Uptake and Retention of Hexakis (2-Methoxyisobutyl Isonitrile) Technetium(I) in Cultured Chick Myocardial Cells
Mitochondrial and Plasma Membrane Potential Dependence

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The fundamental myocellular uptake and retention mechanisms of hexakis (2-methoxyisobutyl isonitrile) technetium(I) (Tc-MIBI), a technetium-99m-based myocardial perfusion imaging agent, are unresolved. Because of the lipophilic cationic nature of Tc-MIBI, it may be distributed across biological membranes in response to transmembrane potential. To test this hypothesis, net uptake and retention of Tc-MIBI in cultured chick embryo ventricular myocytes were determined under conditions known to alter mitochondrial and plasma membrane potentials. Isovolumic depolarization of plasma membrane potentials in 130 mM extracellular K (K_e) 20 mM extracellular Cl buffer reduced net accumulation of Tc-MIBI from 171±16 (control) to 29±3.3 fmol intracellular Tc-MIBI/mg protein · nM extracellular Tc-MIBI. Unidirectional influx of Tc-MIBI in cells depolarized in 30 mM K_e buffer was also reduced; a resting plasma membrane potential of −87±6 mV was calculated from the Goldman flux equation using normal K_e/high K_o Tc-MIBI influx ratios. Addition of the potassium ionophore valinomycin to cells incubated in 130 mM K_e buffer to additionally depolarize mitochondrial membrane potentials further reduced net uptake of Tc-MIBI to levels comparable to that found in nonviable freeze-thawed preparations ([Tc-MIBI]_i/[Tc-MIBI]_o=1). By depolarizing mitochondrial (and in part plasma membrane) potentials with the protonophores 2,4-dinitrophenol and carbonyl cyanide m-chlorophenylhydrazone (CCCP) Tc-MIBI was rapidly depleted from 181±16 (control) to 16±2.6 and 31±4.2 fmol/mg protein · mM, respectively, with kinetics that did not correlate with loss of cellular ATP content. CCCP alone inhibited 90±3% of net accumulation or 66±3% of unidirectional influx of Tc-MIBI in a concentration-dependent manner. By hyperpolarizing mitochondrial membrane potentials with the K^+/H^+ ionophore nigericin or the ATP synthase inhibitor oligomycin, net uptake and retention of Tc-MIBI were increased by 60±9% and 375±20%, respectively. Caffeine, as well as the respiratory chain electron transport inhibitor rotenone, did not significantly alter net cell uptake (p > 0.2). These data indicate that the fundamental myocellular uptake mechanism of Tc-MIBI involves passive distribution across plasma and mitochondrial membranes and that at equilibrium Tc-MIBI is sequestered within mitochondria by the large negative transmembrane potentials. (Circulation 1990;82:1826–1838)

Hexakis (2-methoxyisobutyl isonitrile) technetium(I) (Tc-MIBI) is a myocardial perfusion imaging agent with lipophilic cationic properties designed to exploit the favorable imaging characteristics of technetium-99m. While being clinically used in a manner analogous to thallous chloride (thallium-201) for the noninvasive evalua-
membrane myocardial analysis also demonstrates more than 80% exact concordance between $^{201}$TI and $^{99m}$Tc-MIBI images.5

Despite this initial success of Tc-MIBI as a flow tracer, controversy exists from in vivo and in vitro experience over whether Tc-MIBI is a pure flow tracer by virtue of its lipophilicity or whether altered metabolic states of myocardium may influence Tc-MIBI kinetics and net accumulation. For example, prolonged metabolic blockade in cultured heart cells significantly reduces myocardial unidirectional uptake of Tc-MIBI.6 In buffer-perfused rabbit heart,7 ouabain alters cellular retention of Tc-MIBI, whereas preliminary data with hypoxia at slow flow rates has also been shown to inhibit Tc-MIBI transcapillary exchange in blood-perfused rabbit heart.8 In an open-chest dog model, Tc-MIBI significantly underestimated reperfusion flow to necrotic and perinecrotic regions compared with microsphere analysis that suggested that Tc-MIBI net uptake reflected myocardial viability.9 In addition, image disparity between $^{201}$TI and Tc-MIBI has been reported clinically after lytic therapy.10 Conversely, Tc-MIBI overestimated reperfusion flow to ischemic zones in another open-chest dog model.11 These observations are difficult to reconcile because the fundamental mechanisms of extraction, retention, and washout of Tc-MIBI at the vascular and myocardial levels are not established.

A significant clue to the cellular mechanisms of uptake and retention of Tc-MIBI may be related to its lipophilic cationic nature. This raises the possibility that Tc-MIBI may be distributed across biological membranes in response to transmembrane potential in a manner similar to other well-characterized lipophilic cations such as tetraphenylphosphonium, rhodamine-123, or the cyanine dyes.12-17 Such compounds are sufficiently lipophilic (hydrophobic) to partition into biological membranes but also contain a delocalized charge distributed throughout the molecule, thereby allowing passive distribution of the compound across a bilayer in proportion to an imposed transmembrane electrical potential.12 Because normal myocardial cells maintain a negative plasma membrane potential ($E_m$) that concentrates cations within the cytosol relative to the extracellular buffer and because they also contain a substantial number of mitochondria that generate a large negative potential ($\Delta \psi = -150$ to $-200$ mV$^{14}$) across the inner mitochondrial membrane that will further concentrate cations relative to the cytosol, both potentials would contribute a large driving force for the sequestration of Tc-MIBI within the mitochondrial inner matrix. Therefore, the ratio of Tc-MIBI in the inner matrix to Tc-MIBI in the extracellular spaces at equilibrium would theoretically be related by a form of the Nernst equation15:

$$[\text{Tc-MIBI}]_{\text{mito}}/[\text{Tc-MIBI}]_o = e^{-\Delta \psi / RT}$$

(1) where $RT/F$ is 26 mV at 37°C, and [Tc-MIBI]$_{\text{mito}}$ and [Tc-MIBI]$_o$ are the concentrations of the agent in the mitochondrial and extracellular spaces, respectively.

Previous studies with cultured chick myocardial cells have shown a strong inhibitory effect of prolonged metabolic blockade on Tc-MIBI uptake,6 but the approach did not resolve the fundamental myocardial uptake mechanism. However, preliminary studies in nonmyocardial preparations suggested a potential-dependent uptake mechanism for the agent.16,19 Fully characterizing accumulation in a heart preparation, this report presents new data on potential-dependent uptake of Tc-MIBI in embryonic chick ventricular cells in culture with correlative intracellular sodium, potassium, and ATP content determinations to more directly quantify the contribution of both mitochondrial and plasma membrane potentials. Cultured heart cells represent a preparation that allows manipulation of both mitochondrial and plasma membrane potentials in an otherwise physiologically stable tissue. For the evaluation of myocardial membrane transport processes, this model is characterized by a simple extracellular space unencumbered by complications introduced with intact whole heart preparations by interstitial diffusion delays and dependency on tracer delivery by vascular flow.20,21 The data indicated that equilibrium myocardial accumulation of Tc-MIBI was strongly mitochondrial and plasma membrane potential dependent.

