable covalent labeling. Moving the carboxyls further apart would prevent such unwanted chemical behavior. Indeed, SBFI/AM has proven to load a wide variety of cells with Na^+-responsive indicator, although microscopy still reveals some compartmentation in certain cell types.

SBFI Properties and Calibration in Jurkat Lymphocytes—Excitation spectra of Jurkat cells in suspension were examined in order to study the properties of the indicator in cells. The cells were examined both in normal saline and in solutions which would clamp $[Na^+]_i$ to a fixed value. As shown in Fig. 2*A*, the excitation spectra were sensitive to changes in $[Na^+]_i$, although these spectra differed markedly from excitation spectra of indicator in standard buffer solutions. The excitation maxima were shifted to slightly longer wavelengths, and the quantum efficiency of intracellular dye appeared to be considerably higher than in simple calibration solutions, since release of dye caused a large overall drop in fluorescence.

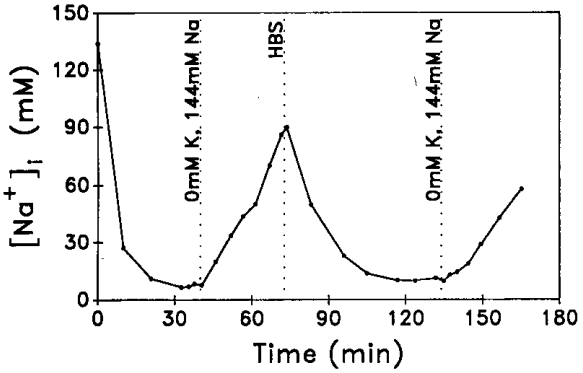

FIG. 1. **SBFP reveals $[Na^+]_i$ increase following blockage of the Na^+ pump by removal of extracellular K^+.** $[Na^+]_i$ was elevated following microinjection of a cell with SBFP but subsequently returned to a steady low resting level as the cell recovered from impalement injury. Removal of extracellular K^+ resulted in a steady increase of $[Na^+]_i$. Return of extracellular K^+ allowed the Na^+ pump to function, resulting in a decrease of $[Na^+]_i$. Removal of extracellular K^+ a second time led again to a steady $[Na^+]_i$ increase. Values for R_0, R_∞, and $(K_d S_{f2}/S_{b2})$ used to calibrate SBFP ratios in terms of $[Na^+]_i$ were determined from other cells injected with SBFP.

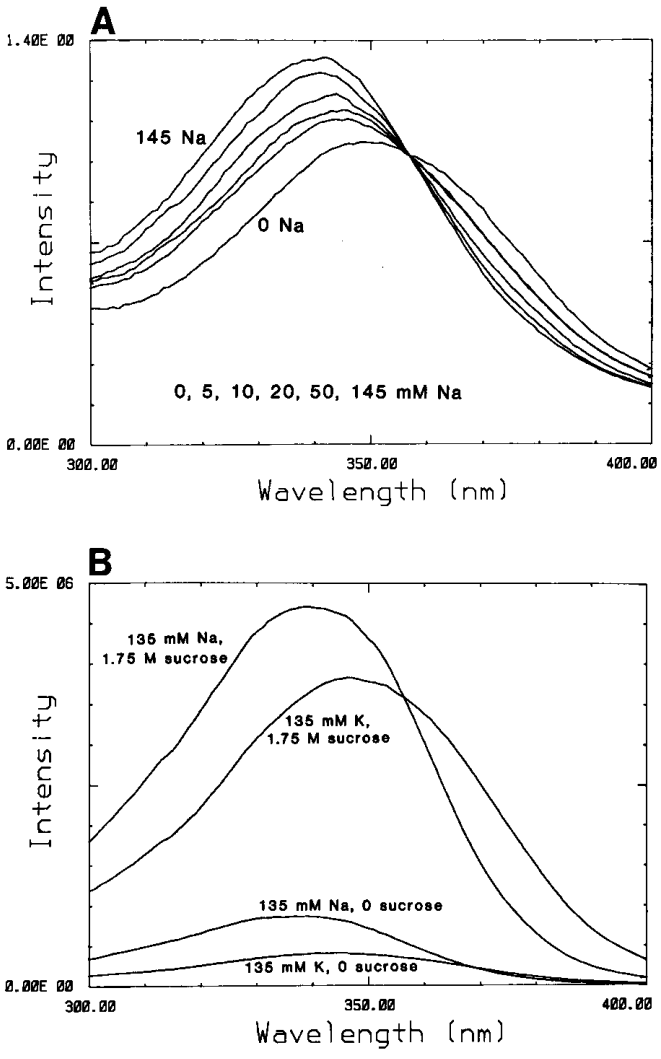

FIG. 2. **Excitation spectra of SBFI.** *A*, excitation spectra of SBFI inside Jurkat lymphocytes titrated in the presence of 1 μM gramicidin with $[Na^+] + [K^+] = 145$ mM at 0, 5, 10, 20, and 50 mM $[Na^+]$. 1 ml of cells at a density of 10^6 cells/ml was incubated with 10 μM SBFI/AM for 1 h at 37 °C in an atmosphere of 5% CO_2. Spectra are normalized to 357 nm excitation. Spectra were taken at 32 °C with an excitation bandwidth of 4.6 nm and emission collected at 500 nm with 19 nm bandwidth. *B*, the effect of different viscosities on excitation spectra of SBFI. Spectra were taken at 135 mM Na^+, 0 mM K^+ and 0 mM Na^+, 135 mM K^+ both in the presence and absence of 1.75 M sucrose. In all cases, the SBFI concentration was 5 μM, the excitation bandwidth was 1.9 nm, and the emission was collected at 500 nm with 9.3 nm bandwidth.

Also, the ability of Na^+ to increase the amplitude of the fluorescence peak was less pronounced inside cells, showing that the quantum efficiencies with and without Na^+ had become more nearly equal. The differences between dye properties in cells from those in buffer solutions were not due to incomplete hydrolysis of the AM ester groups, since titration of lysates showed that dye released from cells had properties essentially identical to never esterified free acid. These effects are somewhat analogous to but more severe than those of cytoplasm on the Ca^{2+} indicator fura-2 (Poenie *et al.*, 1986), and they may again be explainable by the greater microviscosity of cytoplasm compared to simple aqueous solutions. Indeed, these effects can be well simulated simply by the addition of sucrose to increase the viscosity. Fig. 2*B* depicts the excitation spectra of SBFI in 0 and 135 mM Na^+ with and without 1.75 M sucrose present. The excitation maxima, iso-

excitation wavelength, and enhancement of quantum efficiency for the dye in 1.75 M sucrose are quite similar to those for SBFI in Jurkat lymphocytes at 0 and 144 mM $[Na^+]_i$ (Fig. 2*A*). By comparison of the SBFI spectrum in Jurkat lymphocytes in normal medium with the SBFI spectra from cells permeabilized in media with various extracellular Na^+ levels, basal $[Na^+]_i$ of the Jurkat cells appeared to be about 10 mM.

