Regulation of intracellular calcium in N1E-115 neuroblastoma cells: the role of Na\textsuperscript{+}/Ca\textsuperscript{2+} exchange

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Kopper, Kara L., and Joseph S. Adorante. Regulation of intracellular calcium in N1E-115 neuroblastoma cells: the role of Na\textsuperscript{+}/Ca\textsuperscript{2+} exchange. Am J Physiol Cell Physiol 282: C1000–C1008, 2002; 10.1152/ajpcell.00182.2001.—In fura 2-loaded N1E-115 cells, regulation of intracellular Ca\textsuperscript{2+} concentration ([Ca\textsuperscript{2+}]\textsubscript{i}) following a Ca\textsuperscript{2+} load induced by 1 μM thapsigargin and 10 μM carbonyl cyanide p-trifluoromethoxyphenylhydrazone (FCCP) was Na\textsuperscript{+} dependent and inhibited by 5 mM Ni\textsuperscript{2+}. In cells with normal intracellular Na\textsuperscript{+} concentration ([Na\textsuperscript{+}]\textsubscript{i}), removal of bath Na\textsuperscript{+}, which should result in reversal of Na\textsuperscript{+}/Ca\textsuperscript{2+} exchange, did not increase [Ca\textsuperscript{2+}]\textsubscript{i}, unless cell Ca\textsuperscript{2+} buffer capacity was reduced. When N1E-115 cells were Na\textsuperscript{+} loaded using 100 μM veratidine and 4 μg/ml scorpion venom, the rate of the reverse mode of the Na\textsuperscript{+}/Ca\textsuperscript{2+} exchange was apparently enhanced, since an ~4- to 6-fold increase in [Ca\textsuperscript{2+}]\textsubscript{i}, occurred despite normal cell Ca\textsuperscript{2+} buffering. In SBFI-loaded cells, we were able to demonstrate forward operation of the Na\textsuperscript{+}/Ca\textsuperscript{2+} exchanger (net efflux of Ca\textsuperscript{2+}) by observing increases (~ 6 mM) in [Na\textsuperscript{+}]\textsubscript{i}. These Ni\textsuperscript{2+} (5 mM)-inhibited increases in [Na\textsuperscript{+}]\textsubscript{i} could only be observed when a continuous ionomycin-induced influx of Ca\textsuperscript{2+} occurred. The voltage-sensitive dye bis-(1,3-dihexyloxybarbituric acid) trimethine oxonol was used to measure changes in membrane potential. Ionomycin (1 μM) depolarized N1E-115 cells (~25 mV). This depolarization was Na\textsuperscript{+} dependent and blocked by 5 mM Ni\textsuperscript{2+} and 250–500 μM benzamil. These data provide evidence for the presence of an electrogenic Na\textsuperscript{+}/Ca\textsuperscript{2+} exchanger that is capable of regulating [Ca\textsuperscript{2+}]\textsubscript{i}, after release of Ca\textsuperscript{2+} from cell stores.

calcium flux; membrane transport; neuronal calcium regulation; sodium/calcium antiport

The regulation of intracellular Ca\textsuperscript{2+} concentration ([Ca\textsuperscript{2+}]\textsubscript{i}) in mammalian cells is of fundamental importance in physiological and pathophysiological conditions. Ca\textsuperscript{2+} is an important regulator of a variety of physiological functions including processes such as excitation-contraction coupling, fluid secretion in epithelia, and release of neurotransmitters (15). [Ca\textsuperscript{2+}]\textsubscript{i} must be tightly regulated to achieve transient or sustained levels required to trigger specific physiological processes.

Cells have several mechanisms in place to return elevated free [Ca\textsuperscript{2+}]\textsubscript{i} to resting levels. These transport mechanisms may be located at the level of the plasma membrane or reside in the cytosol within organelles such as mitochondria and endoplasmic reticulum (ER). Ca\textsuperscript{2+}-ATPases (Ca\textsuperscript{2+} pumps) located at the plasma membrane (8) and ER (32) are primary active transport mechanisms capable of lowering [Ca\textsuperscript{2+}]\textsubscript{i}, following a stimulus-induced increase and can also help maintain steady-state [Ca\textsuperscript{2+}]\textsubscript{i} levels (8, 20). In addition, many animal cells contain a Na\textsuperscript{+}/Ca\textsuperscript{2+} exchanger, which, depending on the sign of the electrochemical potential (driving force), can operate in the forward or reverse mode. In the forward mode, Ca\textsuperscript{2+} is extruded from the cell. In the reverse mode, the cell is loaded with Ca\textsuperscript{2+} (3, 6).

Although regulation of [Ca\textsuperscript{2+}]\textsubscript{i}, is important for normal cell functioning, its deregulation has been linked to cellular pathologies and even cell death (31). For example, in some central nervous system (CNS) neurons, anoxia results in uncontrolled increases in [Ca\textsuperscript{2+}]\textsubscript{i} that, if unchecked, can eventually lead to cell death (29). Because of the importance of [Ca\textsuperscript{2+}]\textsubscript{i} in neuronal health and disease, a relatively simple cell model system, one where [Ca\textsuperscript{2+}]\textsubscript{i} regulation can be studied fairly easily, is desirable.

N1E-115 neuroblastoma cells have been used for many years as a neuronal model system. They grow very easily and can be used in a variety of assays and cellular investigations. Like other CNS neurons, N1E-115 cells contain voltage-gated Na\textsuperscript{+}, K\textsuperscript{+}, and Ca\textsuperscript{2+} channels (21, 24, 25). In addition, these cells have also been used to investigate Ca\textsuperscript{2+} signaling pathways (7, 23, 33). However, very little is known about how these cells regulate Ca\textsuperscript{2+}\textsubscript{i}. In this study, we examined how N1E-115 neuroblastoma cells regulate [Ca\textsuperscript{2+}]\textsubscript{i}, following the release of Ca\textsuperscript{2+} from intracellular stores. We show that these cells possess 1) an electrogenic Na\textsuperscript{+}/Ca\textsuperscript{2+} exchange mechanism that is responsible for restoring elevated [Ca\textsuperscript{2+}]\textsubscript{i}, to control level following depletion of Ca\textsuperscript{2+} stores and 2) a Na\textsuperscript{+}-independent Ca\textsuperscript{2+} extrusion mechanism that is most evident following [Ca\textsuperscript{2+}]\textsubscript{i} elevation in Na\textsuperscript{+}-free media.