Methods

Cell Culture

Monolayers of spontaneously contractile embryonic chick ventricular myocytes were obtained from disaggregated 10–11-day-old chick hearts by slight modification of previously published methods.22 Briefly, hearts were trimmed of connective tissue and atra, finely minced, and serially exposed to 0.024% (wt/vol) trypsin in Ca$^{2+}$- and Mg$^{2+}$-free Earle’s salt solution for 7 minutes at 37°C. Gentle trituration and agitation on an orbital shaker bath aided disaggregation. Cells released from the first exposure were discarded, and cells aspirated from the next four exposures were then added to an equal volume of trypsin deactivating solution consisting of ice-cold culture medium. Cells were centrifuged at 400g for 10 minutes, resuspended and combined in culture medium, counted with a hemocytometer, and diluted to yield a suspension of $5 \times 10^5$ cells/ml. Twelve milliliters of suspension was incubated in 100-mm plastic culture dishes containing seven coverslips (25-mm diameter) placed on the bottom of each dish to serve as substrate for cell growth. Cells were maintained in a humidified atmosphere of 5% CO$_2$-95% air for 3–4 days, yielding a confluent layer of spontaneously contractile myocytes on each coverslip. Freeze-thawed preparations were produced by incubating confluent cells without media at $-20^\circ$ C for 4–6 hours followed by thawing at room temperature.

Solutions

Control solution for experiments was a modified Earle’s balanced salt solution (MEBSS) containing
(mM): 145 Na+, 5.4 K+, 1.2 Ca2+, 0.8 Mg2+, 152 Cl−, 0.8 H2PO4−, 0.8 SO42−, 5.6 dextrose, 4.0 HEPES, and 1% bovine calf serum (vol/vol), pH 7.4±0.05, 37° C. When bicarbonate-buffered solution was used, 26 mM NaHCO3 replaced an equimolar concentration of sodium chloride; solutions were then maintained at pH 7.4 in a 5% CO2-95% air atmosphere. High K+, low Cl− solution was made by equimolar substitution of potassium-methanesulfonate for sodium chloride. Potassium-methanesulfonate was made by titration of methanesulfonic acid with potassium hydroxide before addition to buffer. Culture medium consisted of the same inorganic salts and dextrose as bicarbonate-buffered MEBSS plus 40% (vol/vol) M199, 6% (vol/vol) fetal bovine serum, and 0.1% (vol/vol) penicillin/streptomycin. Na+−free, K+−free rinse solution contained 145 mM choline-Cl replacing sodium chloride, potassium chloride, NaH2PO4, and serum. For Ca2+−free solution, CaCl2 was deleted, and bovine serum albumin (1.0 g/l) was substituted for serum.

**Preparation of Tc-MIBI**

Synthesis of the radiolabeled compound 99mTc-MIBI was performed with a one-step kit formulation (kindly provided by T.R. Carroll, Cardiolite, E.I. Du Pont, Medical Products Division, Billerica, Mass.) containing solid stannous chloride (0.075 mg) as a reducing agent for the technetium and MIBI as the Cu(MIBI)2BF4 salt.

99mTc-TcO4− (20–30 mCi; 2–25 pmol/mCi) in 1–2 ml saline (0.15 M NaCl) obtained from a commercial molybdenum/technetium generator (New England Nuclear, Billerica, Mass.) was added to the kit reaction vial, heated at 100° C for 15 minutes, and allowed to cool to room temperature producing an almost quantitative yield of the 99mTc-MIBI1+ complex. Excess reducing agent and starting materials were separated from the radiolabeled compound as follows: the contents of the reaction vial were loaded by syringe onto a reversed-phase Sep-Pak cartridge (C-18, Waters Assoc., Milford, Mass.) prewet with ethanol (3 ml, 90%) followed by distilled water (5 ml). Hydrophilic impurities were eluted from the cartridge by washing with saline (10 ml, 0.15 M), and the desired Tc-MIBI was collected by elution with ethanol/saline (2 ml, 9:1, vol:vol). Final total 99mTc activity in the 2-ml effluent (stock) was assayed in a standard dose calibrator (CRC-12, Capintec, Ramsey, N.J.). Radiochemical purity was found to be greater than 97% by thin-layer chromatography (aluminum oxide plates; J.T. Baker, Phillipsburg, N.J.) using ethanol (absolute) as the mobile phase.

**Tc-MIBI Uptake and Retention**

Coverslips with confluent cells were removed from culture media and preequilibrated for 40–60 seconds in buffer. Uptake and retention experiments were initiated by emersion of coverslips in 60-mm glass Petri dishes containing 4 ml loading solution consisting of MEBSS with 0.2–5.0 nM [Tc-MIBI] (0.04–0.67 Ci/nmol, 25–100 μCi/ml). Cells on coverslips were removed at various times, were rinsed three times in 25 ml ice-cold (2° C) isotope-free solution for 8 seconds each to clear extracellular spaces, and were placed in 35-mm plastic Petri dishes. Aliquots of the loading buffer and stock solutions were then obtained and placed in glass scintillation vials for standardizing cellular data with extracellular concentration of Tc-MIBI. Preparations, stock solutions, and extracellular samples were assayed for gamma activity in a well-type sodium iodide gamma counter (Omega 1, Canberra, Meridan, Conn.) after which cells were extracted in 1% sodium dodecysulfate with 10 mM Na-borate before protein assay by the method of Lowry using bovine serum albumin as the protein standard. Appropriate geometric controls allowed normalization of gamma activity assayed in Petri dishes to that assayed in glass vials. Knowledge of the elution history of the generator and activity of stock solutions allowed use of generator equilibrium equations to calculate the absolute concentration of total Tc-MIBI in the solutions. Therefore, most results were expressed as femtomoles cellular Tc-MIBI per milligram of cell protein or normalized to extracellular Tc-MIBI concentration and expressed as femtomoles per milligram protein per extracellular nanomolars. In a few experiments, the elution history was uncertain or unknown, and results were expressed in nanocuries of 99mTc-MIBI per milligram of cell protein. Standard deviations in determination of radioactivity in the loading buffers were estimated to be less than 5% and in determination of absolute concentration of Tc-MIBI were less than 10%.