SBFI fluorescence and $[Na^+]_i$ levels could also be monitored at the single cell level by video imaging. Fig. 3, *A–I*, shows a field of Jurkat lymphocytes viewed at 340 and 385 nm excitation and the pseudocolor ratio images as $[Na^+]_i$ was clamped successively at 0, 10, and 50 mM. Increasing ratios of 340 to 385 nm excitation efficiency are coded as pseudocolors ranging from *blue* through the visible spectrum to *magenta*. With increasing $[Na^+]_i$, the intensity at 340 nm increased while that at 385 nm excitation decreased, so that the 340/385 nm ratio increased as displayed by the *yellow* and *red* pseudocolors. Fig. 3*J* presents the calibration more quantitatively for ten of the cells in the field. The 340/385 intensity ratio fell as extracellular Na^+ was washed out and the plasma membrane was made permeable to Na^+ by the addition of ionophores. As the extracellular solution was changed to higher levels of Na^+ (5, 10, 20, and 50 mM) in the continued presence of the ionophores, the 340/385 intensity ratio rose to successively higher plateaus. Comparison of the ratios before and after permeabilization shows that the resting $[Na^+]_i$ value in the Jurkat cells appeared to be 9.4 mM with a standard deviation of 2.0 mM and a standard error of 0.7 mM. This value is in good agreement with the cuvette measurements, but during contact of the Jurkat cells for tens of minutes with the glass bottom of the chamber, $[Na^+]_i$ gradually rose. Independent measurements show that $[Ca^{2+}]_i$ also rises during prolonged contact with bare glass,[2] suggesting that the receptors on these cells may become activated by adherence to glass. Effects of $[Na^+]_i$ were also observed with a conventional mitogen, the plant lectin phytohemagglutinin (PHA-P). To prevent the effects of bare glass, the coverslip was coated with a thin layer of agarose onto which the cells were allowed to settle. Control experiments[2] have shown that cells are not activated by agarose support. Gentle addition of PHA-P resulted in a detectable $[Na^+]_i$ increase, as shown in Fig. 3*K*. However, a full calibration was not possible, since the cells did not adhere well enough to the agarose to permit multiple changes of solution. If the ratios are calibrated using the data from Fig. 3*J*, the $[Na^+]_i$ appears to rise from 5 to 12 mM, although the absolute values are less certain because the calibration was from a different experimental session.

SBFI Properties and Calibration in REF52 Fibroblasts—Calibration of $[Na^+]_i$ in adherent cell lines was more straightforward than in free-floating cells since the adherent cells could be washed multiple times with calibration solutions during the course of an experiment without significant cell movement or damage. Pseudocolor images of 340/385 nm excitation ratios of a field of REF52 fibroblasts grown on glass are shown in Fig. 4*A*. Once again the 340/385 intensity ratio decreased when extracellular Na^+ was removed and the cells were clamped to 0 mM $[Na^+]_i$ with ionophores, then increased as $[Na^+]_i$ was raised again stepwise to 5, 10, 20, and 50 mM. Clearly the unperturbed cells show a ratio corresponding to 5 mM $[Na^+]_i$ or even somewhat less.

An additional benefit of imaging is the ability to examine differences between subcellular domains and look for either dye compartmentation or $[Na^+]_i$ gradients. As seen in Fig. 5, the 340/385 intensity ratio appeared to be higher in the nuclear region than in the cytoplasmic region when the cells

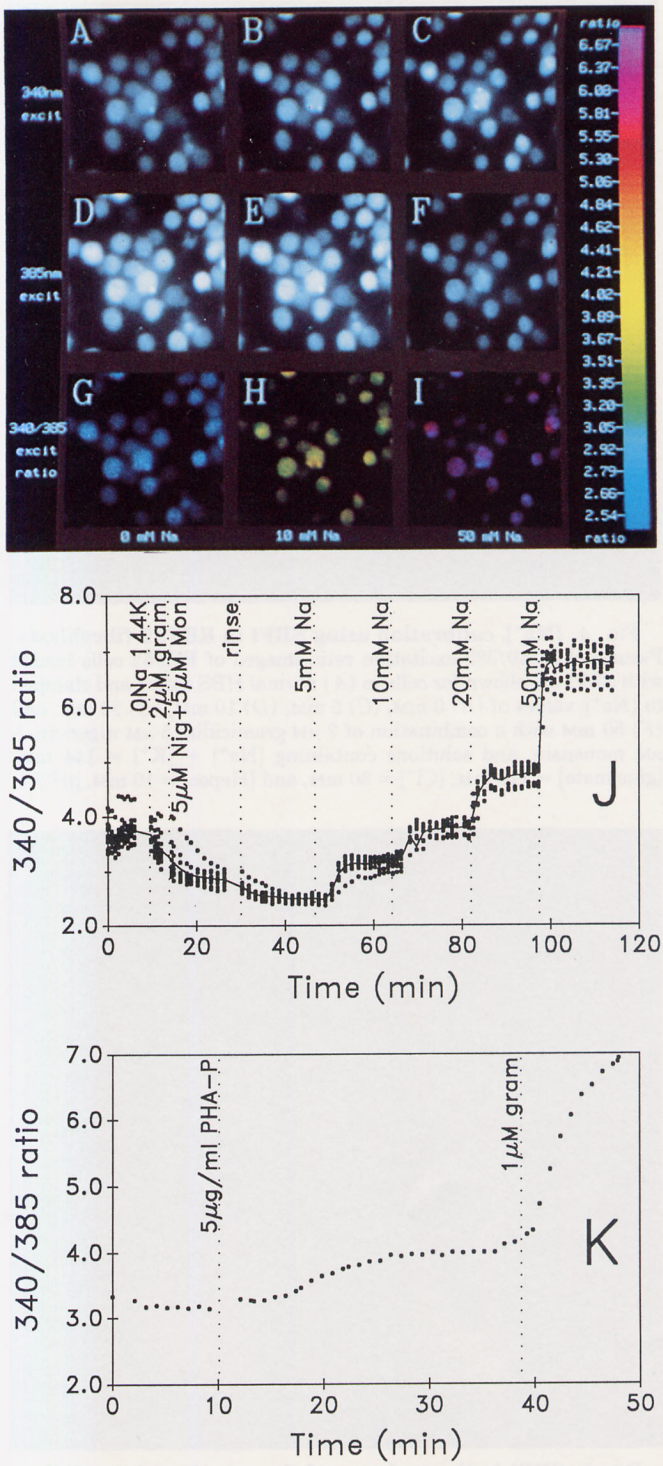

FIG. 3. **$[Na^+]_i$ calibration using SBFI in Jurkat lymphocytes.** Fluorescence video microscopic images of lymphocytes loaded with SBFI. $[Na^+]_i$ was clamped with 2 μM gramicidin, 5 μM nigericin, and 5 μM monensin to 0 mM (*A*, *D*, and *G*), 10 mM (*B*, *E*, and *H*), and 50 mM (*C*, *F*, and *I*). *A*, *B*, and *C* are fluorescence images of a field of cells excited at 340 nm, while *D*, *E*, and *F* show the same cells excited at 385 nm. The corresponding 340/385 nm excitation ratio images in pseudocolor representation are shown in *G*, *H*, and *I*. The relation between pseudocolor hues and 340/385 nm excitation ratios (normalized to 1.0 for Ca^{2+}-free fura-2) is shown on the *right hand color scale*. A plot of the 340/385 nm excitation ratio of 10 of the same cells as in *A–I* during the course of the complete experiment is shown in *J*, where cells initially in HBS were clamped with 2 μM gramicidin, 5 μM nigericin, and 5 μM monensin at 0, 5, 10, 20, and 50 mM $[Na^+]_i$. The *solid line* indicates the mean. A plot of the average 340/385 nm excitation ratio for 25 SBFI-loaded Jurkat lymphocytes resting on an agarose-coated coverslip and treated with 5 μg/ml PHA-P followed by 1 μM gramicidin is presented in *K*.