METHODS

Cell preparation. N1E-115 mouse neuroblastoma cells were purchased from the University of California, San Franc-

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Cisco Cell Culture Facility. They were grown in Dulbecco's modified Eagle's medium (DMEM) high glucose without phenol red supplemented with 2 mM l-glutamine, 50 units penicillin/50 μg streptomycin, 0.2 mM sodium hydroxanthine, 400 nM aminopterin plus 16 μM thymidine (1× HAT; Gibco BRL, Rockville, MD), and 10% fetal bovine serum (FBS, HyClone Laboratories, Logan, UT) at 37°C in a humidified atmosphere of 5% CO₂. The cells were split 1:3 twice a week and were plated on glass coverslips up to 2 days before use. Data are expressed as means ± SE where applicable. Statistical significance was tested, where appropriate, using a paired or unpaired t-test. To determine significance between control and experimental groups, a one-way analysis of variance (one-way ANOVA) followed by Dunnnett's test was performed. Where stated, n is the number of experiments performed.

**Solutions.** HEPES Ringer contained (in mM) 120 NaCl, 10 glucose, 10 HEPES, 5 KCl, 1.2 CaCl₂, 0.6 MgCl₂, and 12 Na-acyclamate. pH was adjusted to 7.40 at 37°C with NaOH, and the osmolarity was adjusted to 291 mosM Na-cyclamate. pH was adjusted to 7.40 at 37°C. The samples were excited at wavelengths of 345 and 390 nm, and the fluorescence were described previously (13). Briefly, SBFI was calibrated by using the voltage-sensitive fluorescent anionic dye bis-(1,3-diethylthiobarbituric acid) trimethine oxonol (bis-oxonol; Molecular Probes). Bis-oxonol partitions into the cell membrane as a Nernstian function of the membrane potential. After depolarization, bis-oxonol enters the membrane and the fluorescence intensity increases. After membrane hyperpolarization, dye leaves the cell and fluorescence intensity decreases (1, 26).

A cell preparation for Eₘ experiments was established as follows: cells were removed from their culture flasks and counted by using trypan blue (Sigma) exclusion to assess viability, which was typically 15–25% dead cells. The cells were divided into aliquots of ~0.42 × 10⁶ living cells per sample, and these were stored on ice for a minimum of 30 min. The pellets were resuspended in 3 ml of HEPES Ringer (37°C) for 10 min in a cuvette before an experiment. Bis-oxonol (30 nM) was added 2 min before the beginning of the run to allow for equilibration. Measurements were made by using an LS-50B spectrofluorometer (Perkin Elmer, Norwalk, CT) at an excitation of 540 nm (±10-nm slit widths) and emission at 580 nm (±5.0-nm slit widths).

Calibration of bis-oxonol was performed as described by Grinstein et al. (12). Calibration solutions were made by substituting Ringer Na⁺ with NMDG. Cells were resuspended in a cuvette containing Ringer with Na⁺ ranging from 0 to 140 mM and were subsequently exposed to 0.5 μM gramicidin. After gramicidin addition, intensity was measured for 2–3 min. Because gramicidin is a nonselective monovalent cation ionophore, permeable to both Na⁺ and K⁺ but not to NMDG, the monovalent cation ratio determines the membrane potential.

The following equation was used to calculate Eₘ at each extracellular Na⁺ concentration (Na⁺o): Eₘ = −60 log([cation]/[cation]o), where i and o denote the intra- and extracellular compartments, respectively. It was assumed that [cation]i = 145 mM. Plotting the intensity for each sample against its calculated Eₘ then generated a calibration curve, which was linear to approximately −75 mV. The equation of this curve allowed the conversion of intensity units to millivolts (see Fig. 2B).

**RESULTS**

**Regulation following Ca²⁺ loading.** Regulation of [Ca²⁺]i in fura 2-loaded N1E-115 cells was investigated following release of Ca²⁺ from intracellular stores and mitochondria by using 1 μM thapsigargin and 10 μM carbonylcyanide p-trifluoromethoxyphenylhydrazone (FCCP), respectively (Fig. 1A). These two compounds allowed sequestered Ca²⁺ to flood into the cytoplasm and also prevented the Ca²⁺ from reloading into the intracellular stores (10). After perfusion of 1 μM thapsigargin and 10 μM FCCP, [Ca²⁺]i rose from 52.9 ± 2.5 nM (215 cells, n = 15) to a peak of 105.3 ± 2.5 nM (153...
cells, \( n = 15 \) and relaxed toward control levels in ~5 min. However, in the absence of extracellular Na\(^+\) (Na\(_{\text{out}}^+\)), [Ca\(^{2+}\)] rose to 129.5 ± 15.4 nM (34 cells, \( n = 8 \)) and remained elevated (Fig. 1A). In the absence of Na\(_{\text{out}}^+\), the increase in [Ca\(^{2+}\)] following 1 \( \mu \)M thapsigargin and 10 \( \mu \)M FCCP was statistically greater than that observed with Na\(_{\text{out}}^+\) present (\( P < 0.05 \), unpaired t-test). When the extracellular solution was replaced with one containing Na\(^+\), [Ca\(^{2+}\)] levels returned to baseline.

