Iodide and bromide inhibit Ca\textsuperscript{2+} uptake by cardiac sarcoplasmic reticulum

GARY J. KARGACIN, ZENOBIA ALI, SHI-JIN ZHANG, NATASHKA S. POLLOCK, AND MARGARET E. KARGACIN

Department of Physiology and Biophysics, University of Calgary, Calgary, Alberta, Canada T2N 4N1

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There has been a great deal of interest recently in the effects of halides and other anions on the function of the sarcoplasmic reticulum (SR). It has been reported, for example, that some anions including Cl\textsuperscript{−} and I\textsuperscript{−} can increase the Ca\textsuperscript{2+} permeability of the skeletal muscle ryanodine receptor over that measured in the presence of organic anions such as propionate\textsuperscript{−} (2, 8, 25, 26, 29). A Cl\textsuperscript{−}-sensitive nonselective channel that allows Ca\textsuperscript{2+} to permeate the SR membrane has also been postulated to exist in skeletal muscle (39). Other investigators (37) suggested that the SR Ca\textsuperscript{2+} pump itself can be directly inhibited by I\textsuperscript{−} and SCN\textsuperscript{−}. In addition to affecting these mechanisms, the anionic environment of the SR could also influence the movement of compensatory charge across the SR membrane during Ca\textsuperscript{2+} uptake or release. That such movement occurs has been inferred primarily from the following two types of observations (reviewed in Refs. 3, 7, 22, 23, 30, and 40): 1) although the Ca\textsuperscript{2+}-ATPase of the SR of muscle cells is believed to be electrogenic, a sustained membrane potential has not been detected across the SR membrane; and 2) both anion and cation channels are found in SR membranes. Thus other ions are thought to cross the membrane to balance charge as Ca\textsuperscript{2+} is released from the SR or actively transported into the SR. In support of this mechanism in smooth muscle, we (33) have shown that SR Ca\textsuperscript{2+} uptake is inhibited by the Cl\textsuperscript{−} channel blockers tamoxifen or I\textsuperscript{−}. The latter effect occurs without an alteration in the ATPase activity of the SR Ca\textsuperscript{2+} pump and without a significant change in the permeability of the SR membrane to Ca\textsuperscript{2+}.

In the work reported here, we studied Ca\textsuperscript{2+} uptake into cardiac SR vesicles when I\textsuperscript{−}, Br\textsuperscript{−}, or SO\textsubscript{4}\textsuperscript{2−} were substituted for extravesicular Cl\textsuperscript{−}. SR Ca\textsuperscript{2+} uptake was not inhibited by the substitution of SO\textsubscript{4}\textsuperscript{2−} for Cl\textsuperscript{−}. Net Ca\textsuperscript{2+} uptake rate was significantly reduced, however, when Br\textsuperscript{−} or I\textsuperscript{−} was substituted for Cl\textsuperscript{−}. This occurred without a detectable effect of I\textsuperscript{−} or Br\textsuperscript{−} on the ATPase activity of the SR Ca\textsuperscript{2+} pump and without an increase in the Ca\textsuperscript{2+} permeability of the SR membrane.

Methods

Preparation of SR vesicles. Animals were euthanized with an overdose of pentobarbital sodium in accordance with procedures approved by the Canadian Council on Animal Care and the University of Calgary Faculty of Medicine Animal Care Committee. SR vesicles were prepared from canine ventricular tissue as previously described (15) according to the method of Chamberlain et al. (1) omitting the sucrose density gradient centrifugation step.

Measurement of SR Ca\textsuperscript{2+} uptake. The fluorometric method for measuring Ca\textsuperscript{2+} uptake into SR vesicles using fura 2 was described previously (15, 16, 18). Briefly, SR vesicles were first diluted to a total protein concentration of 1 mg/ml in uptake buffer containing 100 mM KX (where X is Cl\textsuperscript{−}, I\textsuperscript{−}, or SO\textsubscript{4}\textsuperscript{2−}).

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Address for reprint requests and other correspondence: G. J. Kargacin, Dept. of Physiology and Biophysics, Univ. of Calgary, 3330 Hospital Dr. NW, Calgary, AB, Canada T2N 4N1 (E-mail: kargacin@ucalgary.ca).


\[ \text{I}^- \text{ AND Br}^- \text{ INHIBIT CARDIAC SR UPTAKE} \]

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Br\(^-\), 4 mM MgX\(_2\), and 20 mM HEPES (pH 7.0); or 50 mM K\(_2\)SO\(_4\), 4 mM MgSO\(_4\), and 20 mM HEPES. Vesicle samples were then added to a 4-ml cuvette containing 2 ml of uptake buffer. For most experiments, oxalate was included in uptake buffer to act as a trapping ion for Ca\(^{2+}\) within the SR (28). When oxalate was not included, a higher vesicle protein concentration and other adjustments to the uptake buffers were required to increase measurable SR Ca\(^{2+}\) uptake. Differences in the uptake buffers that were used in experiments done in the presence and absence of oxalate are shown in Table 1. When tetraethylammonium (TEA; 10 mM) was added to either KI or KCl uptake buffer, the K\(^+\) concentration of the buffers was reduced by 10 mM to maintain ionic strength. Uptake was initiated by the addition of Ca\(^{2+}\) to the cuvette. Fluorescence was measured with a SPEX fluorimeter (CMX model; Edison, NJ). Fura 2 was excited at 340- and 380-nm wavelengths, and fluorescence emission was measured through a 510-nm band-pass filter (10-nm bandwidth). Fluorescence ratios (340–380 nm) were obtained every 1 s. The contents of the cuvette were continuously stirred during the course of an experiment.