[2] G. Zlokarnik, personal communication.

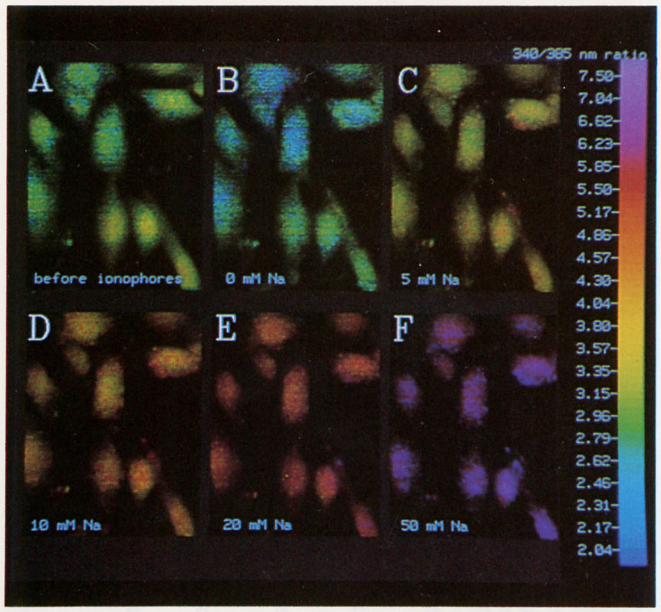

FIG. 4. **$[Na^+]_i$ calibration using SBFI in REF52 fibroblasts.** Pseudocolor 340/385 excitation ratio imaged of REF52 cells loaded with SBFI are shown for cells in (*A*) normal HBS saline and clamped to $[Na^+]_i$ values of (*B*) 0 mM, (*C*) 5 mM, (*D*) 10 mM, (*E*) 20 mM, and (*F*) 50 mM with a combination of 2 μM gramicidin, 5 μM nigericin, 5 μM monensin, and solutions containing $[Na^+] + [K^+] = 144$ mM, [gluconate] = 110 mM, $[Cl^-] = 30$ mM, and [Hepes] = 10 mM, pH 7.4.

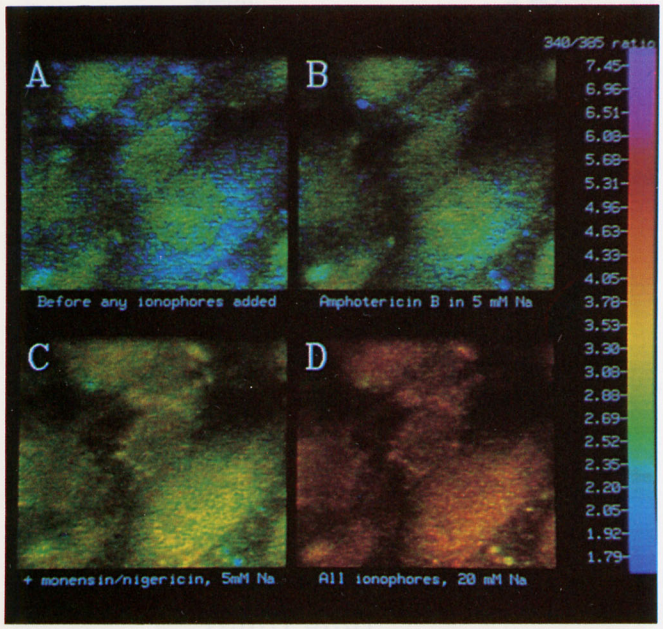

FIG. 5. **SBFI in the nucleus and the cytoplasm have different appearances as revealed by fluorescence imaging.** Nonuniform spatial distribution of 340/385 nm excitation ratios of REF52 cells loaded with SBFI can be reduced with ionophores. Pseudocolor 340/385 nm excitation ratio images are shown for cells in (*A*) HBS; and clamped to different $[Na^+]_i$ levels with low chloride (30 mM Cl^-, 110 mM gluconate, 10 mM Hepes, pH 7.4) solutions containing (*B*) 5 mM Na^+, 139 mM K^+, 1 μM amphotericin B; (*C*) 5 mM Na^+, 139 mM K^+, 1 μM amphotericin B, 4 μM gramicidin, 20 μM nigericin, and 20 μM monensin; and (*D*) 20 mM Na^+, 124 mM K^+, 1 μM amphotericin B, 4 μM gramicidin, 20 μM nigericin, and 20 μM monensin.

were in normal saline (*A*) or after they were permeabilized with amphotericin B (*B*), an antibiotic that forms pores restricted to the plasma membrane (Medoff, 1983). However, addition of the ionophores nigericin and monensin, which should distribute into both surface and intracellular membranes, reduced or eliminated the ratio difference between intracellular domains (*C* and *D*).

Further experiments were performed to ascertain the basis for the difference in 340/385 intensity ratios between the nuclear region and the rest of the cell. Artifacts of imperfect ester hydrolysis did not seem to be responsible, since the ratio differences were also present in REF52 cells directly microinjected with SBFI K^+ salt. Perturbation of SBFI by equilibrium binding to nuclear constituents would not account for the ability of the monensin-nigericin combination to collapse the discrepancy of ratio. Moreover, treatment of cells with digitonin, a detergent relatively specific for the plasma membrane, released 95% of the indicator fluorescence from nuclear regions compared to only 73% of that from cytoplasmic regions of the eight REF52 cells examined. This finding of greater dye compartmentalization in the cytoplasmic region suggested that the dye might be entering organelles. Many organelles such as lysosomes, the Golgi complex, coated vesicles, endosomes, or secretory vesicles (Mellman *et al.*, 1986), are known to have acidic interiors, with pH values as low as 4.6–5.0. Such a low pH would significantly protonate SBFI, probably both on the macrocyclic nitrogens as well as on the terminal carboxylates, and would be expected both to perturb the fluorescence spectrum and to reduce the Na^+ affinity. Fig. 6*A* shows an experiment to test the effect of very low pH on intracellular dye. Lowering the extracellular pH to 4.9 in the presence of gramicidin caused the 340/385 nm ratio to fall well below the values obtained at neutral pH and to be relatively insensitive to Na^+. If the low ratios in cytoplasmic regions of normal cells are due to partial compartmentation into acidic organelles, then neutralization of those compartments should reduce the perturbation. This prediction was tested by applying the weak base chloroquine, which should primarily raise the pH of acidic compartments (de Duve, 1983), as distinct from monensin and nigericin, which should eliminate intracellular gradients of Na^+ and K^+ as well as pH. As shown in Fig. 6*B*, the addition of chloroquine decreased the ratio difference between the nucleus and cytoplasm. Chloroquine was effective in abolishing the difference between the nucleus and the cytoplasm in four out of six cells examined. Since chloroquine was slightly fluorescent, we also used NH_4Cl to alkalinize the acidic compartments. Treatment of cells with 20 mM NH_4Cl also had the result of reducing the difference in ratio between the nucleus and cytoplasm in four out of four cells examined. A final piece of evidence correlating SBFI compartmentation with acidic organelles was that the punctate component of SBFI fluorescence typically colocalized with acridine orange staining outside the nucleus and that both became more pronounced when the fibroblast cultures became confluent. Thus it appears that compartmentalization of SBFI in acidic compartments provides an explanation for some of the difference in ratio between the nucleus and cytoplasm of cells. The origin of the remainder of the difference between intracellular domains is unclear. Autofluorescence could be ruled out, since the fluorescence of both the nuclei and the cytoplasm of unloaded cells was negligible compared to those of loaded cells. Since directly microinjected dye also showed differences in ratio between nucleus and cytoplasm, ester loading was not entirely to blame. Perhaps environmental factors such as viscosity or dye binding to macromolecules contributed to the differences in calibration curves. If so, measurement of fluorescence anisotropies might be informative.