The Na\(^+\) dependence of recovery suggested that Na\(^+\)/Ca\(^{2+}\) exchange may play a role in the recovery process. To determine whether Na\(^+\)/Ca\(^{2+}\) exchange was responsible for the return of [Ca\(^{2+}\)] toward control level, 5 mM Ni\(^{2+}\), an inhibitor of the Na\(^+\)/Ca\(^{2+}\) exchange, was utilized. As in Fig. 1A, cells exposed to 1 \( \mu \)M thapsigargin and 10 \( \mu \)M FCCP in the absence of Na\(_{\text{out}}^+\) (NMDG replacement) failed to regulate the increase in [Ca\(^{2+}\)], caused by these agents until Na\(_{\text{out}}^+\) was added back to the medium. However, in the presence of 5 mM Ni\(^{2+}\) the rate of regulation was slowed following addition of Na\(^+\) to the bath (Fig. 1B). The rate of regulation of Ca\(^{2+}\) in the presence of 5 mM Ni\(^{2+}\) (0.4 × 10\(^{-9}\) M/s, \( n = 6 \)) was significantly slower than the rate in its absence (1.4 × 10\(^{-9}\) M/s, \( n = 5 \); \( P < 0.05 \), unpaired t-test). Substitution of Li\(^+\) for Na\(^+\) was also effective at inhibiting the rate of [Ca\(^{2+}\)], recovery following exposure to 1 \( \mu \)M thapsigargin and 10 \( \mu \)M FCCP (not shown).

**Membrane potential measurements.** It has been previously reported that Na\(^+\)/Ca\(^{2+}\) exchange is electrogenic because 3 Na\(^+\) exchange for every Ca\(^{2+}\), resulting in a net movement of positive charge (3). Under appropriate conditions, this mechanism should be able to alter the membrane potential of the cells during its operation. In the forward mode with net influx of Na\(^+\) and efflux of Ca\(^{2+}\), operation of the exchanger should measurably depolarize the cell provided its relative conductance is significant. To see whether we could detect its contribution to the membrane potential, we employed the voltage-sensitive dye bis-oxonol.

Intracellular Ca\(^{2+}\) is both a substrate and modulator of Na\(^+\)/Ca\(^{2+}\) exchange with a \( K_v \) for activation of Na\(^+\) dependent Ca\(^{2+}\) efflux of 1–3 \( \mu \)M (28). To maximize Na\(^+\)/Ca\(^{2+}\) exchange rate and thus current density, ionomycin, a calcium ionophore, was used to increase [Ca\(^{2+}\)] in a sustained manner. This maneuver should optimize the chances for detecting the electrogenicity of the exchanger.

Addition of 1 \( \mu \)M ionomycin to the cell suspension caused an increase in fluorescence corresponding to a depolarization of ~25 mV. The ionomycin-induced depolarization was reduced by ~80% by the absence of Na\(^+\) in the media and was also blocked by 5 mM Ni\(^{2+}\) (Fig. 2A). In addition, 1 \( \mu \)M tetrodotoxin (TTX), an inhibitor of voltage-gated Na\(^+\) channels, had no effect on the depolarization (Table 1), nor did removal of chloride from the media (data not shown). Furthermore, the removal of Ca\(^{2+}\) from the extracellular media completely inhibited the depolarization, indicating that it was dependent on Ca\(^{2+}\) entering the cell. Benzamil (250–500 \( \mu \)M), a Na\(^+\)/Ca\(^{2+}\) exchange inhibitor (16), also blocked the ionomycin-induced depolarization (Table 1).

In addition, removal of Na\(_{\text{out}}^+\) before 1 \( \mu \)M ionomycin addition resulted in an ~25-mV hyperpolarization (Fig. 2A) consistent with reversal of an electrogenic Na\(^+\)/Ca\(^{2+}\) exchanger. However, this hyperpolarization was transient and a continuous depolarization followed. On addition of 1 \( \mu \)M ionomycin, this depolarization was halted and a slight hyperpolarization followed. Thus the extent of hyperpolarization following ionomycin addition was masked, presumably, by another conductive mechanism activated by removal of media Na\(^+\) (see DISCUSSION).

**Measurement of intracellular Na\(^+\).** In the forward mode of the Na\(^+\)/Ca\(^{2+}\) exchanger, a decrease in [Ca\(^{2+}\)] is accompanied by an increase in [Na\(^+\)]. However, since under physiological conditions nanomolar to micromolar amounts of Ca\(^{2+}\) are removed following a rise
in \([\text{Ca}^{2+}]_i\), the increase in \([\text{Na}^+]_i\) is not detectable using conventional \(\text{Na}^+\) dyes such as SBFI, which has a \(K_d\) of 18 mM \(\text{Na}^+\) (19). However, if a continuous source of \(\text{Ca}^{2+}\) from an infinite reservoir is supplied intracellularly, it should be possible to measure such an increase in \([\text{Na}^+]_i\) (see DISCUSSION and Fig. 3 legend). In the experiments shown in Fig. 3, a continuous source of \(\text{Ca}^{2+}\) was supplied to the cells via ionomycin. Because in our experiments the bath can be considered infinite, in the presence of ionomycin a continuous influx of \(\text{Ca}^{2+}\) occurred.

Addition of ionomycin (2 mM) to the bath increased \([\text{Ca}^{2+}]_i\) to ~475 nM (data not shown) and depolarized \(E_m\) (see Fig. 2A and Table 1). After the addition of ionomycin, there was an increase in \([\text{Na}^+]_i\). In paired experiments, 5 mM \(\text{Ni}^{2+}\) blocked the elevation of \([\text{Na}^+]_i\), following addition of ionomycin. In the absence of \(\text{Ni}^{2+}\), following ionomycin addition, \([\text{Na}^+]_i\) increased by 6.3 ± 0.4 mM, whereas in the presence of 5 mM \(\text{Ni}^{2+}\) there was no increase (−0.2 ± 2.6 mM, \(n = 3; P < 0.05\), see Fig. 3). These observations are consistent with the continuous operation of the \(\text{Na}^+/-\text{Ca}^{2+}\) exchanger in the forward mode.