**Determination of velocity of Ca\(^{2+}\) uptake.** For experiments done in the presence of oxalate, the free Ca\(^{2+}\) concentration ([Ca\(^{2+}\)\(_{\text{free}}\)], total Ca\(^{2+}\) concentration ([Ca\(^{2+}\)\(_{\text{total}}\]), and instantaneous uptake velocity were determined from the fura 2 fluorescence ratio values after correction for background fluorescence and light scatter as described previously (9, 14–18). [Ca\(^{2+}\)\(_{\text{total}}\) was determined from [Ca\(^{2+}\)\(_{\text{free}}\)] as described previously (14–18) using published binding constants (4, 12, 27, 36). The protein concentration of the vesicle preparations was determined by the Bradford protein assay (kit purchased from Bio-Rad; Hercules, CA). The maximum velocity of Ca\(^{2+}\) uptake was determined from plots of uptake velocity versus [Ca\(^{2+}\)\(_{\text{free}}\)] as described by Kargacin and Kargacin (15).

Oxalate is capable of crossing the SR membrane and acts as a Ca\(^{2+}\)-precipitating anion within SR vesicles (28). The advantages of using oxalate in uptake buffers are that it permits greater unidirectional Ca\(^{2+}\) movement and prolongs the initial rapid phase of uptake (28). This allows uptake to be determined from a single vesicle sample over the entire range of physiological [Ca\(^{2+}\)\(_{\text{free}}\]) (15, 18). As discussed below (see RESULTS), however, it was necessary to conduct some experiments in the absence of oxalate. Without oxalate acting as a trapping ion in the SR lumen, as uptake occurs, the free Ca\(^{2+}\) gradient that develops across the vesicle membrane inhibits further uptake. This causes a decline in the rate of uptake to occur at free Ca\(^{2+}\) levels that are higher than those required to define complete uptake velocity curves similar to those obtained in the presence of oxalate. Therefore, for experiments done without oxalate, velocity was determined from the slope of the steepest part of [Ca\(^{2+}\)\(_{\text{total}}\)] versus time curves.

In either the presence or absence of oxalate, the rate of Ca\(^{2+}\) uptake for a specific cardiac SR vesicle preparation is dependent on the purity of the preparation. For this reason, to allow experiments done with different vesicle preparations to be compared, uptake velocities are expressed as a percentage of the average control value (set at 100%) obtained in each experiment. The actual values for the uptake velocities of the control samples done in the presence of oxalate ranged from ~0.3 to ~0.7 µmol·min\(^{-1}\)·mg\(^{-1}\) for the vesicle preparations used in the present study.

**Measurement of SR Ca\(^{2+}\) release.** For Ca\(^{2+}\) release experiments, the uptake buffers described above were used except that 5 mM d-glucose was included as a substrate for hexokinase and creatine phosphate (CP) and creatine phosphokinase (CPK) were omitted from the uptake buffer. After the SR was loaded and net Ca\(^{2+}\) movement into the SR stopped, hexokinase (4.7 U/ml) was added to the cuvette to deplete the buffer of ATP to allow passive Ca\(^{2+}\) release to be measured.

**Measurement of ATPase activity of SR Ca\(^{2+}\) pump using NADH fluorescence.** An enzyme-coupled assay (19) in which ATP hydrolysis by the SR Ca\(^{2+}\) pump is coupled to the conversion of NADH to NAD\(^+\) was adapted to determine whether substitution of Br\(^-\) or I\(^-\) for Cl\(^-\) directly inhibited the SR Ca\(^{2+}\) pump. The ATPase activity of the pump was measured in a 4-ml cuvette in buffers containing 100 mM KX (where X is Cl, I, or Br), 4 mM MgX\(_2\), 0.15 mM NADH, 0.21 U/ml pyruvate kinase, 0.46 mM phosphoenolpyruvate, 2.2 U/ml lactate dehydrogenase (LDH), 1.1 mM ATP, 3.3 mM 4-Br-A23187, and 20 mM HEPES (pH 7.0; [Ca\(^{2+}\)\(_{\text{free}}\)~3 µM]. The Ca\(^{2+}\) ionophore 4-Br-A23187 was included in the buffer to prevent the ATPase activity of the SR Ca\(^{2+}\) pump from being influenced by the reduction of extravesicular Ca\(^{2+}\) or by the establishment of a Ca\(^{2+}\) gradient across the SR membrane (discussed in Ref. 5). Solutions in the cuvette were continuously stirred throughout an experiment. Background fluorescence measurements (made with vesicles and all components except NADH in the cuvette) and measurements of the change in NADH fluorescence (made with all components except ATP in the cuvette) were used to correct the measurements of the changes in NADH fluorescence due to the ATPase activity of the SR Ca\(^{2+}\) pump. Calibration curves for NADH fluorescence were determined (Fig. 1A) by adding known amounts of NADH to KCl, KBr, or KI buffer. There were no effects of I\(^-\) or Br\(^-\) on NADH fluorescence. The calibration curves (which deviate from linearity due to the inner filter effect; see Ref. 24) were fit by the equation

\[
F = a(1 - e^{-b[NADH]})
\]  

where F is the fluorescence intensity in counts per second and a and b are constants. For SR vesicles, the change in NADH content in the sample was linear (see Fig. 5); the rate of change of NADH was therefore determined from linear regression for a 100- to 200-point (50–100 s) segment of the data. For the assay, the sample sizes ranged from 30–190 µg vesicle protein depending on the SR vesicle preparation used. To rule out the possibility that the ATPase assay itself was rate limiting, control experiments were done to show that doubling the amounts of pyruvate kinase and LDH in the assay did not change the Ca\(^{2+}\)-ATPase rates measured by the assay. The ATPase activity of the preparation (when expressed as µmol·min\(^{-1}\)·mg\(^{-1}\) vesicle protein) was also not altered when the amount of vesicle protein was doubled.