Methodological control experiments with Tc-MIBI uptakes are shown in Table 1. Net cellular uptake of Tc-MIBI prepared in normal saline (0.9% NaCl) directly from kit formulations was no different from that of Tc-MIBI prepared by elution with ethanol from a Sep-Pak. Drug carrier alone (dimethylsulfoxide) was without effect on Tc-MIBI net uptake. HCO3−-buffered solution minimally reduced net uptake relative to control HEPES-buffered solution. Changing [Ca2+] from 1.2 to 2.8 mM or eliminating H2PO4− and SO42− from the buffer was without significant effect. Most cultures were routinely grown in the presence of penicillin and streptomycin; however, one series of cultured preparations grown in antibiotic-free media demonstrated no significant difference in 1-minute Tc-MIBI uptake when assayed in MEBSS. In addition, cell uptake of the anion pertechnetate (TcO4− eluted from a Mo/Tc generator and added to the loading buffer at the specific activity routinely used for Tc-MIBI) was minimally detectable. Absolute net uptake of TcO4− (1.7 fmol/mg protein · min) when divided by cell water (6.9 μl/mg protein) yields an intracellular-to-extracellular ratio of 0.24. This value is consistent with cellular exclusion of the TcO4− anion by plasma membrane potentials. Cell death induced by freeze thawing the preparations significantly reduced net uptake of
Table 1. Methodological Controls of Tc-MIBI Myocellular Uptake

<table>
<thead>
<tr>
<th>Condition</th>
<th>Uptake time (min)</th>
<th>$^{99m}$Tc cell content</th>
<th>$n$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Saline Tc-MIBI elution (MEBSS-HEPES buffer)</td>
<td>60</td>
<td>109±3%</td>
<td>4</td>
</tr>
<tr>
<td>MEBSS-HEPES buffer+DMSO (0.25%)</td>
<td>60</td>
<td>100±4%</td>
<td>4</td>
</tr>
<tr>
<td>MEBSS-HCO3 buffer</td>
<td>60</td>
<td>78±4%*</td>
<td>3</td>
</tr>
<tr>
<td>Freeze-thawed preparations (MEBSS-HEPES buffer)</td>
<td>60</td>
<td>2.3±0.2%†</td>
<td>4</td>
</tr>
<tr>
<td>TeO4− loading solution (MEBSS-HEPES buffer)</td>
<td>60</td>
<td>2.4±0.1%†</td>
<td>4</td>
</tr>
<tr>
<td>2.8 mM Ca HEPES buffer</td>
<td>2</td>
<td>113±13%</td>
<td>3</td>
</tr>
<tr>
<td>H2PO4−, SO4-free HEPES buffer</td>
<td>1</td>
<td>96±3%</td>
<td>4</td>
</tr>
<tr>
<td>Antibiotic-free growth media</td>
<td>(MEBSS-HEPES buffer)</td>
<td>1</td>
<td>86±10%</td>
</tr>
</tbody>
</table>

Values are mean±SEM; $n$ is the number of determinations.

Cell-associated $^{99m}$Tc was determined for each condition as fmol/mg protein, then expressed as % of control defined for each experiment as the uptake by cells incubated in standard MEBSS-HEPES buffer (EtoH elution of Tc-MIBI) for the same duration.

Tc-MIBI, hexakis (2-methoxyisobutyl isonitrile) technetium(I); MEBSS, modified Earle's balanced salt solution; DMSO, dimethylsulfoxide.

*p<0.02; †p<0.001; ‡p>0.25 for remaining data points.

Tc-MIBI and was used as one measure of potential-independent uptake of Tc-MIBI into cellular and membrane fragments.

Ion Content Determinations

After experimental manipulations, monolayer preparations were rinsed three times in ice-cold Na+ -free, K+-free rinse solution for 8 seconds each and placed in 35-mm plastic Petri dishes. Ionic contents were extracted by addition of 300 μl 0.75N HNO3 to the dish for at least 60 minutes. Two hundred fifty microliters of extract was removed and diluted with 750 μl Acationox (0.02%) in a polystyrene test tube. Protein was then extracted from the debris in the Petri dish with sodium dodecyl sulphate and sodium-borate solution as described above. Na+ concentration was determined directly from an aliquot of the ionic extract (K+ concentration was determined on an extract further diluted 1 part to 3 parts with Acationox solution) using an atomic absorption spectrophotometer (model 3030, Perkin-Elmer, Norwalk, Conn.) with appropriate Na+/K+ standards and reagent blanks.

ATP Content

In a series of experiments, cell ATP content was assayed fluorometrically by a standard hexokinase reaction on cells that had simultaneously been analyzed for Tc-MIBI retention by a method previously described. Briefly, after equilibration of cells in Tc-MIBI-containing buffer for 30 minutes and subsequent incubation in CCCP for various times, cells on coverslips were extracted in 300 μl 14% perchloric acid for 10–15 minutes in 35-mm plastic Petri dishes on ice. A 250-μl aliquot was then transferred to a microfuge tube, neutralized with 200 μl 1.5 M K2CO3 on ice, and the sample assayed for Tc-MIBI content in a well-type gamma counter before freezing (−20°C). Cell debris remaining in the Petri dish from the ATP extraction procedure was also assayed for gamma activity, then protein was extracted in sodium dodecylsulfate and sodium-borate for protein determination as above. Appropriate geometric corrections allowed total cell-associated gamma activity to be calculated from the paired microfuge tubes and Petri dishes. Within 4 days, previously frozen extracts were then thawed and spun for 5 minutes in an air centrifuge, and 150 μl of the supernatant was assayed fluorometrically for ATP content (Kontron Instruments SFM 25, Zurich, Switzerland; excitation, 340 nm; emission, 460 nm). Reactions were initiated by addition of hexokinase. ATP standards were assayed every third to fourth sample to allow internal calibration. ATP content is expressed as nanomoles per milligram of cell protein.

Materials

Sources of material were as follows: certified fetal bovine serum, M199, and penicillin (5,000 units/ml)-streptomycin (5,000 μg/ml) solution (GIBCO, Grand Island, N.Y.); bovine calf serum (Flow Laboratories, McLean, Va.); HEPES, trypsin, bovine serum albumin (V), valinomycin, nigericin, ouabain, 2,4-dinitrophenol, rotenone, carbonyl cyanide-m-chlorophenyl-hydrazone (CCCP), caffeine, and amiloride (Sigma Chemical Co., St. Louis, Mo.); Acationox (Scientific Products, McGraw Park, Ill.); methanesulfonic acid (Eastman Kodak, Rochester, N.Y.); and nitric acid (reagent grade; Fisher Scientific, Medford, Mass.).

Statistical Analysis

Values are mean±SEM. $n$ is the number of observations. Statistical significance was determined by
one-way analysis of variance or the two-tailed Student’s t test as indicated.

Results

Collapse of $E_m$ and $\Delta \psi$

Incubation of cultured chick embryo heart cells in physiological 5.4 mM $K_0$ buffer containing Tc-MIBI resulted in a time-dependent myocellular accumulation of the agent toward an asymptote (Figure 1). Nominal intracellular-to-extracellular equilibrium concentration ratios of Tc-MIBI varied from culture to culture ([Tc-MIBI]$_i$/[Tc-MIBI]$_o$ range, 25–50). Half-times ($t_{1/2}$) of net cellular uptake demonstrated in experiments from this report were consistent with previous determinations of 9.3±1.5 minutes. Equilibrium levels of Tc-MIBI accumulated by cultured myocytes incubated in normal $K_0$ buffer were linearly proportional to total cell protein per coverslip (Figure 2A). In addition, equilibrium accumulation was linearly related to Tc-MIBI extracellular concentration from 0.08 to 1.2 nM (Figure 2B).