As shown in Fig. 7, $[Na^+]_i$ was calibrated in both the nuclear and cytoplasmic domains of REF52 cells in normal saline

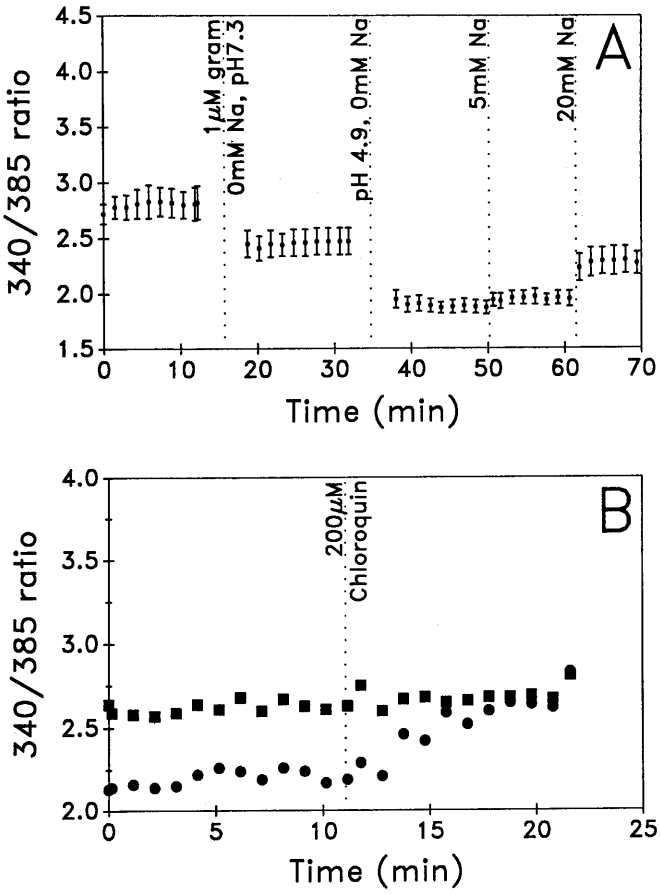

FIG. 6. **SBFI 340/385 nm excitation ratio is altered in acidic intracellular environments.** *A*, 340/385 excitation ratios of REF52 cells loaded with SBFI are shown in normal saline at pH 7.4 and in low chloride solutions containing 1 μM gramicidin and 0 mM Na^+, pH 7.4; 0 mM Na^+, pH 4.9; 5 or 20 mM Na^+, pH 4.9. The mean 340/385 nm excitation ratio is plotted with the *error bars* representing the standard deviation of the 10 cells examined. *B*, 340/385 nm excitation ratio taken from cytoplasmic (*circles*) and nuclear (*squares*) regions of a single cell before and after treatment with 200 μM chloroquine.

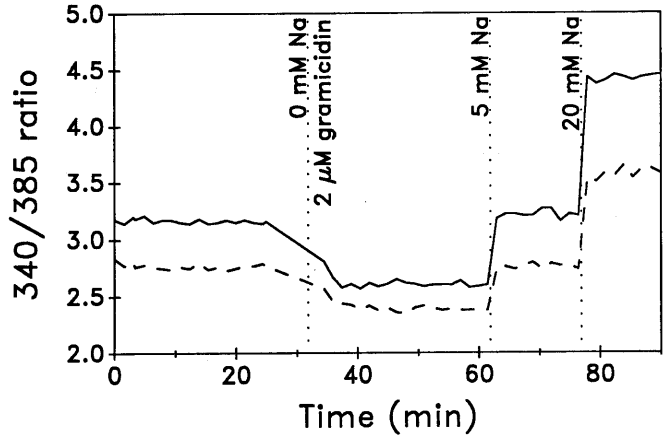

FIG. 7. **Comparison of 340/385 nm excitation ratios from different intracellular locales of REF52 cells loaded with SBFI.** Excitation ratios from cytoplasmic (*dashed line*) and nuclear (*solid line*) regions of an individual cell in HBS and clamped to specific $[Na^+]_i$ levels with low chloride saline solutions containing 2 μM gramicidin and 0, 5, or 20 mM Na^+.

solution in order to determine whether a true difference in $[Na^+]$ contributed to the difference in intensity ratio between the two domains. Despite the offset between the two calibrations, the value for $[Na^+]_i$ was found to be about 5 mM in both regions of cells in normal saline when calibrated with solutions containing 0, 5, and 20 mM extracellular Na^+ and 2 μM gramicidin. Calibrations using nigericin and monensin in addition to or in place of gramicidin resulted in lower apparent values for $[Na^+]_i$ in the cytoplasm. Thus, it is probable that more accurate calibrations of cytosolic $[Na^+]_i$ may be obtained when using gramicidin or amphotericin, since they should permeabilize only the plasma membrane (Rink *et al.*, 1980) and thereby affect mainly cytosolic dye, whereas monensin and nigericin should additionally perturb the dye in acidic compartments.

The effect of varying amounts of extracellular $[K^+]$ and $[Cl^-]$ upon the $[Na^+]_i$ calibration was also examined, since the cuvette data indicate that SBFI is somewhat sensitive to $[K^+]$ in calibration media. As shown in Fig. 8*A* for REF52 cells permeabilized to monovalent cations with ionophores, no change in ratio was seen when the external K^+ concentration was changed in 10 mM steps while large changes in ratio in response to 10 mM Na^+ were easily observed. A more stringent test of the insensitivity of the dye to changes in $[K^+]_i$ is shown in Fig. 8*B* which shows a lack of indicator response to additions of 40 mM KCl but a high degree of indicator sensitivity to Na^+ additions of 4 and 5 mM. Insensitivity to changes in extracellular chloride is also implied by these data since KCl and not potassium gluconate was used to change the K^+ values in this instance. An additional experiment shown in Fig. 8*C* shows that when Cl^- was exchanged for gluconate, no change in the $[Na^+]_i$ calibration is seen at 5 mM extracellular $[Na^+]$ with 1 μM gramicidin present.