To determine whether substitution of Cl\(^-\) with Br\(^-\) or I\(^-\) had a direct effect on the enzyme-coupled ATPase assay, NADH fluorescence changes (Fig. 1B) were measured by adding ADP to KCl, KBr, or KI buffers plus assay components but without ATP or SR vesicles present. The results of these experiments showed that Br\(^-\) and to a greater extent I\(^-\) inhibit the assay itself. As shown in Fig. 1C, the initial rate of change of NADH fluorescence in the ATPase assay in response to ADP addition was reduced to 65.2% of control in

Table 1. Differences in buffers used in experiments done in presence and absence of oxalate

<table>
<thead>
<tr>
<th>Buffer</th>
<th>Vesicle Protein, µg</th>
<th>K(_{\text{ATP}}), mM</th>
<th>CP, mM</th>
<th>CPK, U/ml</th>
<th>Fura 2, µM</th>
</tr>
</thead>
<tbody>
<tr>
<td>+ Oxalate (10 mM)</td>
<td>25</td>
<td>1.3</td>
<td>1.2</td>
<td>3.0</td>
<td>2.9</td>
</tr>
<tr>
<td>− Oxalate</td>
<td>240–360</td>
<td>2.6</td>
<td>2.4</td>
<td>6.0</td>
<td>2.9</td>
</tr>
</tbody>
</table>

CP, creatine phosphate; CPK, creatine phosphokinase.
KBr buffer and to a greater extent (29.4% of control) in KI buffer. These results were used to correct the measurements of ATPase activity of the cardiac SR Ca\(^{2+}\) pump.

Measurement of ATPase activity of SR Ca\(^{2+}\) pump using purine-nucleoside phosphorylase. A second spectrophotometric assay (43, 44) in which the inorganic phosphate (P\(_i\)) generated by the ATPase activity of the SR Ca\(^{2+}\) pump was measured was also used to determine whether substitution of I\(^-\) for Cl\(^-\) had a direct inhibitory effect on the SR Ca\(^{2+}\)-ATPase. In the assay, purine-nucleoside phosphorylase (PNP) converts 2-aminopyridine-6-mercaptopurine riboside (MESG) and P\(_i\) to ribose 1-phosphate (R1-P) and 2-aminopyridine-7-methylpurine (AMM) (Eq. 2)

\[
\text{MESG} + P_i \leftrightarrow \text{R1-P} + \text{AMM}
\]

As the SR Ca\(^{2+}\) pump hydrolyzes ATP and generates P\(_i\), conversion of MESG (maximum absorbance at 330 nm) to AMM (maximum absorbance at 360 nm) results in an absorbance increase at 360 nm that is proportional to the change in P\(_i\). The generation of P\(_i\) by turnover of the cardiac SR Ca\(^{2+}\) pump was measured in buffer containing 100 mM KX (where X is Cl or I), 4 mM MgX\(_2\), 1.1 mM ATP, 1 U/ml PNP, 150 mM MESG, and 20 mM HEPES (pH 7.0; [Ca\(^{2+}\)])\(_{in}\) = 3 \(\mu\)M). 4-Br-A23187 (3.3 \(\mu\)M) was included in the buffer for the reasons discussed above. Measurements were made in a 4-ml cuvette with an ultraviolet/visible spectrophotometer (model Lambda 3B, Perkin-Elmer; Norwalk, CT); solutions in the cuvette were continuously stirred during the experiments. The assay system was calibrated by adding known amounts of P\(_i\) to the buffers. The calibration curves in Fig. 2A show that the magnitude of the change in absorbance for a given change in P\(_i\) was slightly less in KI buffer than it was in KCl buffer. The rate at which the assay responded to step changes in P\(_i\) was also compared in KCl and KI buffers. Figure 2B shows that the response of the assay to step changes in P\(_i\) was slightly slower in the KI buffer. The mean initial rate of response in KI buffer was determined as shown in Fig. 2B for a variety of step changes and starting P\(_i\) concentrations was 79.4 \pm 4.6% \((n = 7)\) of that measured in KCl buffer. Figure 2C shows that the rate at which the enzyme system could respond to changes in P\(_i\) was not limited by the rate at which solutions could be stirred into the cuvette.

For the measurement of the ATPase activity of the SR Ca\(^{2+}\) pump, all assay components except cardiac SR vesicles were first added to the buffer and the reaction described in Eq. 2 was allowed to proceed to remove contaminating P\(_i\) in the buffer. Vesicles (30 \(\mu\)g) were then added, and the additional P\(_i\) generated by the SR Ca\(^{2+}\) pump was determined as a function of time from the change in absorbance at 360 nm (measured at 1-s intervals) with the use of the calibrations shown in Fig. 2. The slopes of the P\(_i\) versus time curves (determined by a linear fit to the steepest linear part of the curve; typically 100 data points = 100 s) were determined and used to compare the ATPase activity of the SR Ca\(^{2+}\) pump in different solutions. Figure 2D shows a curve of P\(_i\) versus time for an experiment with SR vesicles in KCl buffer.

Reagents. ADP (potassium salt), K\(_2\)ATP, CP, CPK, NADH, phosphoenol pyruvate, pyruvate kinase, dithiothreitol, 4-aminopyridine, D-glucose, and histidine were purchased from Sigma Chemical (St. Louis, MO). Aristar grade KCl, KOH, and sucrose, Suprapur KI, and AnalR KBr were purchased from BDH (Edmonton, AB, Canada). Hexokinase was purchased from Roche Diagnostics (Laval, QC, Canada). Microselelg MgCl\(_2\).6H\(_2\)O and MgBr\(_2\).6H\(_2\)O, puriss grade oxalic acid and MgSO\(_4\).7H\(_2\)O, purum grade TEA hydroxide, and MgL\(_2\), ruthenium red, and HEPES (potassium salt) were purchased from Fluka (Ronkonkona, NY). LDH was purchased from Worthington (Freehold, NJ). Fura 2 free acid,
4-Br-A23187, and the reagents for the purine-nucleoside phosphorylase assay (ENZCheck kit) were purchased from Molecular Probes (Eugene, OR).

Experiments were carried out at 22°C. In RESULTS, errors are expressed as ±1 SD.