**Effect of \(\text{Na}^+\) removal.** The direction of the \(\text{Na}^+/-\text{Ca}^{2+}\) exchanger is determined by the sign of the driving force and therefore is a function of the membrane potential and the ion gradients for \(\text{Na}^+\) and \(\text{Ca}^{2+}\). One way of reversing the driving force for \(\text{Na}^+/-\text{Ca}^{2+}\) exchange is to favor net efflux of \(\text{Na}^+\) and influx of \(\text{Ca}^{2+}\) to remove \(\text{Na}^+_o\). Figure 4A shows the effect of removing \(\text{Na}^+_o\) on \([\text{Ca}^{2+}]_i\). The removal of \(\text{Na}^+_o\) from the media resulted in little (2.6 ± 1.9 nM, \(n = 6\), if any, increase in \([\text{Ca}^{2+}]_i\), (Fig. 4A). One explanation for the insignificant \([\text{Ca}^{2+}]_i\) increase was that the cell buffered the \(\text{Ca}^{2+}\) as it was brought in by the exchanger. To test this hypothesis, the cells were exposed to 1 \(\mu\text{M}\) thapsigargin and 10 \(\mu\text{M}\) FCCP to reduce their ability to buffer \([\text{Ca}^{2+}]_i\). Thapsigargin plus FCCP caused \([\text{Ca}^{2+}]_i\) to rise transiently and then recover. After recovery, \(\text{Na}^+_o\) was removed from the media in the presence of 1 \(\mu\text{M}\) thapsigargin and 10 \(\mu\text{M}\) FCCP. This maneuver resulted in a significant increase in \([\text{Ca}^{2+}]_i\) to a final concentration of 79.8 ± 6.8 nM (69 cells, \(n = 5\)). This increase in \([\text{Ca}^{2+}]_i\) was significantly greater (\(P < 0.05\), paired t-test) than that seen in the absence of 1 \(\mu\text{M}\) thapsigargin and 10 \(\mu\text{M}\) FCCP. Reperfusion with \(\text{Na}^+_o\) returned \([\text{Ca}^{2+}]_i\) to its previous level. These data suggest that the buffer capacity of the cell was able to keep pace with the influx of \(\text{Ca}^{2+}\) following \(\text{Na}^+_o\) removal (Fig. 4A).

The increase in \([\text{Ca}^{2+}]_i\) following removal of \(\text{Na}^+_o\) is consistent with reversal of a \(\text{Na}^+/-\text{Ca}^{2+}\) exchanger such that net \(\text{Na}^+\) efflux results in net \(\text{Ca}^{2+}\) influx, thus the countercoupling of \(\text{Na}^+\) and \(\text{Ca}^{2+}\). If \(\text{Na}^+\) and \(\text{Ca}^{2+}\) fluxes are countercoupled as predicted, then the increase in \([\text{Ca}^{2+}]_i\), following \(\text{Na}^+_o\) removal should be dependent on \(\text{Ca}^{2+}\). Figure 4B shows that, after recovery of the \([\text{Ca}^{2+}]_i\) increase induced by 1 \(\mu\text{M}\) thapsigargin and 10

### Table 1. Effect of inhibitors on the ionomycin-induced depolarization

<table>
<thead>
<tr>
<th>Condition</th>
<th>Ionomycin Depolarization, mV</th>
<th>%Inhibition</th>
<th>(n)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ionomycin (1 (\mu\text{M}))</td>
<td>28.3 ± 2.5</td>
<td>10</td>
<td></td>
</tr>
<tr>
<td>Ionomycin (2 (\mu\text{M}))</td>
<td>36.0 ± 3.9</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td>Nickel (5 (\mu\text{M}))</td>
<td>11.3 ± 5.3*</td>
<td>60.1</td>
<td>5</td>
</tr>
<tr>
<td>TTX (1 (\mu\text{M}))</td>
<td>25.9 ± 3.6 (NS)</td>
<td>8.4</td>
<td>4</td>
</tr>
<tr>
<td>NMDG (0 (\text{Na}^+))</td>
<td>6.3 ± 5.5*</td>
<td>77.7</td>
<td>5</td>
</tr>
<tr>
<td>Benamil (250 (\mu\text{M}))</td>
<td>17.3 ± 2.7 (SD)</td>
<td>38.8</td>
<td>2</td>
</tr>
<tr>
<td>Benamil (500 (\mu\text{M}))</td>
<td>0.5 ± 0.6*</td>
<td>98.2</td>
<td>3</td>
</tr>
<tr>
<td>EGTA (0 (\text{Ca}^2+))</td>
<td>0.9 ± 1.1*</td>
<td>96.5</td>
<td>3</td>
</tr>
</tbody>
</table>

Values are means ± SE unless otherwise indicated; \(n\), no. of experiments. N1E-115 cells were suspended in a HEPES Ringer containing 30 mM bis-oxonol. Percent inhibition is the inhibition of the depolarization induced by 1 \(\mu\text{M}\) ionomycin. NMDG (0 \(\text{Na}^+\)), \(\text{Na}^+\) was isosmotically replaced with \(N\)-methyl-d-glucamine (NMDG). EGTA (0 \(\text{Ca}^2+\)), 0.5 mM EGTA was added to a \(\text{Ca}^{2+}\)-free buffer. To determine statistical significance between all data groups a one-way ANOVA was performed yielding a \(P\) value < 0.001. To determine if the specific experimental groups were different from control (1 \(\mu\text{M}\) ionomycin), a one-tailed Dunnett’s test was utilized. *Value obtained for a given experimental condition was significantly different from control (\(P < 0.05\)). NS, no statistical significant difference.
2mM ionomycin + 5mM Nickel

Fig. 3. Continuous influx of Ca^{2+} resulted in a measurable increase in [Na^{+}]. Sodium-binding benzofuran isophthalate (SBFI)-loaded N1E-115 cells were perfused with control HEPES buffer, and at the arrows the solution was changed to one containing 2 μM ionomycin with or without 5 mM Ni^{2+}. Ionomycin was used to elevate [Ca^{2+}], and to allow for continuous influx of Ca^{2+} into cells. Under these experimental conditions, a sustained level of [Ca^{2+}] was maintained (see text for added details). The ionomycin and the ionomycin + Ni^{2+} curves were obtained by averaging the response from 26 and 24 cells, respectively, and are representative of at least 3 similar experiments.