Addition of the protonophore CCCP (5 μM) to heart cells containing plateau values of Tc-MIBI caused the rapid and near-complete release of the previously retained Tc-MIBI (Figure 1A). Furthermore, addition of CCCP (5 μM) to the buffer at time zero inhibited 90±3% of net Tc-MIBI uptake (Figure 1B). CCCP is known to rapidly uncouple mitochondria by depolarizing the inner mitochondrial membrane potential and has also been reported to depolarize (at least in part) plasma membrane potentials of vertebral cells such as mouse NG108-15. Not only did CCCP depress net accumulation of Tc-MIBI in heart cells, but unidirectional uptake kinetics of Tc-MIBI, as estimated by 1-minute uptake rates, were also inhibited in a concentration-dependent manner. Half maximal inhibition (IC$_{50}$) of 1-minute Tc-MIBI uptake occurred at approximately 0.3 μM CCCP (Figure 3). To estimate potential-independent Tc-MIBI accumulation, freeze-thawed myocellular preparations were used that yielded results comparable to the residual Tc-MIBI retained in intact heart cells exposed to CCCP (Figure 1A).

In addition to the rapidity of the response, further evidence that the release of cellular Tc-MIBI by CCCP was mediated directly by depolarization of membrane potentials per se and not by secondary effects on high-energy phosphate metabolism was provided by the simultaneous determination of cell ATP content and Tc-MIBI retention (Figure 4). Consistent with expectations for the uncoupler, CCCP depleted ATP content from 29.7±0.6 nmol/mg pro-

\begin{figure}
\centering
\includegraphics[width=0.8\textwidth]{figure1.png}
\caption{Plots of effect of CCCP (5 μM) on retention and net uptake of Tc-MIBI by cultured chick heart cells in normal $K_0$ buffer. Panel A: Tc-MIBI net uptake by intact heart cells (■); CCCP added to the buffer at the arrow. Tc-MIBI uptake by disrupted freeze-thawed heart cells in normal $K_0$ buffer (□). Panel B: Tc-MIBI uptake by heart cells in the absence (■) and presence (□) of CCCP. Each point represents the mean of three determinations. Error bars represent SEM when larger than symbol. Solid lines drawn by hand. Difference in control uptake of Tc-MIBI in each experiment reflects different extracellular concentrations of Tc-MIBI. CCCP, carbonyl cyanide m-chlorophenylhydrazone; Tc-MIBI, hexakis (2-methoxyisobutyl isonitrile) technetium(I).}
\end{figure}

\begin{figure}
\centering
\includegraphics[width=0.8\textwidth]{figure2.png}
\caption{Plots of effect of cell protein (panel A) and extracellular concentration of Tc-MIBI (panel B) on 60-minute Tc-MIBI uptake into cultured heart cells. Symbols represent single determinations each in panel A and the mean±SEM of three determinations in panel B. Solid lines are linear regressions of the data (r=0.72 and 0.97, panels A and B, respectively). CCCP, carbonyl cyanide m-chlorophenylhydrazone; Tc-MIBI, hexakis (2-methoxyisobutyl isonitrile) technetium(I).}
\end{figure}
tein (n=6) to a new quasi-steady state of 16±1.8 nmol/mg protein (n=3) (54±3% of control) within 3 minutes of addition. However, cell content of Tc-MIBI was reduced from 1,971±190 (n=6) to 302±13 (n=3) nCi/mg protein (15±1% of control) within the same time interval. Unexpectedly, a slight recovery of ATP content over time occurred, but Tc-MIBI content remained persistently low. Therefore, the inability of myocytes to retain Tc-MIBI under these uncoupled conditions correlated poorly with their ATP content.

Separation of $E_m$ and $\Delta\psi$ Effects

The influence of $E_m$ on Tc-MIBI uptake could be separated from that of mitochondrial $\Delta\psi$ by use of high $K_o$ buffer and the potassium ionophore valinomycin. For $K_o$ greater than 10 mM, the plasma membrane potential of cultured chick myocytes can be approximated by the Nernst equation for a potassium diffusion potential.\(^{30,31}\) Steady-state $K_i$ was 891±37 nmol/mg protein (Table 2), which corresponds to a concentration of 130±5 mM, assuming our previously determined myocellular water space of 6.9 µl/mg protein.\(^{18}\) Therefore, the equalization of $K_o$ and $K_i$ concentrations in cultured chick myocytes by incubation in 130 mM $K_o$ 20 mM Cl\(_o\) buffer would almost completely depolarize $E_m$ ($E_m$ has been determined to be less than −4.5 mV under similar conditions\(^{32}\)). Sixty-minute net uptake of Tc-MIBI was decreased by high $K_o$ buffer from 170.7±15.5 to 28.7±3.3 fmol/mg protein · nM\(_o\) (calculated from Figure 5A). In addition, the Tc-MIBI initially accumulated in 5.4 mM $K_o$ buffer was depleted by subsequent exposure to high $K_o$ buffer toward levels comparable to the Tc-MIBI accumulated when high $K_o$ buffer was present throughout (Figure 5B); this demonstrated the reversibility of Tc-MIBI uptake driven by $E_m$. Use of low Cl\(_o\)

![Figure 3](http://circ.ahajournals.org/)

**Figure 3.** Panel A: Concentration-effect curve of CCCP inhibition of potential-dependent 1-minute Tc-MIBI uptake into cultured heart cells. Preparations were preincubated for 1 minute in normal $K_o$ buffer containing the desired CCCP concentration before exposure to Tc-MIBI uptake solution containing the same concentration of CCCP. Data were corrected for potential-independent Tc-MIBI uptake estimated from 1-minute Tc-MIBI uptake into freeze-thawed preparations (34±3% of the total 1-minute Tc-MIBI uptake into intact cells). Each point represents the mean±SEM of three determinations. Solid line represents a simple titration curve assuming an $IC_{50}$ of approximately 0.3 µM. Panel B: Dixon plot of the data. Intercept (apparent $K_i$) is 0.25 µM and the correlation coefficient is 0.98. The maximum rate of Tc-MIBI uptake ($V_0$) calculated from this plot is 52 nCi/mg protein-min. CCCP, carbonyl cyanide m-chlorophenylhydrazone; Tc-MIBI, hexakis (2-methoxyisobutyl isonitrile) technetium(I).