RESULTS

Effect of anion substitutions on Ca\(^{2+}\) uptake in SR vesicles in presence of oxalate. Substitution of K\(_2\)SO\(_4\) for KCl in the uptake buffer did not significantly change the rate at which Ca\(^{2+}\) was taken up into the SR in the presence of oxalate. A comparison of uptake rates in K\(_2\)SO\(_4\) and KCl buffers is shown in Fig. 3A. The uptake rate in KBr uptake buffer was less than that measured in KCl uptake buffer (Fig. 3B) and was slower still in KI uptake buffer (Fig. 3C). For the experiments shown, the maximum uptake rate was 71.9% of control in KBr and 44.4% of control in KI buffer. Consistent with the results shown in Fig. 3, A–C, the results of several similar paired experiments showed that the maximum velocity was significantly
lower in KBr (69.8 ± 4.6% in KBr, n = 7; 100.0 ± 7.8% in KCl, n = 7) and KI (39.3 ± 2.6% in KI, n = 6; 100.0 ± 13.4% in KCl, n = 5) buffers. Uptake velocity was not significantly different from control in K2SO4 buffer (94.4 ± 8.8% in K2SO4, n = 5; 100.0 ± 13.4% in KCl, n = 8). Figure 3D shows that the inhibitory effects of Br− and I− on uptake velocity were concentration dependent.

Inhibition of Ca2+ uptake by I− in absence of oxalate. As noted above (see METHODS), measurement of SR Ca2+ uptake in the presence of oxalate allows one to determine uptake rate for a single sample over a range of physiological [Ca2+]free. However, although the relative concentration of oxalate to the other anions used in the experiments described above was low (10 mM oxalate:108 mM other anions), the presence of oxalate in the KBr and KI buffers may have partially masked the full inhibitory effect of these anions on Ca2+ uptake. To test this possibility, the magnitude of the inhibitory effect of I− (the anion that inhibited SR uptake to the greatest extent) on Ca2+ uptake was measured in the absence of oxalate. Figure 3E shows that in the ab-
Inhibitory effect of I\textsuperscript{−} on SR Ca\textsuperscript{2+} uptake in presence of ruthenium red. It is possible that SR Ca\textsuperscript{2+} uptake could be inhibited in the presence of Br\textsuperscript{−} or I\textsuperscript{−} if these anions increased the permeability of the SR membrane to Ca\textsuperscript{2+}. The increased leak of Ca\textsuperscript{2+} out of the SR would reduce the net amount of Ca\textsuperscript{2+} moved into the SR by the Ca\textsuperscript{2+} pump over any time interval. This possibility is consistent with recent results indicating that the permeability of the skeletal muscle SR membrane to Ca\textsuperscript{2+} is greater in the presence of some inorganic anions than it is in the presence of organic anions such as propionate\textsuperscript{−} (2, 8, 25, 26, 29, 31, 39). These reported increases in permeability, whether they occur through the ryanodine receptor (2, 8, 25, 26, 29) or through a different SR channel (39), are blocked by ruthenium red (see Refs. 8, 26, and 39). Therefore, if Br\textsuperscript{−} or I\textsuperscript{−} inhibited net SR Ca\textsuperscript{2+} uptake in our experiments by increasing the permeability of the cardiac SR membrane through one of these mechanisms, one would expect ruthenium red to block this inhibition. Because the Ca\textsuperscript{2+} sensitivity of the cardiac SR Ca\textsuperscript{2+}-ATPase is reduced by ruthenium red (17), Ca\textsuperscript{2+}−ATPase measurements made in KI uptake buffer containing ruthenium red were compared with control experiments done in KCl buffer with the same concentration of ruthenium red. As shown in Fig. 4A, SR Ca\textsuperscript{2+} uptake was reduced by I\textsuperscript{−} even in the presence of ruthenium red. The extent of inhibition by I\textsuperscript{−} was the same when ruthenium red (20 \textmu M) was present in the uptake buffers (summarized in Fig. 4B) as it was in the absence of ruthenium red (mean uptake rate in KI with ruthenium red was 32.0 ± 6.1% of the rate in KCl with ruthenium red; mean uptake rate in KI without ruthenium red was 39.3 ± 2.6% of the rate in KCl buffer without ruthenium red).

Our results with ruthenium red show that the inhibitory action of I\textsuperscript{−} on SR Ca\textsuperscript{2+} uptake that was seen in our experiments cannot be explained by an I\textsuperscript{−}-induced increase in the permeability of the SR membrane to Ca\textsuperscript{2+} that involves one of the mechanisms discussed above. It is possible, however, that I\textsuperscript{−} or Br\textsuperscript{−} increased the permeability of a SR Ca\textsuperscript{2+} channel that is not sensitive to ruthenium red. To test this possibility, we compared the leakiness of the cardiac SR membrane to Ca\textsuperscript{2+} in the presence and absence of I\textsuperscript{−} by measuring the passive rate of release of Ca\textsuperscript{2+} from the SR when the Ca\textsuperscript{2+} pump was rapidly deprived of ATP. This was done by first allowing SR vesicles to take up Ca\textsuperscript{2+} to the same extent in either KCl or KI buffer and then adding hexokinase to rapidly hydrolyze the ATP available to the Ca\textsuperscript{2+} pump (see Ref. 5). The results of one of these experiments are shown in Fig. 4, C and D. It can be seen that the rate of release of Ca\textsuperscript{2+} from the SR is faster in KCl buffer than it is in KI buffer (release rate in KCl buffer was 1.94 ± 0.19 nmol·min\textsuperscript{−1}·mg\textsuperscript{−1}, n = 5; release rate in KI buffer was 1.52 ± 0.06 nmol·min\textsuperscript{−1}·mg\textsuperscript{−1}, n = 5). This is inconsistent with an I\textsuperscript{−}-induced increase in the permeability of the SR membrane to Ca\textsuperscript{2+}.