Responses of Fibroblast $[Na^+]_i$ to Mitogenic Stimuli—Once SBFI was shown to be able to monitor $[Na^+]_i$, it was of interest to examine changes in $[Na^+]_i$ elicited by mitogens or hormones. Fig. 9 depicts the response of a population of cells to serum stimulation. The average value of $[Na^+]_i$ initially was 4.2 mM when each cell was calibrated individually; the standard deviation of $[Na^+]_i$ was 1.0 mM for the eight cells. Upon addition of 5% fetal calf serum $[Na^+]_i$ increased steadily over the course of several minutes to a mean of 8.4 mM with a standard deviation of 1.4 mM. These results are summarized in Table I along with results for vasopressin, platelet-derived growth factor, and ionomycin, a Ca^{2+} ionophore, which elicited similar increases in $[Na^+]_i$. No changes in $[Na^+]_i$ were seen when cells were treated with phorbol 12,13-dibutyrate, a well characterized activator of protein kinase C. Although these results were taken at room temperature, similar results were seen for cells observed at 37 °C. While most experiments continued for only 30 min after stimulation, $[Na^+]_i$ remained elevated for over 90 min in the presence of 50 nM vasopressin. $[Na^+]_i$ decreased upon removal of the vasopressin (at $t = 90$ min), although $[Na^+]_i$ after vasopressin removal was slightly higher than $[Na^+]_i$ before vasopressin addition.

Since mitogenic activation of cells is often accompanied by an increase in pH_i of between 0.05 and 0.3 pH units due to activation of the Na^+/H^+ exchanger, we examined the effect of increased intracellular pH upon the SBFI 340/385 nm excitation ratio. Successive applications of NH_4Cl were used to alkalinize the cells. The use of the fluorescent pH indicator BCECF confirmed that extracellular application of 20 mM NH_4Cl increased pH_i by 0.4 pH unit. As shown in Fig. 9*B*, no significant changes in SBFI ratio could be detected from NH_4Cl application. Thus the increases in pH_i which are usually associated with mitogenic activation of the Na^+/H^+

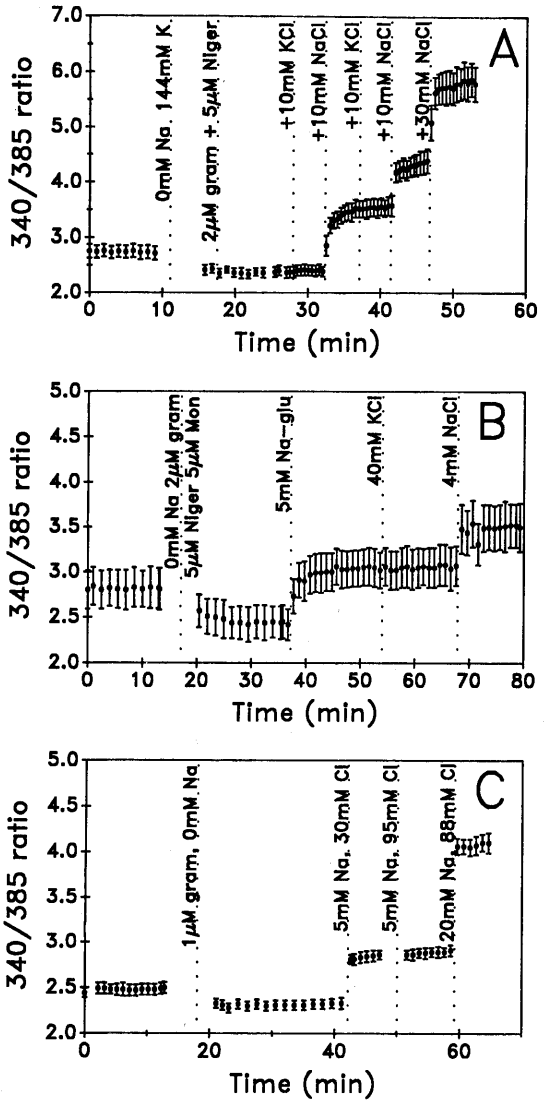

FIG. 8. **340/385 excitation ratio of SBFI in REF52 cells is insensitive to changes in $[K^+]$ or $[Cl^-]$.** *A*, 340/385 nm excitation ratio is shown of SBFI-loaded cells, initially in normal HBS, which is then replaced with an experimental medium containing 0 mM Na^+, 144 mM K^+, 110 mM gluconate, 30 mM Cl^-, and 10 mM Hepes, at pH 7.4. The cells were subsequently treated with 2 μM gramicidin (*gram*), 5 μM nigericin (*Niger*), and 5 μM monensin (*Mon*) to facilitate equilibration of monovalent cations. At the times marked by *dotted lines*, 10 mM KCl, 10 mM NaCl, 10 mM KCl, 10 mM NaCl, and 30 mM NaCl were added sequentially to the experimental medium by thoroughly mixing in requisite small volumes of 3 M stock solutions of either NaCl or KCl. *B*, 340/385 nm excitation ratio from SBFI-loaded cells initially in HBS, which is then replaced with an experimental medium containing 2 μM gramicidin, 5 μM nigericin, 5 μM monensin, 0 mM Na^+, 144 mM K^+, 110 mM gluconate, 30 mM Cl^-, and 10 mM Hepes, at pH 7.4. At the times indicated by *dotted lines*, 5 mM sodium gluconate, 40 mM KCl, and 4 mM NaCl were added sequentially to the experimental medium. *C*, 340/385 nm excitation ratio of SBFI-loaded cells initially in HBS but subsequently exchanged into experimental medium containing 1 μM gramicidin, 0 mM Na^+, 144 mM K^+, 110 mM gluconate, and 30 mM Cl^-. At the indicated times, the cells were exchanged into solutions containing 1) 5 mM Na^+, 139 mM K^+, 110 mM gluconate, and 30 mM Cl^-, 2) 5 mM Na^+, 139 mM K^+, 45 mM gluconate, and 95 mM Cl^-, and 3) 20 mM Na^+, 124 mM K^+, 52 mM gluconate, and 88 mM Cl^-. All solutions were buffered at pH 7.4 with 10 mM Hepes and contained 1 μM gramicidin.