![Figure 4](http://circ.ahajournals.org/)

**Figure 4.** Plot of effect of addition of CCCP at concentrations of 1 µM (○, ○) and 5 µM (■, ●) on ATP (○, ○) and Tc-MIBI (■, ●) contents as a function of time. Preparations were preincubated in Tc-MIBI in normal $K_o$ buffer for 30 minutes before addition of CCCP; points at time 0 minutes on the graph were obtained immediately before CCCP addition. Paired ATP and Tc-MIBI contents were then determined on each preparation (see "Methods"). Each point represents the mean±SEM of three observations. CCCP, carbonyl cyanide m-chlorophenylhydrazone; Tc-MIBI, hexakis (2-methoxyisobutyl isonitrile) technetium(I).
Table 2. Effect of Nigericin, Amiloride, and Ouabain on Intracellular Sodium, Potassium, and Net Uptake of Tc-MIBI in Cultured Chick Heart Cells

<table>
<thead>
<tr>
<th>Buffer</th>
<th>Na⁺ (nmol/mg protein)</th>
<th>K⁺ (nmol/mg protein)</th>
<th>Tc-MIBI net uptake (fmol/mg protein · nM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Culture Medium</td>
<td>123.3±14.4</td>
<td>937.5±40.8</td>
<td>—</td>
</tr>
<tr>
<td>HEPES-MEBSS</td>
<td>180.9±43.3</td>
<td>890.7±36.7</td>
<td>194.6±13.3</td>
</tr>
<tr>
<td>+ nigericin</td>
<td>423.8±30.7</td>
<td>534.5±31.8</td>
<td>306.8±45.3</td>
</tr>
<tr>
<td>+ nigericin+amiloride</td>
<td>390.1±17.2</td>
<td>493.4±9.3</td>
<td>338.3±26.6</td>
</tr>
<tr>
<td>+ nigericin+ouabain</td>
<td>715.5±16.8</td>
<td>46.0±2.2</td>
<td>29.5±3.8</td>
</tr>
<tr>
<td>+ nigericin+amiloride+ouabain</td>
<td>859.2±41.5</td>
<td>52.5±10.6</td>
<td>60.6±5.0</td>
</tr>
<tr>
<td>+ amiloride</td>
<td>101.6±21.2</td>
<td>783.4±26.4</td>
<td>154.1±10.3</td>
</tr>
<tr>
<td>+ amiloride+ouabain</td>
<td>416.8±31.4</td>
<td>416.9±10.8</td>
<td>245.4±12.9</td>
</tr>
<tr>
<td>+ ouabain</td>
<td>473.9±21.2</td>
<td>402.5±12.8</td>
<td>301.2±16.4</td>
</tr>
</tbody>
</table>

Values are mean±SEM of four determinations.
Preparations were incubated for 60 minutes in various buffers containing Tc-MIBI and combinations of nigericin (5 µg/ml), amiloride (100 µM), or ouabain (100 µM) as indicated. Preparations were washed in ice-cold sodium-potassium-free buffer without isotope to clear extracellular spaces and intracellular sodium, potassium, and Tc-MIBI determined on each preparation.

MEBSS, modified Earle’s balanced salt solution; Tc-MIBI, hexakis (2-methoxyisobutyl isonitrile) technetium(1).
Each value of Tc-MIBI is significantly different from control net uptake in HEPES-MEBSS (p ≤ 0.05).

In high K₀ buffer by equimolar substitution with the impermeant anion methanesulphonate prevented the high Kᵢ-induced cell swelling usually observed in cultured chick myocytes when normal Clᵢ is present.32 Furthermore, myocyte volume in this solution has been determined to be 7.4 µl/mg protein, which is not significantly different from control.18 Methanesulphonate (5.4 mM) had no effect on Tc-MIBI uptake in normal K₀ buffer (data not shown). Overall, these results were consistent with the expectation that reduction of the plasma membrane potential should decrease the driving force for net uptake of Tc-MIBI. In addition, unidirectional influx of Tc-MIBI estimated from 1-minute uptakes was decreased by high Kᵢ-induced plasma membrane depolarizations (4.5 mM Kᵢ, 352±20 versus 30 mM Kᵢ, 196±15 nCi/mg protein; n = 3).

Cells depolarized in high Kᵢ buffer and exposed to valinomycin (1 µg/ml) showed further depression of Tc-MIBI net uptake to 6.0±1.2 fmol/mg protein · nM₀ (calculated from Figure 5A). This value, which is normalized to the extracellular concentration of Tc-MIBI, can be divided by the cell water space of 6.9 µl/mg protein to yield an uptake ratio ([Tc-MIBI]/[Tc-MIBI]₀) of 0.9±0.2. This is close to unity and implied a lack of potential-independent accumulation of Tc-MIBI by cells under these conditions. Furthermore, the valinomycin-induced incremental inhibition of net uptake of Tc-MIBI in high Kᵢ buffer may have isolated the consequence of complete depolarization of mitochondrial ΔΨ on net accumulation of the agent in heart cells (see “Discussion”).

Mitochondrial Inhibitors and ΔΨ

Further evidence supporting mitochondrial participation in cellular accumulation of the agent was derived from the effect of other ionophores and metabolic inhibitors. Myocytes were allowed to accu-

Figure 5. Plots of effect of high Kᵢ buffer and valinomycin on Tc-MIBI net uptake. Panel A: control net uptake in normal Kᵢ buffer (●); 130 mM Kᵢ, 20 mM Clᵢ buffer (○); 130 mM Kᵢ, 20 mM Clᵢ buffer plus valinomycin (1 µg/ml) (▲). Panel B: Reversibility of Tc-MIBI accumulation. Uptake in normal Kᵢ buffer for 30 minutes (●), then buffer switched to 130 mM Kᵢ, 20 mM Clᵢ buffer containing same specific activity of Tc-MIBI (○). Tc-MIBI net uptake in 130 mM Kᵢ, 20 mM Clᵢ buffer throughout (▲). Each point is the mean±SEM of three determinations. Lines were drawn by hand. Extracellular concentrations of Tc-MIBI in panels A and B were 0.60 and 0.25 nM, respectively. Tc-MIBI, hexakis (2-methoxyisobutyl isonitrile) technetium(I).
mulate Tc-MIBI to plateau levels (181±16 fmol/mg protein·nMox) at which point a test compound was added to the buffer (Figure 6). The mitochondrial uncoupler 2,4-dinitrophenol (1 mM) rapidly and nearly completely depleted cellular Tc-MIBI within 3 minutes to levels comparable to CCCP (5 µM) and slightly above the accumulation demonstrated by freeze-thawed preparations (5.7±1.2 fmol/mg protein·nMox). In contrast, rotenone (10 µM), a specific inhibitor of site I of mitochondrial electron transport, is not expected to rapidly collapse Δψ and, indeed, showed no significant effect on Tc-MIBI retention during a 20-minute exposure (p>0.25). In addition, the ionophore nigericin (5 µg/ml), which mediates electroneutral K+/H+ exchange and is known to collapse the pH gradient across mitochondrial inner membranes, thereby producing a secondary hyperpolarization of Δψ, modestly increased Tc-MIBI content in myocytes within 3 minutes (also, see below). Control experiments confirmed that the drug carrier dimethylsulfoxide had no effect on Tc-MIBI retention (p>0.5). An additional pharmacological control experiment with caffeine (2 mM), which alters Ca2+ flux across sarcoplasmic reticulum, had no significant effect on 1-minute Tc-MIBI uptake (control versus caffeine, 125±10 versus 151±13 fmol/mg protein; n=4, p=0.2).