μM FCCP, simultaneous removal of Na^{+} and Ca^{2+} did not cause [Ca^{2+}], to increase; in fact, it decreased, an effect most likely due to the presence of EGTA in the bathing solution. In experiments conducted in Ca^{2+}-containing Ringer, removal of media Na^{+} following a 1 μM thapsigargin/10 μM FCCP pulse was always followed by an increase in [Ca^{2+}]; (126.8 ± 19.3 nM, n = 6). On the other hand, this same protocol in the absence of media Ca^{2+} caused [Ca^{2+}], to decrease by −37.6 ± 12.9 nM (n = 5). This decrease was significantly different (P < 0.01, paired t-test) from the [Ca^{2+}], increase seen following Na^{+} removal in a Ca^{2+}-containing Ringer. This result strongly suggests that the increase in [Ca^{2+}], following Na^{+} removal is due to a Na^{+}/Ca^{2+} exchange mechanism operating in a reverse mode favoring net Na^{+} efflux and Ca^{2+} influx.

Effect of Na^{+} loading. The intracellular Ca^{2+} buffers were able to prevent a detectable increase in [Ca^{2+}], following Na^{+} removal (Fig. 4A). Such intracellular buffers were therefore able to keep pace with the influx of Ca^{2+} via the exchanger following Na^{+} removal. However, it is possible that the putative Na^{+}/Ca^{2+} exchanger was not running at maximum velocity because of insufficient concentration of a kinetic modulator. For example, [Na^{+}], is known to modulate the reverse mode of the Na^{+}/Ca^{2+} exchanger with a K_{d} of ~25–60 mM (28).

To test this hypothesis, neuroblastoma cells were loaded with Na^{+} by using the voltage-gated Na^{+} channel activator veratridine (100 μM) and scorpion venom (4 μg/ml, Leiurus quinquestriatius from North Africa), a toxin that prevents Na^{+} channels from inactivating (5). To prevent net efflux of Na^{+} via active transport, ouabain (100 μM), which poisons the Na^{+}-K^{+}-ATPase, was also added. This treatment increased [Na^{+}], from 11.5 ± 1.4 mM (188 cells, n = 11) to 25.8 ± 2.1 mM (18 cells, n = 3) as measured using SBFI (not shown) and was statistically significant (P < 0.01, unpaired t-test).

The increase in [Ca^{2+}], following the removal of Na^{+} in Na^{+}-loaded cells was transient (Fig. 5A). This [Ca^{2+}], increase (~4- to 6-fold over resting level) peaked at 410.5 ± 95.4 nM (43 cells, n = 4) and was inhibited by 5 mM Ni^{2+} (Fig. 5B) but not by 1 μM TTX (Fig. 5C). In the presence of 5 mM Ni^{2+}, the rate of rise of [Ca^{2+}], following Na^{+} removal (0.5 ± 0.3 × 10^{-9} M/s, n = 4) was significantly less than that in its absence (5.0 ± 1.1 × 10^{-9} M/s; P < 0.01, paired t-test). In the presence of 1 μM TTX, the increase in [Ca^{2+}], (∆[Ca^{2+}]), was 178.2 ± 18.1 nM; n = 5) was not significantly different.
from that seen in its absence ($\Delta [\text{Ca}^{2+}]_i = 198.5 \pm 28.8 \text{nM}, n = 5$; unpaired $t$-test). Compared with the $[\text{Ca}^{2+}]_i$ increase following $\text{Na}^+$ removal after $\text{Ca}^{2+}$ store depletion (Fig. 4A), the increase in $[\text{Ca}^{2+}]_i$ shown in Fig. 5A was significantly greater ($P < 0.05$, unpaired $t$-test).

These results suggest that the $[\text{Ca}^{2+}]_i$ increase was caused by the reversal of the $\text{Na}^+/\text{Ca}^{2+}$ exchanger and not by the influx of $\text{Ca}^{2+}$ through $\text{Na}^+$ channels following perfusion with $\text{Na}^+$-free media. Moreover, these results are consistent with the notion that increasing $[\text{Na}^+]_i$ kinetically stimulates the putative $\text{Na}^+/\text{Ca}^{2+}$ exchanger in N1E-115 cells.

However, Fig. 5A also revealed that $[\text{Ca}^{2+}]_i$ returned to near baseline levels of $99.0 \pm 14.9 \text{nM}$ (43 cells, $n = 4$) after a few minutes despite the absence of $\text{Na}^+$. The recovery of $[\text{Ca}^{2+}]_i$ in the absence of $\text{Na}^+$ was apparently the result of a $\text{Na}^+$-independent extrusion mechanism that was activated or kinetically stimulated by the relatively large increase in $[\text{Ca}^{2+}]_i$ following $\text{Na}^+$ removal (compare Figs. 1A and 4A with Fig. 5A; see DISCUSSION).

On the basis of results shown in Figs. 4A and 5A, we predicted that reducing N1E-115 cell $\text{Ca}^{2+}$ buffering and kinetically stimulating the putative $\text{Na}^+/\text{Ca}^{2+}$ exchanger by elevating $[\text{Na}^+]_i$ should give the most robust increase in $[\text{Ca}^{2+}]_i$ seen so far. To reveal the full magnitude of the $[\text{Ca}^{2+}]_i$ increase due to the reversal of the $\text{Na}^+/\text{Ca}^{2+}$ exchange, we utilized 1 $\mu$M thapsigargin and 10 $\mu$M FCCP to compromise the cell’s buffer capacity and a 100 $\mu$M veratridine/4 $\mu$g/ml scorpion venom cocktail to elevate $[\text{Na}^+]_i$. (Fig. 6). After $\text{Na}^+$ removal, the increase in $[\text{Ca}^{2+}]_i$ was dramatic with $[\text{Ca}^{2+}]_i$ peaking at $607.3 \pm 207.4 \text{nM}$ (47 cells, $n = 3$).