ATPase activity of SR Ca\textsuperscript{2+} pump in presence of Br\textsuperscript{−} or I\textsuperscript{−}. A second mechanism that could account for the inhibitory effects of I\textsuperscript{−} and Br\textsuperscript{−} on SR Ca\textsuperscript{2+} uptake is a direct effect of these ions on the SR Ca\textsuperscript{2+} pump. This possibility is consistent with some results in the literature (37) but not with others (11). To determine whether I\textsuperscript{−} or Br\textsuperscript{−} directly inhibited the SR Ca\textsuperscript{2+} pump in our experiments, two methods were used to determine the ATPase activity of the SR Ca\textsuperscript{2+} pump in the presence of Cl\textsuperscript{−}, Br\textsuperscript{−}, and I\textsuperscript{−}.

With the use of the ATPase assay in which NADH fluorescence is measured (see METHODS), Stefanova et al. (37) reported a direct inhibition of the skeletal muscle SR Ca\textsuperscript{2+}-ATPase by I\textsuperscript{−}; however, these authors did not discuss any effect of I\textsuperscript{−} on the assay itself. Therefore, because we found that the rate at which the assay responded to fixed concentration of ADP was reduced in KBr buffer to 65.2% of the rate seen in KCl and, to a greater extent (29.4%), in KI buffer (see METHODS and Fig. 1), we examined the ATPase activity of the cardiac SR Ca\textsuperscript{2+} pump in the presence of Br\textsuperscript{−} and I\textsuperscript{−} and made corrections for the effects of these ions on the enzyme-coupled assay itself. Before correction for the direct inhibition of the assay by Br\textsuperscript{−}, the maximum ATPase rate of the SR vesicles in KBr buffer was 72.9% of the maximum rate measured in KCl; before correction, the maximum rate in KI buffer was 38% of control (see Fig. 5B). The apparent reductions in the rates in Br\textsuperscript{−} and I\textsuperscript{−} could be completely accounted for by the inhibitory effects of Br\textsuperscript{−} and I\textsuperscript{−} on the enzyme-coupled ATPase assay (Fig. 5, A and B). Although the corrected results in Fig. 5B suggest that the ATPase rates in KBr and KI buffers may be somewhat faster than those measured in KCl buffer (significantly higher than those measured in KCl buffer at P = 0.026 in KBr and P = 0.034 in KI), the second ATPase assay (see below) indicates that this was probably not the case.

Because the rate of oxidation of NADH was reduced for the assay itself in the presence of Br\textsuperscript{−} or I\textsuperscript{−} (as shown in Fig. 1), it might be argued that an additional effect of inhibitors on the SR Ca\textsuperscript{2+}-ATPase could not be detected with this assay. To rule out this possibility, thapsigargin [a specific inhibitor of sarco(endo)plasmic reticulum Ca\textsuperscript{2+}-ATPase (SERCA) Ca\textsuperscript{2+} pumps; see Ref. 42] was used to reduce the ATPase activity of the pump in the presence of Cl\textsuperscript{−} or I\textsuperscript{−}, and the rate of change in NADH fluorescence was determined. In two experiments in KCl buffer, 5 \textmu M thapsigargin reduced the maximum ATPase rate of the cardiac SR vesicle preparation to 27.6% (range = 5%) of the rate measured in its absence. In KI buffer, the inhibitory effect of 5 \textmu M thapsigargin on the measured ATPase rate was detectable and was 29.3 ± 9.7% of the rate mea-
sured in KI buffer without thapsigargin (n = 4). In the presence of 10 μM thapsigargin, the measured ATPase rate in KCl buffer was 20 ± 8% (n = 3) of that measured in its absence and was not significantly different from that measured in 5 μM thapsigargin.

Because of the inhibitory effect of I− on the enzyme-coupled assay utilizing pyruvate, pyruvate kinase, LDH, and NADH, a second method was also used to compare the ATPase activity of the cardiac SR Ca2+ pump in KCl and KI buffers. The purine-nucleotide absorbance assay, described by Eq. 2 (see METHODS), was also affected by the presence of I− but to a far lesser extent than the first assay (compare Figs. 1 and 2). Figure 6 summarizes the results obtained with the absorbance assay and indicates that, after the correction of the results for the direct inhibition of the assay by I−, there were no differences in the ATPase rates of the SR Ca2+ pump in KCl and KI buffers. The ATPase rates measured for vesicles from the same preparation in KCl buffer with this assay (0.398 μmol·min⁻¹·mg⁻¹ in KCl buffer and 1.61 nmol·min⁻¹·mg⁻¹ in KI buffer) are in good agreement with those obtained using the NADH fluorescence assay (0.395 μmol·min⁻¹·mg⁻¹ in KCl buffer and 1.86 × 10⁻³ μM/s for KI (solid line). These rates, normalized to total protein, were 1.97 nmol·min⁻¹·mg⁻¹ in KCl buffer and 1.61 nmol·min⁻¹·mg⁻¹ in KI buffer.

Because of the inhibitory effect of thapsigargin on the enzyme-coupled assay utilizing pyruvate, pyruvate kinase, LDH, and NADH, a second method was also used to determine the ATPase rates of the SR Ca2+ pump in KCl and KI buffers. The NADH fluorescence assay (see METHODS), was also affected by the presence of I− but to a far lesser extent than the first assay (see above). Figure 6 shows the results obtained with the NADH fluorescence assay (0.395 μmol·min⁻¹·mg⁻¹ in KCl buffer and 1.86 × 10⁻³ μM/s for KI (solid line). These rates, normalized to total protein, were 1.97 nmol·min⁻¹·mg⁻¹ in KCl buffer and 1.61 nmol·min⁻¹·mg⁻¹ in KI buffer.