Oligomycin (5 µg/ml), an inhibitor of the ATP synthase of mitochondria, caused a marked increase in plateau retention of Tc-MIBI (Figure 7). The nominal Tc-MIBI accumulation ratio was 140 and was rising under this condition. Azide (10 mM NaN3), like rotenone, an inhibitor of electron transport, slightly reduced Tc-MIBI net uptake. However, when azide and oligomycin were together added to the buffer at time 0 minutes, cellular accumulation of Tc-MIBI was markedly inhibited (p<0.001).

Interaction of E and Δψ on Tc-MIBI Accumulation

To further examine the physiological interaction of mitochondrial and plasma membrane potentials on Tc-MIBI uptake, we exposed cells to transport inhibitors and ionophores. Nigericin (5 µg/ml) was added at time 0 minutes to cultured heart cells and caused an increase in plateau (60 minutes) accumulation of Tc-MIBI from 148±13 to 248±5 fmol/mg protein (60±9% increase) (Figure 8). This was consistent with expectations for a nigericin-induced hyperpolarization of Δψ. However, ouabain (0.1 mM), a specific inhibitor of the plasma membrane Na+/K+ ATPase known to cause minimal effects on Tc-MIBI 1-minute uptake kinetics, increased plateau accumulation of Tc-MIBI when added alone, yet reduced Tc-MIBI net uptake far below control values when added together with nigericin (Table 2). This implies that an action of nigericin could additionally have been mediated at the plasma membrane, thereby interacting either directly or indirectly with the electrogentic Na+ pump.

To determine whether loss of K1 (and subsequent loss of EK) or mitochondrial gain of Ca2+ dominates net cellular uptake of Tc-MIBI under these conditions, amiloride (0.1 mM), an inhibitor of Na+/H+ exchange, was added to nigericin-treated cells, and Tc-MIBI and cation contents were determined. Amiloride plus nigericin slightly increased net uptake of Tc-MIBI beyond levels induced by nigericin alone (Table 2). Amiloride also partially attenuated the rise in Na+, produced by nigericin that, in turn, would
be expected to relatively reduce Ca\textsuperscript{2+} loading of mitochondria, thereby minimizing Ca\textsuperscript{2+} effects on mitochondrial accumulation of Tc-MIBI. Because the significant loss of K\textsubscript{i} in nigerin or nigerin plus amiloride (Table 2) implied a decline in E\textsubscript{m} this confirmed that the increase in net uptake of Tc-MIBI must have reflected hyperpolarization of $\Delta \psi$ when the Na\textsuperscript{+} pump was functional.

However, Na\textsuperscript{+} pump inhibition with ouabain during nigerin-induced K\textsuperscript{+}/H\textsuperscript{+} exchange (even in the presence of amiloride) caused near-complete inhibition of Tc-MIBI accumulation and reversal of Na\textsuperscript{+} and K\textsubscript{i} to nearly extracellular values (Table 2). Nominally Ca\textsuperscript{2+}-free buffer only partially reversed the inhibition of Tc-MIBI net uptake under these conditions (Table 3). These data suggest that the lack of Tc-MIBI accumulation in the presence of Na\textsuperscript{+} pump inhibition and nigerin was dominated by the nigerin-induced depletion of K\textsubscript{i}, resulting in depolarization of E\textsubscript{m} rather than Ca\textsuperscript{2+} loading of mitochondria.

Discussion

Lipophilic ions are compounds that in general are sufficiently lipophilic (hydrophobic) to partition into biological membranes and also possess a delocalized charge that imparts the characteristic potential-dependent transmembrane distribution.\textsuperscript{12} Detailed theoretical and kinetic models of the individual reactions involved in adsorption, translocation, and desorption across membrane bilayers for hydrophobic ions such as tetraphenylphosphonium and tetraphenylborate have been recently reported.\textsuperscript{36,37} Hexakis (alkylisonitrile) technetium(I) complexes are monovalent cations with a central Tc(I) core octahedrally surrounded by six identical lipophilic ligands coordinated through the isonitrile carbon.\textsuperscript{1,38} Data from the present study indicate that Tc-MIBI, a clinically promising agent from this class, appears to fortuitously possess the appropriate balance of delocalized charge and hydrophobicity to render the agent a permeant probe responsive to membrane potentials in heart cells.

Effect of E\textsubscript{m}

The evidence that myocellular uptake of Tc-MIBI was at least plasma membrane potential dependent was derived from potassium-clamping cells. Equalizing K\textsubscript{i} and K\textsubscript{o} by exposing cultured heart cells to 130 mM K\textsubscript{o} buffer is known to reduce E\textsubscript{m} to approximately zero\textsuperscript{32} and predictably decreased Tc-MIBI plateau uptake. Our data indicate that Tc-MIBI unidirectional influx was also decreased by high K\textsubscript{o}-induced depolarizations. Therefore, one way to estimate plasma membrane potential is to assume that Tc-MIBI influx follows the Goldman flux equation\textsuperscript{39,40}:

$$J_1/J_2=X_1(1-e^{-\Delta\psi})/X_2(1-e^{-\Delta\psi})$$  \hspace{1cm} (2)

where $X$ is $E_{m}F/RT$ and $J_1$ and $J_2$ are the Tc-MIBI influx rates at $E_{m1}$ and $E_{m2}$, respectively. Because the plasma membrane potential of cultured chick heart cells is equal to the potassium diffusion potential when K\textsubscript{o} is 30 mM ($E_{m2}=-38$ mV),\textsuperscript{31} then resting membrane potential can be estimated by solving Equation 2 for $E_{m1}$. The influx data indicated that $J_1/J_2$ is equal to 1.80±0.13, which yields an $E_{m1}$ equal to $-87±6$ mV. This compares favorably with previous determinations of resting E\textsubscript{m} in cultured heart cells using direct electrophysiologic measurements.\textsuperscript{31} Further quantitative analysis of errors produced by any potassium-dependent and potential-dependent membrane adsorption of Tc-MIBI will be required. Nonetheless, Tc-MIBI estimated correctly the plasma membrane potential of cultured cardiac myocytes, thereby supporting the fundamental hypothesis of this study.