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**Fig. 5.** Kinetic stimulation of the reverse mode of $\text{Na}^+/\text{Ca}^{2+}$ exchange by elevating $[\text{Na}^+]_i$. Fura 2-loaded N1E-115 cells were perfused with control HEPES buffer and then were exposed to a solution containing 100 $\mu$M veratridine (Verat), 4 $\mu$g/ml scorpion venom (SV), and 100 $\mu$M ouabain (Ouab). This solution was used to load the cells with $\text{Na}^+$ in $\text{A}$–$\text{C}$. $\text{A}$: after 5 min of $\text{Na}^+$ loading, the perfusate was changed to a $\text{Na}^+$-free (NMDG replacement) buffer containing 100 $\mu$M ouabain. After an additional 5 min, $\text{Na}^+$ was returned to the medium. The curve was generated by data averaged from 6 cells and is representative of 4 experiments. $\text{B}$: $\text{Ni}^{2+}$ blocked the $[\text{Ca}^{2+}]_i$ increase. After 5 min of $\text{Na}^+$-loading, the perfusate was changed to a $\text{Na}^+$-free (NMDG replacement) buffer with 100 $\mu$M ouabain. For the first 5 min, the buffer also contained 5 mM NiCl$_2$. After the 5 min, the $\text{Ni}^{2+}$ was washed out. At the end of the experiment, the medium was changed to one containing $\text{Na}^+$ (average response from 7 cells). Data are representative of 4 similar experiments. $\text{C}$: $\text{Ca}^{2+}$ does not move into the cell through the $\text{Na}^+$ channels. After 5 min of $\text{Na}^+$ loading, $\text{Na}^+$ was isosmotically replaced with NMDG and 1 $\mu$M TTX was added to the perfusate. At the end of the experiment, the medium was changed to one containing $\text{Na}^+$ (average response from 12 cells). Data are representative of 5 similar experiments.

**Fig. 6.** $\text{A}$: effect of $\text{Na}^+$ loading and decreased $\text{Ca}^{2+}$ buffer capacity on the reverse mode of $\text{Na}^+/\text{Ca}^{2+}$ exchange. Fura 2-loaded N1E-115 cells were perfused with control HEPES buffer and then were exposed to 1 $\mu$M thapsigargin and 10 $\mu$M FCCP to compromise the $\text{Ca}^{2+}$ buffer capacity. After 4 min the cells were loaded with $\text{Na}^+$ by using 100 $\mu$M veratridine, 4 $\mu$g/ml scorpion venom, and 100 $\mu$M ouabain. After 6 min of $\text{Na}^+$ loading, $\text{Na}^+$ was removed from the medium (isosmotically replaced with NMDG). At the end of the experiment, the medium was changed to one containing $\text{Na}^+$; part of the recovery was $\text{Na}^+$ dependent. The scale has been magnified and starts 4 min into the $\text{Na}^+$ load after the $\text{Ca}^{2+}$ buffer capacity has been reduced (average response from 12 cells and representative of 3 similar experiments).
However, compared with the peak increase in [Ca\(^{2+}\)], shown in Fig. 5A where cells were simply loaded with Na\(^+\) (−400 nM), the increase was not significant, as indicated by the relatively large SE. Again, a significant portion of the [Ca\(^{2+}\)] increase recovered in the absence of Na\(^{+}\), indicating the presence of a Na\(^{+}\)-independent Ca\(^{2+}\) regulatory mechanism.

Although significant, the recovery in Na\(^{-}\)-free media was not complete because [Ca\(^{2+}\)] remained elevated. This sustained phase of the [Ca\(^{2+}\)] increase (144 ± 11 nM; 47 cells, n = 3) was significantly greater than resting level (P < 0.05, paired t-test) and Na\(^{+}\) dependent because introduction of Na\(^{+}\) into the bath reduced [Ca\(^{2+}\)] to control levels (See Fig. 6B).

**DISCUSSION**

Under normal physiological conditions, the Na\(^{+}\)/Ca\(^{2+}\) exchanger runs in the forward direction, extruding one Ca\(^{2+}\) for three Na\(^{+}\) taken in (3). By elevating [Ca\(^{2+}\)], one can observe the cells’ ability to return [Ca\(^{2+}\)] to control levels. After addition of 10 μM FCCP and 1 μM thapsigargin, N1E-115 cells were able to recover from a [Ca\(^{2+}\)] load induced by releasing Ca\(^{2+}\) from intracellular stores. Because Ca\(^{2+}\) sequestering mechanisms were inhibited (22, 32) in the presence of 10 μM FCCP and 1 μM thapsigargin, the [Ca\(^{2+}\)] recovery was presumably achieved through extrusion mechanisms. The restoration of [Ca\(^{2+}\)] to control level following its elevation with FCCP and thapsigargin was exclusively Na\(^{+}\) dependent (Fig. 1A). Consistent with a Na\(^{+}\)/Ca\(^{2+}\) exchanger, the rate of recovery was slowed by 5 mM Ni\(^{2+}\) and blocked by replacing Na\(^{+}\) with NMDG (Fig. 1B).