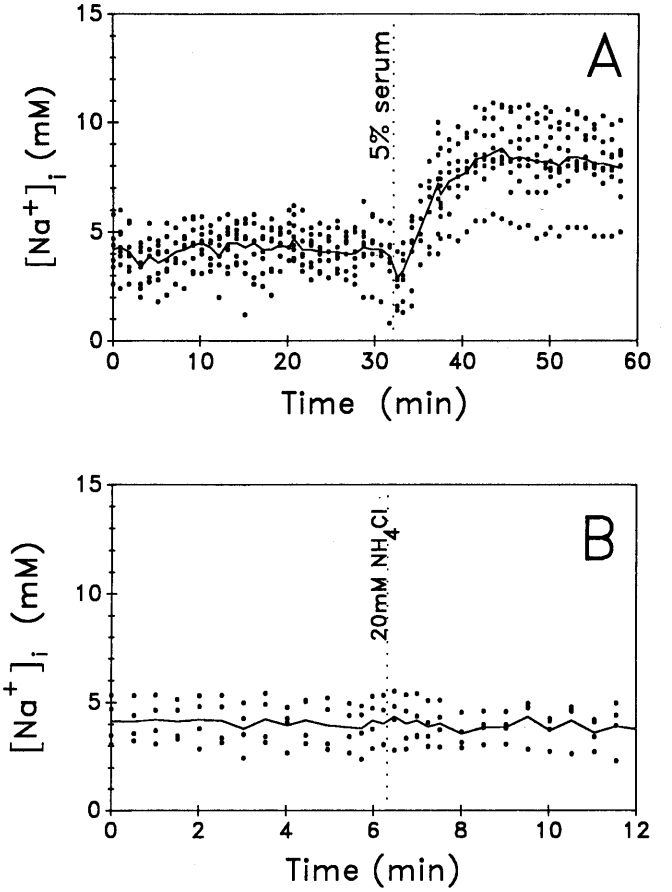

FIG. 9. **Serum elicits an increase in $[Na^+]_i$ in REF52 cells.** *A*, $[Na^+]_i$ as calibrated *in situ* is shown both before and after addition of 5% fetal bovine serum for eight cells. Also shown (*B*) is the lack of effect of increasing pH_i with 20 mM NH_4Cl on $[Na^+]_i$ as determined using SBFI in four cells.

TABLE I

Serum, vasopressin, PDGF, and ionomycin elicit increases in $[Na^+]_i$

Treatment	Average[a] $[Na^+]_i$	
	Before treatment	After treatment
	mM	*mM*
5% serum	4.8	8.5
5% serum	4.0	8.3
5% serum	3.7	8.4
5% serum	4.2	8.2
5% serum	4.4	13.2
30 nM vasopressin	3.5	7.8
50 nM vasopressin	4.0	6.0
50 nM vasopressin	4.0	7.0
1 μM vasopressin	2.9	7.7
40 ng/ml PDGF	2.7	4.3
40 ng/ml PDGF	2.7	5.7
500 nM ionomycin	3.5	6.1
1 μM ionomycin	3.5	10.5
3 μM ionomycin	3.6	5.2
3 μM ionomycin	3.5	6.1
200 μM PDB[b]	3.2	3.6

[a] Each line in the table represents a single experimental session. Each number is the average $[Na^+]_i$ value from a population of cells before or 20 min after application of the stimulant.

[b] PDB, 4β-phorbol-12,13-dibutyrate.

exchanger should not affect the $[Na^+]_i$ measurements made with SBFI.

One might analogously be concerned whether the rise in $[Na^+]_i$ seen with agents that elevate $[Ca^{++}]_i$ could be an artifact of the dye responding directly to Ca^{2+}. Such a selec-

tivity problem is very unlikely, for at least three reasons: the dissociation constant for SBFI and Ca^{2+} in standard saline solution is 38 mM, which is so far above cytosolic $[Ca^{2+}]_i$ to be quite safe; the time courses for the $[Ca^{2+}]_i$ and $[Na^+]_i$ rises are quite different, the former being a fast spike decaying back to a lower plateau, the latter being a gradual rise to a plateau (Harootunian *et al.*, 1988); and if gramicidin is applied to clamp $[Na^+]_i$ near its resting level, no SBFI signal is seen in response to $[Ca^{2+}]_i$ elevation.

Analysis of Na^+ Fluxes Due to Vasopressin—An increase in $[Na^+]_i$ can occur either through an increase in Na^+ influx, a decrease in Na^+ efflux, or both. To determine which of these possibilities was occurring in the REF52 cells upon stimulation, experiments were performed in which the Na^+ pump was blocked with 1 mM ouabain and changes in the rate of $[Na^+]_i$ increase were observed when the cells were exposed to either serum or vasopressin. If the agonist boosts Na^+ influx, it should increase the rate of rise of $[Na^+]_i$. As shown in Fig. 10 and summarized in Table II, stimulation with vasopressin or serum did increase Na^+ influx. Ionomycin did not elicit an increase in Na^+ influx even though in the absence of ouabain (Table I) it elicited an increase in $[Na^+]_i$. Thus the increase in $[Na^+]_i$ upon stimulation with serum or vasopressin is due at least in part to an increase in Na^+ influx, whereas the $[Na^+]_i$ elevation due to ionomycin probably stems from a decrease in Na^+ efflux.

The increase in $[Na^+]_i$ in response to serum or vasopressin can be magnified by partially inhibiting the Na^+ pump through reduction of extracellular K^+ from 5.80 to 1.45 mM. The result of such an experiment is shown in Fig. 11, which shows that 50 nM vasopressin can increase average $[Na^+]_i$ from 4 to 28 mM over 30 min. Note that in 1.45 mM external K^+, $[Na^+]_i$ remains steady at a level only slightly higher than in normal external K^+, but changes drastically upon exposure of the cell to vasopressin. The use of low but nonzero levels of potassium should be of use in amplifying very small changes in $[Na^+]_i$.

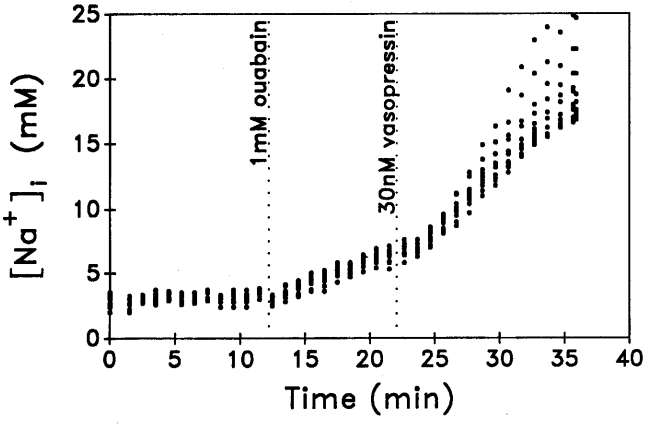

FIG. 10. **Vasopressin causes an increase in the rate of Na^+ influx.** $[Na^+]_i$ as calibrated with SBFI is shown for 12 REF52 cells in normal HBS saline, in HBS with 1 mM ouabain, and in HBS with 1 mM ouabain and 30 nM vasopressin.

TABLE II

Serum or vasopressin elicit increases in Na^+ influx in REF52 fibroblasts

1 mM ouabain was continually present in the bathing medium throughout the experiment. Each line in the table represents a single experimental session, and each numerical entry is the average value from a population of cells.