Effect of $\Delta\psi$

The myocellular data with ionophores indicated that Tc-MIBI was also concentrated within mitochondria in response to mitochondrial $\Delta\psi$ (Figure 9). Several
approaches were used: 1) The addition of valinomycin to myocytes incubated in high K\textsubscript{o} buffer reduced plateau uptake of Tc-MIBI further than did high K\textsubscript{o} buffer alone. Because K\textsubscript{f} and K\textsubscript{c} were equal and the potassium permeability of the plasma membrane is already high in 130 mM K\textsubscript{o} buffer,\textsuperscript{31} any further increase in potassium permeability produced by valinomycin had no further effect on E\textsubscript{m}. Thus, the valinomycin-induced reduction of Tc-MIBI uptake in high K\textsubscript{o} buffer must have represented the consequences of additionally depolarizing mitochondrial membrane potentials. 2) The protonophores CCCP and 2,4-dinitrophenol both caused the rapid release of Tc-MIBI in intact myocytes to levels slightly greater than the accumulation found in disrupted freeze-thawed preparations. This suggests that these protonophores depolarized both E\textsubscript{m} and Δψ in the preparation. 3) The IC\textsubscript{50} of 0.3 μM for CCCP inhibition of 1-minute Tc-MIBI uptake is comparable to the IC\textsubscript{50} for CCCP as a protonophore. Last, 4) the CCCP-induced release of Tc-MIBI correlated poorly with ATP content, further supporting the hypothesis that Tc-MIBI responds directly to membrane potential and not to a secondary metabolic consequence of mitochondrial uncoupling.

Other classes of metabolic inhibitors of oxidative phosphorylation also demonstrated effects on net uptake and retention of Tc-MIBI, which were consistent with their known modes of action on mitochondrial Δψ rather than indirect actions on high-energy phosphate metabolism (Figure 9). For example, the mode of action of both NaN\textsubscript{3} and rotenone is to block respiratory chain electron transport, which is not expected to produce a rapid decline in Δψ (Δψ may slowly decrease over time but still be relatively maintained by F\textsubscript{1}F\textsubscript{0} ATPase activity). Indeed, both agents minimally altered Tc-MIBI myocardial retention up to 60 minutes. However, oligomycin, a specific inhibitor of the F\textsubscript{1}F\textsubscript{0} ATP synthase together with electron transport inhibition by NaN\textsubscript{3} reduced Tc-MIBI to nearly potential-independent levels as expected. Oligomycin alone increased net uptake of Tc-MIBI, which is consistent with the known action of oligomycin to block proton translocation (influx) through the synthase and allow hyperpolarization of mitochondria by ADP-stimulated electron transport on the cytochromes. Similar results with inhibitors have been reported with other lipophilic cations applied to isolated mitochondria and cultured cells.\textsuperscript{14,15,41,42}

Nigericin mediates electroneutral K\textsuperscript{+}/H\textsuperscript{+} exchange and is known to equilibrate the pH gradient across mitochondrial inner membranes.\textsuperscript{33} In mitochondria in which most of the total proton-motive energy (Δµ) is stored in the pH gradient, a secondary increase in Δψ occurs; conversely, the nigericin-induced hyperpolarization of Δψ is thought to be less in mitochondrial phenotypes with relatively low pH gradients across their inner membranes.\textsuperscript{43} The relative nigericin-
induced increase in Tc-MIBI net uptake observed in cardiac myocytes is significantly less than that previously reported in BALB/c 3T3 mouse fibroblasts or the nigericin-induced increase in tetracynaphospho-

nium net uptake in CV1 cells. The attenuated response of Tc-MIBI to nigericin in cardiac myocytes would be consistent with the notion that the total proton-motive energy of myocardial mitochondria is derived primarily from $\Delta \psi$.

Note that the rate of net cellular efflux of Tc-MIBI into 130 mM K$_0$ buffer from myocytes previously loaded with Tc-MIBI in normal K$_0$ buffer ($\Delta \psi$ polarized; E$_m$ 0 in high K$_0$ buffer) (Figure 5B) was significantly slower than the rate of CCCP-induced net efflux into normal K$_0$ buffer ($\Delta \psi$ 0; E$_m$ polarized) (Figures 1, 4, and 6). The 130-mM K$_0$ buffer, which rapidly depolarizes E$_m$ and has a less-direct effect on $\Delta \psi$, may have isolated the net efflux rate of Tc-MIBI from intact charged mitochondria previously loaded with Tc-MIBI. This rate (t$_{1/2}$ about 10 minutes) more closely matched the unidirectional washout rate of Tc-MIBI from intact myocytes in normal K$_0$ buffer ($\Delta \psi$ and E$_m$ polarized) (t$_{1/2}$ about 8 minutes) and implied that the rate-limiting step for Tc-MIBI cellular efflux was localized to the mitochondrial membrane rather than the plasma membrane.

Physiological Consequences of Transport and Metabolic Inhibition

Ouabain markedly inhibited the nigericin-induced increase in Tc-MIBI accumulation. Possible mechanisms for this may involve the following (Figure 9): 1) Nigericin-mediated K$^+$/H$^+$ exchange occurred at the plasma membrane (as well as the mitochondrial inner membrane) to produce a decrease in K$_i$ and cytosolic pH. Secondary activation of the plasma membrane Na$^+$/H$^+$ exchanger, known to be abundant and stimulated by cytosolic acidification in heart cells, could secondarily increase cytosolic Na$^+$ that would, in turn, stimulate the electrogenic Na$^+$ pump to hyperpolarize E$_m$. Ouabain would, therefore, prevent the component of Tc-MIBI net uptake responding to hyperpolarization of E$_m$. 2) Cell Ca$^{2+}$ content could also increase with any substantial rise in Na$_i$ (by Na$^+$/Ca$^{2+}$ exchange), and this effect should be enhanced during inhibition of the Na$^+$ pump with ouabain. The net result could be enhanced Ca$^{2+}$ uptake by mitochondria during Na$^+$ pump inhibition that may depolarize $\Delta \psi$ because Ca$^{2+}$ uptake by the mitochondrial uniporter is a constructive process.

The data best support the loss of K$_i$ (and depolarization of E$_m$) as dominating the effect of ouabain on nigericin-induced enhancement of Tc-MIBI accumulation. Blocking Na$^+$/H$^+$ exchange with amiloride or preventing Ca$^{2+}$ loading of mitochondria with Ca$^{2+}$-free buffer slightly attenuated, but did not reverse, the strong inhibitory effects of ouabain. However, nigericin-induced K$^+$/H$^+$ exchange across the plasma membrane lead to large losses in K$_i$ only during pump inhibition that appeared to overwhelm any nigericin-induced mitochondrial hyperpolarization.