Fig. 4. Substitution of Cl− with I− does not alter the permeability of the SR membrane to Ca2+. A and B: inhibition of SR Ca2+ uptake by I− in the presence of ruthenium red. A: SR Ca2+ uptake as a function of time in KCl buffer with 20 μM ruthenium red (bottom trace) and KI buffer with 20 μM ruthenium red (top trace). For this experiment, uptake rate was 0.248 μmol·min⁻¹·mg⁻¹ in KCl buffer and 0.098 μmol·min⁻¹·mg⁻¹ in KI buffer. B: summary of results with ruthenium red. Maximum rate of SR Ca2+ uptake in KI buffer + 20 μM ruthenium red was 32 ± 6% (n = 8; crosshatched bar) of the maximum rate of SR Ca2+ uptake in KCl buffer + 20 μM ruthenium red (100 ± 18%, n = 8; open bar). Error bars are ±1 SD. C and D: Ca2+ release in the presence of Cl− and I−. C: cardiac SR samples (0.145 mg) were loaded with Ca2+ in the presence of ATP (K0.76 mM) in KCl (○) and KI (●) uptake buffer. Ca2+ uptake was allowed to proceed until [Ca2+] free declined to 60 nM in the cuvette. Hexokinase was then added (at 335 s for KCl; 500 s for KI) to rapidly deplete the buffer of ATP, and the rates of the resulting Ca2+ release were compared in the two buffers. D: Ca2+ release segments from the curves in C are shown for KCl (○) and KI (●) aligned on the time axis for comparison. Release rates were determined by a linear regression fit to the first 100 points of the release curves. Release rates were 2.28 × 10⁻³ μM/s in KCl (regression line shown by dashed line) and 1.86 × 10⁻³ μM/s for KI (solid line). These rates, normalized to total protein, were 1.97 nmol·min⁻¹·mg⁻¹ in KCl buffer and 1.61 nmol·min⁻¹·mg⁻¹ in KI buffer.
SR uptake in presence of TEA or 4-aminopyridine. Although it has been suggested (see Refs. 22 and 23) that Cl$^-$ may be the primary ion involved in compensating for the net positive charge moved into the SR by the Ca$^{2+}$-ATPase, movement of K$^+$ out of the SR lumen may also occur and could become more important under conditions in which anion movement is inhibited to the greatest extent (i.e., KI buffer). To test the possibility that efflux of K$^+$ out of the SR occurred during SR Ca$^{2+}$ uptake, uptake rates in KCl and KI buffers were compared in the presence and absence of 4-aminopyridine or TEA. These blockers were used because they are known to block skeletal muscle SR K$^+$ channels from the cytoplasmic side of the SR membrane at the concentrations used in our experiments (6, 7). Figure 7 shows that 1 mM 4-aminopyridine had no significant effect on the maximum rate of SR Ca$^{2+}$ uptake in KCl buffer or on the reduced uptake rate measured in KI buffer. The maximum rates of uptake in KCl or KI buffers were also unaffected by 10 mM TEA (results not shown).

DISCUSSION

Table 2 summarizes the results of our study showing that, although the substitution of extravesicular Cl$^-$ with either Br$^-$ or I$^-$ inhibits Ca$^{2+}$ uptake into cardiac SR vesicles, neither ion has a direct inhibitory effect on the SR Ca$^{2+}$ pump nor does the inhibition of uptake appear to be accompanied by an increase in the Ca$^{2+}$ permeability of the SR membrane. As discussed below, our results are consistent with the hypothesis that the anionic environment of the SR can modulate Ca$^{2+}$ up-

Fig. 5. Hydrolysis of ATP by the SR Ca$^{2+}$ pump in KCl, KBr, and KI buffers determined with the NADH fluorescence assay. A: NADH as a function of time monitored by the NADH fluorescence assay for 190-μg SR vesicle samples in KCl and KI buffers. Raw data obtained before correction for the effect of I$^-$ on the ATPase assay are shown (solid symbols). The maximum rates of change in NADH for the raw data (red lines) were determined by line fits to the data segments between the yellow symbols. These slopes were −1.25 nmol/s in KCl buffer and −0.457 nmol/s in KI buffer. These values correspond to ATPase rates of 0.395 μmol P$_i$·min$^{-1}$·mg$^{-1}$ in KCl and 0.144 μmol P$_i$·min$^{-1}$·mg$^{-1}$ in KI buffer, respectively. The maximum uncorrected rate of change in NADH in KI was 36.8% of that measured in KCl buffer; this difference in rate could be entirely accounted for by the inhibition of the ATPase assay itself by I$^-$ (response of the assay in KI buffer to a step change in ADP was reduced to 29.4 ± 9.8% of control; see Fig. 1). The change in NADH in KI buffer after correction of the raw data for the inhibition of the ATPase assay itself by I$^-$ is shown by the blue line (the slope of the corrected KI data = −1.55 nmol/s). B: ATPase activity of the SR Ca$^{2+}$ pump in KCl, KBr, and KI buffers. ATPase rates, measured for SR vesicle samples, are expressed as a percentage of the maximum rates in KCl buffer (red bars) before (open bars) and after (blue bars) correction for the effects of Br$^-$ or I$^-$ on the ATPase assay (n values are shown above the bars). Error bars are ±1 SD. Br$^-$ and I$^-$ denote uncorrected results; *Br$^-$ and *I$^-$ denote corrected results.

Fig. 6. Hydrolysis of ATP by the SR Ca$^{2+}$ pump in KCl and KI buffers determined with the PNP (absorbance) assay. ATPase activity expressed as a percentage of the mean activity in KCl buffer (crosshatched bar) before (open bar) and after (hatched bar) correction for the effect of I$^-$ on the ATPase assay itself (corrected rate in I$^-$ = measured rate/0.808). The mean corrected ATPase rate of the SR Ca$^{2+}$ pump in KI buffer was not significantly different from that measured in KCl (P = 0.68; n = 5 for all experiments). Error bars are ±1 SD. I$^-$ denotes uncorrected results in KI buffer; *I$^-$ denotes corrected results.
take by altering the movement of negative charge into the SR that occurs as Ca^{2+} is actively transported by the Ca^{2+} pump.