Treatment	Rate of increase of $[Na^+]_i$		
	Before treatment	After treatment	Rate enhancement
	mM/min		*-fold*
5% serum	0.5	0.9	1.8
5% serum	0.4	1.2	3.0
5% serum	0.5	1.4	2.8
5% serum	0.5	1.3	2.5
30 nM vasopressin	0.4	0.9	2.3

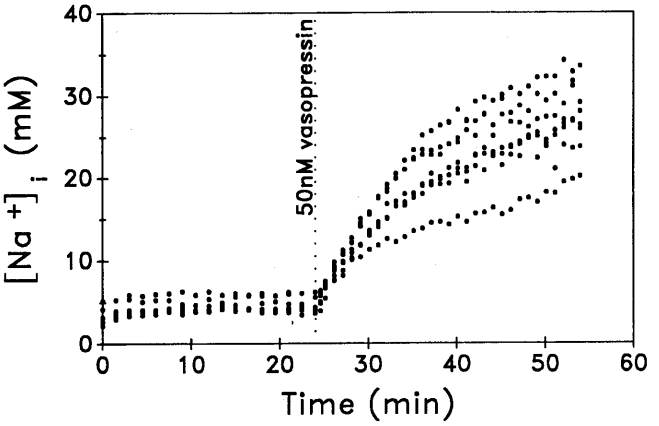

FIG. 11. **The $[Na^+]_i$ increase elicited by vasopressin is amplified by reducing extracellular $[K^+]$ to 1.45 mM.** The $[Na^+]_i$ measured using SBFI is shown for seven cells in 1.45 mM $[K^+]$ HBS saline both before and after addition of 50 nM vasopressin.

We attempted to use amiloride and amiloride analogs to block Na^+/H^+ exchange to reveal its contribution to the serum induced Na^+ influx. Unfortunately, these drugs were themselves too fluorescent. The fluorescence intensity of cells with or without SBFI loading increased instantaneously upon exposure of the cells to 0.1 mM amiloride and continued to rise progressively, preventing reliable subtraction of the fluorescence due to the drug. Problems with amiloride entering or binding to cells have been reported previously and are thought to be due at least in part to accumulation of amiloride as a weak base into acidic compartments (Benos, 1988). Amiloride analogues with hydrophobic substituents are reported to be more potent at blocking Na^+/H^+ exchange, but seemed also to produce fluorescence artifacts at lower concentrations (20 μM). Clearly development of a nonfluorescent amiloride analog or development of a visible excitation wavelength Na^+ indicator would be of great benefit in this instance.

Another approach to block the Na^+/H^+ exchanger was to raise intracellular pH. Alkalinization is reported to inhibit the Na^+/H^+ exchanger via an intracellular allosteric H^+ binding site (Aronson *et al.*, 1982; Aronson, 1985). Intracellular pH was increased either by extracellular application of NH_4Cl or by increasing extracellular pH to 8.0. The fluorescent pH indicator BCECF was used to confirm increases in pH caused by either of the above treatments. Increasing extracellular pH to 8.0 resulted in a sustained increase in intracellular pH to approximately 8.0 with only a slight recovery over 40 min of observation. Application of 20 mM NH_4Cl caused an abrupt increase in pH_i from 7.13 to 7.58; the pH of the cell slowly recovered to a value of 7.21 after 25 min. 50 nM vasopressin was applied to these cells a short time (2–5 min) after NH_4^+ application while the cells were still alkalinized. Surprisingly, the increase in $[Na^+]_i$ elicited by 50 nM vasopressin remained intact at elevated pH_i using either method of alkalinization. These results suggest that the increase in $[Na^+]_i$ and Na^+ influx is primarily mediated by a pathway other than the conventional Na^+/H^+ exchanger.

Yet another possible contributor to Na^+ fluxes is Na^+/Ca^{2+} exchange. However, this process appears unlikely to be sig-

nificant in REF52 fibroblasts, since elevation of $[Na^+]_i$ with ouabain has no effect on $[Ca^{2+}]_i$ measured with fura-2 (results not shown), whereas cells known to contain the Na^+/Ca^{2+} exchanger typically show at least a 3-fold increase in $[Ca^{2+}]_i$ in response to ouabain (Sheu and Fozzard, 1982; Pritchard and Ashley, 1986). Furthermore, vasopressin could still elicit sustained increases in $[Na^+]_i$ when the cells were in nominally Ca^{2+}-free media, which prevented any sustained $[Ca^{2+}]_i$ increase.

DISCUSSION

Although this paper is restricted to the use of the new Na^+ indicators in fibroblasts and lymphocyte cell lines, SBFI has also shown promise in smooth muscle (Moore *et al.*, 1988), gastric glands (Negulescu *et al.*, 1988), hepatocytes, and the LLC-PK1 kidney cell line.[3] Na^+:K^+ selectivity appears to be usable in all these cell types. The quantitative spectral properties of the indicator in the cytosol appeared to differ from the dye in simple calibration solutions, but this phenomenon could be bypassed by *in situ* $[Na^+]_i$ calibration. Changes in $[Na^+]_i$ of less than 1 mM could be seen, since the noise level of the population mean over time was typically about 0.5 mM. Except when $[Na^+]_i$ was grossly elevated by blocking the Na^+ pump, the standard deviation of populations of cells ranged from less than 0.5 mM to slightly greater than 2 mM with 1 mM being typical. Measurement of $[Na^+]_i$ may be inherently easier than $[Ca^{2+}]_i$ in at least three aspects: $[Na^+]_i$ is large enough so that buffering by the indicator is unlikely to be worrisome and so that significant percentage changes in $[Na^+]_i$ will probably be relatively slow, and absolute calibration of the indicator is much easier because of the availability of several antibiotics and ionophores to reliably clamp $[Na^+]_i$ equal to extracellular $[Na^+]$.

Our overall impression is that the quality of loading (judged by lack of compartmentation and maximum Na^+ responsiveness of the 340/385 nm ratio) decreases in the following order: microinjected SBFI > ester-loaded SBFI ≥ microinjected SBFP ≫ ester loaded SBFP. We have not further investigated why SBFP is inferior to SBFI both when microinjected and when administered as an AM ester. When SBFI was introduced either by microinjection or by ester loading, the nucleus was often brighter than the cytoplasm and showed a slightly different relationship between 340/385 nm ratio and $[Na^+]$ (Fig. 5), although when that difference was taken into account, the free $[Na^+]$ in nucleus and cytoplasm was indistinguishable (Fig. 7). When SBFI was loaded via its AM ester, some further compartmentation was visible within the cytoplasm. This could be detected by failure of all the dye outside the nucleus to be released with digitonin. Much of the compartmentation appeared to be in acidic organelles, since weak bases that should neutralize the pH of such organelles reduced the difference in dye calibration between nucleus and cytoplasm (Fig. 6*B*). The perturbing effect of compartmentation could be minimized (although probably not completely eliminated) by calibrating with antibiotics that primarily permeabilize the plasma membrane and leave the acidic organelles relatively unaffected. By this procedure the contribution of the Na^+-unresponsive dye in the organelles should be nearly constant despite permeabilization, so that it is approximately cancelled out by the *in situ* calibration. Obviously, compartmentation would need to be checked for whenever applying SBFI to a new cell type.

The ability to determine the heterogeneity of $[Na^+]_i$ within a population is only one advantage of using microscopy and ratio imaging in combination with the Na^+ indicator. Other advantages of microscopy include the minimal number of cells required, the ability to see indicator compartmentation directly, the inherent discrimination against dye that may have leaked out of the cells, the ease of completely changing solutions when the cells are immobilized, and the compatibility with electrophysiological techniques. Cuvette measurements by contrast are population averages which are susceptible to leaked dye and require on the order of 10^6 cells/ml. Other methods of measuring $[Na^+]_i$ such as NMR may require as many as 5×10^8 cells/ml, packed inside a relatively inaccessible magnet cavity.