We saw no evidence of a sustained Ca\(^{2+}\) entry following the initial rise in [Ca\(^{2+}\)], with 1 μM thapsigargin/10 μM FCCP addition (see Figs. 1A and 4A). Thus a capacitative Ca\(^{2+}\) entry (CCE) following Ca\(^{2+}\) release from stores was apparently absent or negligible. However, Mathes and Thompson (18) did find evidence for CCE in DMSO-differentiated N1E-115 cells. In addition, in those authors’ studies, addition of thapsigargin (1 μM) was found to increase [Ca\(^{2+}\)], by ~150 nM (18), whereas in our hands a 1 μM thapsigargin/10 μM FCCP led only to a ~60 nM increase in [Ca\(^{2+}\)]. Unlike the studies of Mathes and Thompson (18), our N1E-115 cells were not differentiated, and this difference may account for the apparent absence of CCE and the smaller [Ca\(^{2+}\)] elevation in response to thapsigargin/FCCP. In support of this notion, it has been shown that the Ca\(^{2+}\)-gated K\(^{+}\) channel in N1E-115 cells is only expressed following differentiation using DMSO (27).

**Effect of ionomycin on membrane potential.** Because the Na\(^{+}\)/Ca\(^{2+}\) exchanger has been reported to be electrogenic (3), operation of the exchanger is expected to result in current flow across the cell membrane. In addition, depending on the relative conductance of the exchanger, a measurable change in membrane potential may also occur. When [Ca\(^{2+}\)] was raised by using 1 μM ionomycin, the membrane potential depolarized by ~27 mV. In the absence of Ca\(^{2+}\), there was no depolarization, which indicates the necessity for Ca\(^{2+}\) to enter the cell for the depolarization to occur. The ionomycin-induced depolarization was Na\(^{+}\) dependent and inhibited by 5 mM Ni\(^{2+}\) and 250–500 μM benzamil (Table 1). The depolarization was not blocked by 1 μM TTX, ruling out a Na\(^{+}\) channel contribution. These results suggest that depolarization was most likely mediated by the Na\(^{+}\)/Ca\(^{2+}\) exchanger.

After Na\(^{+}\) removal, E\(_{m}\) went from approximately −60 mV to −85 mV (at t = 0 min, see Fig. 2A) and then slowly drifted upward. In normal Na\(^{+}\), E\(_{m}\) did not depolarize over time. Thus the cells hyperpolarize by as much as 25 mV following Na\(^{+}\) removal. The upward drift in E\(_{m}\) in Na\(^{+}\)-free Ringer is not understood. One possibility is that removal of Na\(^{+}\) acidifies the cells via reverse Na\(^{+}\)/H\(^{+}\) exchange and that this decrease in pH blocks a K\(^{+}\) conductance (34). On addition of ionomycin in Na\(^{+}\)-free Ringer, the linear increase in E\(_{m}\) was halted and, in fact, there was a slight hyperpolarization. Two factors may be responsible for this small effect on E\(_{m}\) following ionomycin addition in Na\(^{+}\)-free media. First, the secondary depolarization in Na\(^{+}\)-free Ringer following the initial hyperpolarization (see Fig. 2A) would tend to offset the ionomycin-induced hyperpolarization. Second, after Na\(^{+}\) removal, intracellular Na\(^{+}\) (10–15 mM) drops precipitously within 1–2 min to a few millimoles per liter (data not shown). Thus, when ionomycin was added some minutes after the cells were in Na\(^{+}\)-free Ringer, [Na\(^{+}\)], most likely was greatly reduced, and this would tend to reduce the large expected ionomycin-induced hyperpolarization.

In any event, N1E-115 cells do, in fact, hyperpolarize following Na\(^{+}\) removal as expected for reversal of a Na\(^{+}\)/Ca\(^{2+}\) exchange mechanism. However, without knowing the relative conductances of all current-generating/electrogenic mechanisms in these cells, there is no way a priori to predict what the magnitude of the hyperpolarization should be.

**Detection of the forward mode of the Na\(^{+}\)/Ca\(^{2+}\) exchanger during continuous influx of Ca\(^{2+}\) using SBFI.** During forward operation of the Na\(^{+}\)/Ca\(^{2+}\) transporter, three Na\(^{+}\) exchange for one Ca\(^{2+}\) (3). Because Ca\(^{2+}\) is a tightly regulated second messenger, it is highly unlikely that, under physiological conditions, millimolar amounts of Ca\(^{2+}\) are moved by the Na\(^{+}\)/Ca\(^{2+}\) exchange. Therefore, detecting less-than-micromolar increases in [Na\(^{+}\)] during the forward operation of the Na\(^{+}\)/Ca\(^{2+}\) exchanger is not technically possible. However, under special conditions (see Fig. 3), it is possible to measure millimolar increases in [Na\(^{+}\)], provided 1) the exchanger is fed with a continuous supply of Ca\(^{2+}\) and 2) the parameters determining the driving force favor net influx of Na\(^{+}\) (forward mode of the exchanger).

To determine what values of [Na\(^{+}\)], [Ca\(^{2+}\)], and E\(_{m}\) were needed to allow forward operation of the exchanger, the equation below was used to calculate the driving force

\[
\Delta \mu_{Na,Ca} = RT/F \log ([Na^+]_o/[Na^+]_i)^3 - \log ([Ca^{2+}]_o/[Ca^{2+}]_i) + E_m
\]
where $\Delta \mu_{Na-Ca}$ is the electrochemical potential (driving force, in mV) and $R$, $T$, and $F$ are the gas constant, the absolute temperature, and Faraday’s constant, respectively. $E_m$ and the subscripts o and i denote the membrane potential (mV) and the extra- and intracellular ion concentrations, respectively. A plot of the driving force as a function of $E_m$ at $[Na]^+$ ranging from 10 to 25 mM shows what values are required, at $[Ca^{2+}]_i$ of 475 nM, for net $Na^+$ influx to occur (Fig. 7).

Under our experimental conditions (see Fig. 7 legend), driving force calculations show that the exchanger will reverse when $[Na]^+$ reaches ~20 mM and/or when $E_m$ depolarizes to ~15 mV (Fig. 7). Because both $[Na]^+$ and $E_m$ depolarizes following ionomycin addition, the increase in $[Na]^+$ was, as expected, short lived (Fig. 3). After $[Ca^{2+}]_i$ was increased and held at ~475 mM, $[Na]^+$ levels increased ~6 mM, whereas cells treated with 5 mM Ni$^{2+}$ had no $[Na]^+$ gain (Fig. 3).