Recent interest in the influence that the anionic environment of the SR may have on its ability to regulate intracellular Ca^{2+} in muscle cells has been focused primarily on the modulation of Ca^{2+} channels in the SR that mediate Ca^{2+} release or control the leakiness of the SR membrane to Ca^{2+}. In skeletal muscle, Cl\(^{-}\) and some other inorganic anions (including I\(^{-}\); reviewed in Ref. 25) can increase the rate of release of Ca^{2+} through the ryanodine receptor Ca^{2+} channel over the release rates measured in the presence of inorganic anions such as propionate, methanesulfonate, or gluconate (2, 8, 25, 26, 29, 31, 39); however, similar effects on the cardiac muscle ryanodine receptor have not been consistently observed (8, 25). It has also been proposed (39) that a nonselective channel mediates a Cl\(^{-}\)-induced increase in Ca^{2+} permeability in skeletal muscle SR. This permeability increase was not seen with I\(^{-}\) or Br\(^{-}\) (39). The anion-dependent increases in Ca^{2+} release from skeletal muscle SR, whether mediated through the ryanodine receptor or through a Cl\(^{-}\)-sensitive nonselective channel (39), have been shown to be blocked by ruthenium red (see Refs. 26 and 39). In our experiments, the inhibition of SR uptake by the substitution of I\(^{-}\) for Cl\(^{-}\) was the same in the presence and absence of ruthenium red (Table 2 and Fig. 4). This is inconsistent with the hypothesis that inhibitory effects I\(^{-}\) and Br\(^{-}\) on net Ca^{2+} uptake are due to an increased Ca^{2+} efflux through an SR Ca^{2+} channel similar to those discussed above. We also showed (Table 2 and Fig. 4) that I\(^{-}\) did not increase the leakiness of the SR membrane to Ca^{2+}, as evidenced by the rate at which Ca^{2+} was released from the SR when the ATP available to the SR Ca^{2+} pump was rapidly removed by hexokinase. From these experiments, we conclude that cardiac SR Ca^{2+} uptake is inhibited by I\(^{-}\) by a mechanism that does not involve an increase in the Ca^{2+} permeability of the SR membrane.

A second mechanism that might explain our results is a direct inhibitory effect of I\(^{-}\) or Br\(^{-}\) on the SR Ca^{2+} pump. Our results, however, do not support this conclusion. Our results showing that I\(^{-}\) does not directly inhibit the cardiac SR Ca^{2+}-ATPase are consistent with those of Highsmith (11), who measured the ATPase activity of the skeletal muscle SR Ca^{2+} pump in the presence of I\(^{-}\) (at concentrations as high as 40 mM) with the use of molybdate to monitor phosphate release. On the other hand, as noted above (see RESULTS), Stefanova et al. (37) reported an inhibition of the skeletal muscle SR Ca^{2+}-ATPase by I\(^{-}\) when ATPase activity was monitored with the same enzyme-coupled ATPase assay (based on NADH fluorescence) that was used in some of our experiments. Although there may be differences in the influence of I\(^{-}\) on the activity of the skeletal and cardiac SR Ca^{2+} pumps, we believe that the discrepancy between the results of Highsmith (11) and those of Stefanova et al. (37) and between those of the latter authors and our own results can be accounted for by the effect of I\(^{-}\) on the NADH-based assay itself. The conclusion that I\(^{-}\) and Br\(^{-}\) have no direct inhibitory effects on the cardiac SR Ca^{2+} pump is further supported by our results with the absorbance assay of Ca^{2+}-ATPase activity. It might be argued, nevertheless, that I\(^{-}\) and Br\(^{-}\) do not affect the ability of the SR Ca^{2+} pump to hydrolyze ATP but uncouple this activity from the binding and transport of Ca^{2+}. To our knowledge, this type of uncoupling has not been reported for any known direct inhibitors of the SR Ca^{2+} pumps. Our previous work (33) showing that the rate of Ca^{2+} uptake into the SR of smooth muscle is

![Graph](http://ajpheart.physiology.org/)

**Table 2. Summary of results with Br\(^{-}\) and I\(^{-}\)**

<table>
<thead>
<tr>
<th>Buffer</th>
<th>Ca^{2+} Uptake Velocity, %</th>
<th>ATPase Activity NADH Assay, %</th>
<th>ATPase Activity P(_{i}) Assay, %</th>
<th>Ca^{2+} Release with Hexokinase, nmol min(^{-1}) mg(^{-1})</th>
</tr>
</thead>
<tbody>
<tr>
<td>KCl</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>1.94 ± 0.19</td>
</tr>
<tr>
<td>KI</td>
<td>39.3 ± 2.6(^*)</td>
<td>129 ± 11(^*)</td>
<td>102 ± 7</td>
<td>1.52 ± 0.16(^\dagger)</td>
</tr>
<tr>
<td>KBr</td>
<td>69.8 ± 4.6(^*)</td>
<td>112 ± 12</td>
<td></td>
<td></td>
</tr>
<tr>
<td>KI without oxalate</td>
<td>32.0 ± 7.9(^*)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>KI + RR</td>
<td>32.0 ± 6.1(^*)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>KI + 4-AP</td>
<td>35.3 ± 1.8(^*)</td>
<td></td>
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</table>