Several other physiological consequences of transport inhibition were also suggested by the data (Table 2). 1) Addition of amiloride to myocytes incubated in control buffer slightly reduced Na$_i$ and K$_i$ content as well as Tc-MIBI accumulation. These data confirm previous reports indicating a small contribution to net sodium influx from Na$^+$/H$^+$ exchange operating in myocytes at steady-state values of pH$_e$. The lower value for Tc-MIBI accumulation may reflect slight net depolarization of E$_m$ as K$_i$ declined (possibly secondary to reduced sodium pump activity as Na$_i$ decreased). 2) Prolonged ouabain exposure alone caused the anticipated increase in Na$_i$ (partially attenuated by amiloride) and loss of K$_i$ but also produced an unexpected increase in Tc-MIBI accumulation. The sum of Na$_i$ + K$_i$ suggested a 13% loss of cell volume in ouabain that, when combined with the K$_i$ data, indicated a depolarization of resting E$_m$ from $-83$ mV to at least $-65$ mV. Therefore, the large increase in Tc-MIBI accumulation during prolonged ouabain exposure may reflect secondary hyperpolarization of $\Delta \psi$ under these conditions, although contributions from E$_m$ (for example, Ca$^{2+}$-dependent changes in potassium permeability) cannot be excluded.

Membrane potential-dependent accumulation of Tc-MIBI may explain findings from our previous study regarding the inhibitory effects of metabolic blockade produced by prolonged exposure to the glycolytic inhibitor iodoacetate. Heart cells demonstrated the ability up to 60 minutes under metabolic inhibition to maintain 1-minute Tc-MIBI uptake rates despite near-complete depletion of cellular ATP content. However, for times greater than 60 minutes, metabolic blockade did eventually inhibit significantly the unidirectional uptake of Tc-MIBI. Results from the present study indicate that E$_m$ or $\Delta \psi$ remained polarized during the first hour of metabolic blockade but declined thereafter. This is consistent with microanalytic and ultrastructural data in this model of cell injury as well as the recent finding of delayed anoxia-induced injury in mitochondria.

Implications for Clinical Imaging

The membrane potential-dependent net uptake of Tc-MIBI is time dependent. Previous studies in cultured chick heart and rat myocytes with Tc-MIBI have indicated a t$_{1/2}$ of 9–15 minutes for net uptake to myocellular equilibrium. This has consequences for clinical imaging. Usual patient protocols for myocardial perfusion imaging involve the intravenous injection of a bolus of 30–45 mCi of Tc-MIBI. This produces a transient rise in intravascular and interstitial activity of Tc-MIBI followed by a decline. Blood clearance studies typically show a t$_{1/2}$ of 2–5 minutes. Because net myocardial uptake of Tc-MIBI is time dependent, a bolus of extracellular (interstitial) activity in vivo would only allow a small component of the potential-dependent myocellular net uptake of Tc-MIBI observed in the present study to occur. This situation in vivo would be analogous to only the first 1–3 minutes of the myocellular accumulation curve shown in Figure 1.
Although no significant potential-independent uptake into myocardial cells was found, such nonspecific binding to the interstitium in vivo cannot be excluded. Therefore, a component of potential-independent binding may still contribute to myocardial localization of the agent during clinical imaging as the transient bolus of blood activity passes through the whole heart. Conversely, this model would predict increased importance of the potential-dependent myocardial component of uptake during constant infusion protocols.

Discovery of the fundamental cellular mechanism of localization of Tc-MIBI may provide a framework for understanding better some observed properties of Tc-MIBI in vivo. For example, Tc-MIBI activity increases linearly for 2 hours in Langendorff-perfused rat hearts during constant infusion of Tc-MIBI, which led previous investigators\(^4\) to conclude that the volume of distribution of Tc-MIBI is large. Data from our study equate this large volume of distribution to mitochondrial concentration of the agent. In addition, maintaining elevated blood levels of Tc-MIBI in a dog model by a constant 30-minute infusion results in enhanced Tc-MIBI washout.\(^4^8\) Our data suggest that the washout is from mitochondria and cells previously loaded with Tc-MIBI. The effects of Na\(^+\) pump inhibition on myocardial accumulation of Tc-MIBI are also consistent with results reported from perfused rabbit hearts.\(^7\)

Any manipulation that decreases myocardial \(E_m\) or \(\Delta \psi\) would be expected to inhibit the component of Tc-MIBI uptake and retention responsive to membrane potential in vivo. For example, in a reperfused dog model of myocardial ischemia, Tc-MIBI underestimated reflux flow compared with microspheres in regions of ischemia.\(^9\) In severe ischemia, \(E_m\) and \(\Delta \psi\) would be expected to decline significantly and, therefore, result in less Tc-MIBI accumulation within the myocytes. However, previous evidence indicates that mitochondria within ischemic zones depolarize relatively slowly during the course of an ischemic insult,\(^4^6\),\(^4^9\) and therefore, zones of myocardium can continue to accumulate Tc-MIBI within intact cells and mitochondria during times of mild-to-moderate injury. This may provide an explanation for the conflicting results reported for the effect of ischemic injury in vivo on Tc-MIBI myocardial uptake. Depending on the severity of myocardial injury induced by a particular experimental protocol, \(E_m\) and \(\Delta \psi\) may or may not have declined, and therefore, that component of total Tc-MIBI uptake responsive to myocardial energetics may vary considerably between protocols. In addition, in any one patient with severe coronary artery disease, regional differences in membrane potential throughout the myocardium in response to chronic ischemia may lead to spatial inhomogeneity in the final myocardial image independent of flow. Thus, the lack of myocardial or mitochondrial sequestration of Tc-MIBI could be used as a monitor of severely (possibly irreversibly) injured myocardium.

In summary, myocardial uptake and retention of Tc-MIBI are strongly dependent on mitochondrial and plasma membrane potentials both qualitatively and quantitatively. Therefore, although Tc-MIBI has been shown to be a flow-dependent tracer, our data indicate that the agent also has properties of a viability agent. This insight may provide a framework for improved myocardial image analysis in clinical settings and indicate new uses for this class of agents as probes of tissue energetics in vivo and in vitro.

Acknowledgments

We thank B. Leonard Holman, Alan Davison, and Alan G. Jones for their support and encouragement, Mitzy Cannesa for use of the atomic absorption spectrophotometer, Jeff Cone and Kathleen Taylor for their assistance in preparing Tc-MIBI, and Robert A. Otis and Georgia Washington for their secretarial assistance.

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**Key Words** • isonitriles • membrane potential • myocardial perfusion • carbonyl cyanide m-chlorophenylhydrazone • ouabain • nigericin • oligomycin • valinomycin • mitochondria
Uptake and retention of hexakis (2-methoxyisobutyl isonitrile) technetium(I) in cultured chick myocardial cells. Mitochondrial and plasma membrane potential dependence.
D Piwnica-Worms, J F Kronauge and M L Chiu

Circulation. 1990;82:1826-1838
doi: 10.1161/01.CIR.82.5.1826

Circulation is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0009-7322. Online ISSN: 1524-4539

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://circ.ahajournals.org/content/82/5/1826

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