Since the use of SBFI to measure $[Na^+]_i$ is a new technique, it is worth comparing our results with previous measurements in lymphocytes and fibroblasts. The 6 mM increase in $[Na^+]_i$ elicited by phytohemagglutinin in Jurkat tumor lymphocytes agrees surprisingly well with previous studies showing that lectin or concanavalin A elevated total $[Na^+]$ by 4–6 mM in lymphocytes freshly isolated from a variety of sources (Segal *et al.*, 1979; Felber and Brand, 1983). However, our estimate for basal free $[Na^+]_i$, ≤10 mM, is somewhat lower than previous values for total intracellular Na^+ (15–29 mM) and even a little below the only previous estimate of free $[Na^+]_i$ (13.8 mM) in lymphocytes (Smith *et al.*, 1986), obtained by an NMR method on pig mesenteric lymphocytes. The higher value in pig cells might be a real difference between preparations, since the pig lymphocytes also appear to be much higher in total $[Na^+]$ than mouse or human cells (Segal *et al.*, 1979; Felber and Brand, 1983), or it might reflect the difficulty of preserving dense cell suspensions in optimal condition in the cavity of an NMR spectrometer. The results for fibroblasts exhibit a similar pattern, in that our value for free $[Na^+]_i$, 4.2 mM, is much lower than previous measurements of total Na^+ (*e.g.* 14 mM), yet the magnitude of the increases due to serum are similar, 5.1 *versus* 5.0 mM, respectively (Frantz *et al.*, 1981).

There is some previous evidence that free $[Na^+]_i$ is much less than total Na^+. A large number of ^{23}Na NMR studies reviewed by Springer (1987), show that the signal from intracellular Na^+ is less than half that expected from the total Na^+ content, as if much of the intracellular Na^+ is perturbed by quadrupolar splittings from intracellular macromolecules. In several tissues in which both free and total Na^+ have been measured, the free $[Na^+]_i$ or sodium activity is always substantially less than total, regardless of the method used for measuring free $[Na^+]_i$, for example ^{19}F NMR in lymphocytes (Smith *et al.*, 1986), Na^+-selective microelectrodes in *Xenopus* embryos (Slack *et al.*, 1973), or microdialysis against gelatin droplets in salamander oocytes (Horowitz and Paine, 1979). The latter two cases are particularly interesting in that the ratio of free to total Na^+ changed during development of the embryos and was a nonlinear function of free $[Na^+]_i$ in oocytes. Even at low free $[Na^+]_i$, a considerable amount of Na^+ was bound, but this binding was saturable, so that further increments in free and total Na^+ were comparable in magnitude. If a similar binding isotherm applied to the fibroblasts in this study, it would qualitatively explain why the basal free $[Na^+]_i$ is much less than the total Na^+, yet the increments in free and total Na^+ due to mitogens are roughly equal.

The fluorescent Na^+ indicator can be used not only to measure $[Na^+]_i$ but also to assess unidirectional Na^+ influxes by the rate of $[Na^+]_i$ increase after Na^+ pump blockage. Our finding that vasopressin and serum increase unidirectional influx by 2.3–2.6-fold agrees well with other influx measurements showing 2–3-fold enhancements (Mendoza *et al.*, 1980a, 1980b; Villereal, 1981; Cassel *et al.*, 1983; Chang-Sing and

[3] A. T. Harootunian, unpublished results.

Lechene, 1983). Na^+/H^+ exchange is an obvious candidate for the mechanism of the influx, since the exchanger is known to be activated by a variety of mitogenic stimuli. However, the simplest test of this hypothesis, to block the exchanger with amiloride or analogous drugs, was hindered by the considerable fluorescence of these agents. Elevation of intracellular pH, an alternative means of inhibiting the Na^+/H^+ exchanger, did not interfere with the mitogen-induced $[Na^+]_i$ increase, suggesting that other mechanisms of Na^+ influx must be operating. Indeed, amiloride independent increases in Na^+ influx have been seen in fibroblasts in response to thrombin (Paris and Pouyssegur, 1986), PDGF (Cassel *et al.*, 1983), and serum (Villereal, 1981; Cassel *et al.*, 1983). A reasonable candidate would be the opening of nonspecific cation channels, which have been found electrophysiologically in many cells including lymphocytes (Kuno and Gardner, 1987), fibroblasts (Chen *et al.*, 1988), mast cells (Penner *et al.*, 1988), and neutrophils (von Tscharner *et al.*, 1986), and which may also contribute to sustained $[Ca^{2+}]_i$ elevation.

Application of a Ca^{2+} ionophore, ionomycin, produced increases in $[Na^+]_i$ without a significant increase in Na^+ influx. Presumably, elevated $[Ca^{2+}]_i$ acts by decreasing the Na^+ efflux from the cell. Previous work showed that the Ca^{2+} ionophore A23187 also had no effect on the Na^+ influx or $[Na^+]_i$ (Smith and Rozengurt, 1978). Although further work would be needed to determine the exact mechanism by which $[Ca^{2+}]_i$ affects Na^+ homeostasis, our data are consistent with proposals that the Na^+ pump is inhibited by high levels of Ca^{2+} (Yingst, 1988). Such an inhibition of Na^+ efflux could also contribute to the $[Na^+]_i$ increases due to other mitogens, all of which are known to elevate $[Ca^{2+}]_i$.

The exact role of the increase in $[Na^+]_i$ elicited by mitogens remains uncertain. Increasing $[Na^+]_i$ with melittin, a channel-forming component of bee venom (Tosteson *et al.*, 1985), has been reported to synergize with insulin or epidermal growth factor to elicit DNA synthesis (Rozengurt *et al.*, 1981). This result is not simple to interpret, since other workers have claimed melittin to be an inhibitor of phospholipase A_2 (Shier, 1979; Morgan *et al.*, 1985) and of calmodulin (Comte *et al.*, 1983; Baudier *et al.*, 1987), and mellitin has also been reported to increase $[Ca^{2+}]_i$ (Mix *et al.*, 1984). Increasing $[Na^+]_i$ directly with monensin or ouabain has also been reported to elicit differentiation in a pre-B cell line (Rosoff and Cantley, 1983). It is unclear whether such effects are directly due to $[Na^+]_i$ or are due to secondary changes in $[Ca^{2+}]_i$ or pH_i. Perhaps the most detailed evidence for the importance of $[Na^+]_i$ in cell growth and differentiation is in *Xenopus* embryos, where formation of the neural plate appears to depend critically on a decrease in $[Na^+]_i$ of about 3-fold (Breckinridge and Warner, 1982; Blackshaw and Warner, 1978). Since this factor is only slightly larger than the increases in $[Na^+]_i$ observed in this study, the possibility that such changes may have significant biochemical and developmental consequences should be given serious consideration. Experiments to test such hypotheses as well as other potential functions of $[Na^+]_i$ should be greatly facilitated by the sensitive, relatively noninvasive optical technique introduced here.

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