Values are means ± SD. Values for Ca^{2+} uptake velocity are expressed relative to the maximum velocity (V\(_{\text{max}}\)) of the appropriate control KCl buffer. Values for the ATPase activity NADH assay and P\(_{i}\) assay are expressed relative to the ATPase activity measured in KCl buffer and corrected for the effect of Br\(^{-}\) or I\(^{-}\) on the NADH fluorescence assay (NADH assay) or the purine-nucleoside phosphorylase assay (P\(_{i}\) assay) of ATPase activity. RR, 10 \(\mu\)M ruthenium red; 4-AP, 1 mM 4-aminopyridine. \(^*\)Significantly different from V\(_{\text{max}}\) of control. \(^\dagger\)Significantly higher from the ATPase rate of control but not verified by the P\(_{i}\) assay. (See text for details.) \(^\ddagger\)Significantly different from release rate in KCl buffer.
not detectably altered when Cl\(^-\) is replaced by either \(\text{Br}^-\) or I\(^-\) also argues against an effect of these ions on the coupling between Ca\(^{2+}\) binding and ATPase activity. The SERCA2b Ca\(^{2+}\) pump in smooth muscle is identical to the SERCA2a form of the pump of cardiac muscle for most of its amino acid sequence (the 4-amino acid COOH-terminal sequence of SERCA2a is replaced by a 49-amino acid COOH-terminal addition in SERCA2b that is hydrophobic and thought to insert into the smooth muscle SR membrane; reviewed in Ref. 34). Any direct effects of I\(^-\) or \(\text{Br}^-\) on the binding of Ca\(^{2+}\) to the cardiac SR Ca\(^{2+}\) pump would, therefore, have to be mediated by the four COOH-terminal amino acids unique to the cardiac form of the pump or, conversely, the 49-amino acid tail of the SERCA2b form of the pump would have to prevent this from occurring in smooth muscle. It is thus unlikely that \(\text{Br}^-\) and I\(^-\) would have this type of a direct inhibitory effect on the SR Ca\(^{2+}\) pump in cardiac muscle but not smooth muscle.

The results of our experiments are most consistent with the hypothesis that I\(^-\) and \(\text{Br}^-\) inhibit cardiac SR Ca\(^{2+}\) uptake by reducing the rate at which negative charge moves into the SR as Ca\(^{2+}\) is actively transported by the Ca\(^{2+}\)-ATPase. The conclusion that such an anion influx occurs during cardiac SR Ca\(^{2+}\) uptake is also consistent with our recent work (14) showing that tamoxifen, an agent known to block some types of Cl\(^-\) channels, inhibits cardiac SR uptake without directly inhibiting the SR Ca\(^{2+}\) pump and without inducing a significant increase in the permeability of the SR membrane to Ca\(^{2+}\).

The fact that \(\text{Br}^-\) and I\(^-\) have no effect on smooth muscle SR Ca\(^{2+}\) transport suggests that the anion-permeant pathways in the SR are different in the two muscle types. This conclusion is consistent with our findings (33) that SR Ca\(^{2+}\) uptake in smooth muscle is inhibited by two Cl\(^-\) channel blockers [NPPB and R(+)]-IAA-94] that have no effect on cardiac muscle Ca\(^{2+}\) uptake.

We did not see effects of TEA (at a concentration high enough to block many types of K\(^+\) channels) or of 4-aminopyridine on Ca\(^{2+}\) uptake in KI or KCl uptake buffers. If K\(^+\) movement significantly contributed to charge compensation during SR Ca\(^{2+}\) uptake, we might have expected the K\(^+\) channel blockers to inhibit uptake. The results with TEA and 4-aminopyridine are generally consistent with those of Fink and Stephen son (6; see also Ref. 7) who found that the amount of force that could be produced by Ca\(^{2+}\) released from the SR of skinned amphibian skeletal muscle fibers was not decreased but instead increased slightly when the SR was loaded in the presence of TEA (10 mm), 4-aminopyridine (6 \(\mu\)M–2 mM), or other K\(^+\) channel blockers. Although our results cannot be used to completely rule out the involvement of a K\(^+\)-permeant pathway in the cardiac SR membrane that allows cations to leave the SR during Ca\(^{2+}\) uptake, they do indicate that if cation movement does occur during uptake, it occurs through a channel or channels that are TEA and 4-aminopyridine insensitive. It is important to note in this regard that canine cardiac SR K\(^+\) channels reconstituted into lipid bilayers were found to be insensitive to pharmacological manipulation by lemakalim, glyburide, and charybdotoxin (32). It is also possible that the involvement of K\(^+\) channels during uptake is more complex than would be expected for a channel that simply allowed K\(^+\) efflux from the SR (discussed in Refs. 6 and 7). Our results do not address the possibility that K\(^+\) movement is important during SR Ca\(^{2+}\) release.

Comparison of the properties of the striated muscle SR anion channels that have been studied in isolation with the functional evidence from our study suggests some possible candidates for the channel(s) that mediates anion movement during SR Ca\(^{2+}\) uptake. Although a number of the SR Cl\(^-\) channels that have been characterized in electrophysiological experiments have permeabilities to I\(^-\) and/or \(\text{Br}^-\) that are greater than or equal to that of Cl\(^-\) (see Refs. 10, 13, and 41), a recent report by Kawano et al. (20) describes a cardiac muscle SR anion channel that is less permeable to I\(^-\) than Cl\(^-\). This channel is, however, more permeable to \(\text{Br}^-\) than Cl\(^-\). There are a number of plasma membrane anion channels found in muscle and nonmuscle cells that have permeabilities to \(\text{Br}^-\) and/or I\(^-\) that are less than their permeability to Cl\(^-\) (reviewed in Ref. 38). In our experiments, substitution of Cl\(^-\) with SO\(_4^{2-}\) did not alter the maximum rate of SR Ca\(^{2+}\) uptake, a functional property that is consistent with the involvement of a channel with properties similar to those of the small conductance Cl\(^-\) channel identified by Kourie et al. (23) in rabbit skeletal muscle SR. To our knowledge, the permeability of this channel to \(\text{Br}^-\) or I\(^-\) has not been determined. In attempting to correlate single-channel studies with functional studies of the intact SR, one must also consider the possibility that different Cl\(^-\) channels may function during SR Ca\(^{2+}\) uptake and release or that the 49-amino acid COOH-terminal sequence of SERCA2a is unique to the cardiac form of the pump or, conversely, that the involvement of K\(^+\) channels during uptake is more complex than would be expected for a channel that simply allowed K\(^+\) efflux from the SR (discussed in Refs. 6 and 7). Our results do not address the possibility that K\(^+\) movement is important during SR Ca\(^{2+}\) release.

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