Reversal of $Na^+$/Ca$^{2+}$ exchange following removal of media $Na^+$. The $Na^+$ and Ca$^{2+}$ transmembrane gradients and $E_m$ determine whether net Ca$^{2+}$ efflux (forward mode) or influx (reverse mode) is occurring via the Na$^+$/Ca$^{2+}$ exchanger. Altering any of these components can change the direction of the exchanger. With the removal of external Na$^+$, the driving force favoring the reverse mode of the exchanger is infinite. However, simply removing Na$^+$ did not increase $[Ca^{2+}]_i$ (Fig. 4A). In contrast, when the buffer capacity was compromised before Na$^+$ removal, with 10 $\mu$M FCCP and 1 $\mu$M thapsigargin, the contribution of the Na$^+$/Ca$^{2+}$ exchange was readily seen. Reducing the Ca$^{2+}$ buffer capacity of the cell followed by Na$^+$ removal caused $[Ca^{2+}]_i$ to increase. This increase was completely dependent on the presence of Ca$^{2+}$ (Fig. 4B).

To investigate a possible kinetic effect of $[Na]^+$, as demonstrated by others (28), veratridine, scorpion venom, and ouabain were used to increase $[Na]^+$. This cocktail increased $[Na]^+$, by ~14 mM. When bath Na$^+$ was removed following $[Na]^+$ loading, $[Ca^{2+}]_i$ increased, transiently peaking to ~410 nM (Fig. 5A).

Compared Fig. 4A with Fig. 5A demonstrates that $[Na]^+$ can kinetically modulate the Na$^+$/Ca$^{2+}$ exchanger because an increase in $[Ca^{2+}]_i$ was seen only after $[Na]^+$ was elevated. The kinetic modulation increased Ca$^{2+}$ uptake via the putative Na$^+$/Ca$^{2+}$ exchanger such that it was able to swamp the cells’ Ca$^{2+}$ buffer capacity.

Interestingly, $[Ca^{2+}]_i$ returned to near baseline levels even in the absence of Na$^+$ in the experiments shown in Fig. 5 but not in those of Fig. 4. One difference in these two experiments was the level of $[Ca^{2+}]_i$, increase. On average, kinetically stimulating the Na$^+$/Ca$^{2+}$ exchanger ($Na^+$ loading) caused $[Ca^{2+}]_i$ to increase fivefold during Na$^+$ removal compared with simply reducing the cells’ buffer capacity. It appears that increasing $[Ca^{2+}]_i$ above ~200 nM stimulates or kinetically modulates another Ca$^{2+}$ extrusion mechanism that is Na$^+$ independent and functioning at a much lower rate at lower $[Ca^{2+}]_i$.

It is possible that the Na$^+$-independent recovery may be due to an ATP-driven Ca$^{2+}$ extrusion mechanism. Others have reported that Ca$^{2+}$-ATPases (pumps) may play an important role in $[Ca^{2+}]_i$ regulation (20). That is, instead of the traditional role of maintaining basal levels of $[Ca^{2+}]_i$, new evidence suggests that some isoforms of the plasma membrane Ca$^{2+}$-ATPase may play an active role in $[Ca^{2+}]_i$ regulation after cellular stimulation and may be involved in shaping Ca$^{2+}$ signals (8). The $K_m$ for the Ca$^{2+}$-ATPase (~0.5–1 $\mu$M; Ref. 9) is within the range of the Ca$^{2+}$ increases shown in Fig. 5, A and B, and this may explain why regulation is so rapid in the above experiments despite the absence of media Na$^+$.

Because the stimulatory effect of elevating $[Na]^+$ allows the Na$^+$/Ca$^{2+}$ exchanger to exceed the cells’ Ca$^{2+}$ buffer capacity, one would predict that reducing the buffer capacity and kinetically stimulating the exchanger would also yield a large $[Ca^{2+}]_i$ increase. Figure 6 shows when bath Na$^+$ was removed following FCCP/thapsigargin and veratridine/scorpion toxin treatments, $[Ca^{2+}]_i$ increased to a peak value that was significantly greater than that following FCCP/thapsigargin treatment alone ($P < 0.05$). Here again, there was a significant recovery in the absence of Na$^+$. However, there was also a significant sustained increase of ~90 nM above baseline $[Ca^{2+}]_i$ (see Fig. 5C). The sustained increase in $[Ca^{2+}]_i$ was $Na^+$ dependent because addition of Na$^+$ caused $[Ca^{2+}]_i$ to return to control levels. This Na$^+$-dependent decrease in $[Ca^{2+}]_i$ is presumably the result of activation of the Na$^+$/Ca$^{2+}$ exchanger in the forward mode.

In summary, our data strongly suggest that there are at least two extrusion mechanisms involved in $[Ca^{2+}]_i$ regulation in N1E-115 neuroblastoma cells: a Na$^+$/Ca$^{2+}$ exchanger, which has been our primary focus; and a second, Na$^+$-independent mechanism (possibly a Ca$^{2+}$-ATPase) that is further stimulated/kinetically modulated following relatively large increases in $[Ca^{2+}]_i$. Under pathophysiological conditions, increases...
ing $[\text{Na}^+]_i$ may set into motion a destructive pathway of events (29). $[\text{Na}^+]_i$ overload via voltage-gated Na$^+$ channels can lead to $[\text{Ca}^{2+}]_i$ overload via reversal of the Na$^+$/Ca$^{2+}$ exchanger (30). The existence of both a Na$^+$/Ca$^{2+}$ exchanger and Na$^+$ channels may make the N1E-115 neuroblastoma a potentially valuable model for studying neurotoxicity from $[\text{Na}^+]_i$ and $[\text{Ca}^{2+}]_i$ overload.

REFERENCES


