Enhancement by Tetraphenylborate of Technetium-99m-MIBI Uptake Kinetics and Accumulation in Cultured Chick Myocardial Cells

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Myocellular uptake and retention of technetium-99m-hexakis (2-methoxyisobutylisonitrile) (Tc-MIBI), a lipophilic cationic myocardial perfusion and viability imaging agent, is dependent on both mitochondrial and plasma-membrane potentials. To test for enhancement of uptake kinetics by lipophilic anions, cultured chick heart cells were exposed to tetraphenylborate (TPB), which produced a concentration-dependent maximal 15-fold increase in Tc-MIBI uptake kinetics (at 3×10^{-5} *M*) and enhanced peak accumulation of Tc-MIBI from 165.4 ± 26.3 to 705.6 ± 61.3 fmoles/mg protein · nM_o ($P < 0.001$). Carbonyl cyanide-m-chloro phenylhydrazone (CCCP; 10^{-5} *M*), a mitochondrial uncoupler, rapidly depleted cellular content of Tc-MIBI in the presence of TPB (10^{-5} *M*) from 300.0 ± 30.0 to 42.5 ± 1.9 fmole/mg protein · nM_o ($p < 0.001$). TPB enhanced both uptake rates and net accumulation of Tc-MIBI at all buffer K_o concentrations between 130 mM and 0.54 mM. Tc-MIBI influx rates allowed estimation of plasma-membrane potential as a function of K_o in the presence of valinomycin with a slope of −67 mV/decade ($r = -0.99$). The results further support a potential-dependent mechanism for cell uptake of Tc-MIBI and suggest a rational approach for increasing tissue extraction fraction in vivo.

J Nucl Med 1991; 32:1992–1999

Hexakis (2-methoxyisobutyl isonitrile) technetium (I) (Tc-MIBI) is a lipophilic, cationic myocardial perfusion imaging agent that exploits the favorable emission characteristics of ^{99m}Tc (*1,2*). While the agent has proven highly successful as a clinical flow tracer (*3,4*), evidence has demonstrated a significant component of myocardial localization dependent on tissue viability (*5–8*). In this regard, cellular physiological studies with cardiac myocytes have indicated that the fundamental biophysical mechanism of uptake and retention of Tc-MIBI is dependent on both mitochondrial and plasma membrane potentials (*5, 9*), similar to other lipophilic cations (*10,11*). Membrane potential-dependent accumulation of Tc-MIBI has also been observed in NIH 3T3 mouse fibroblasts (*9*), v-src transformed NIH fibroblasts (*9*), BALB/c cells (*9*) and several human carcinoma cell lines in vitro (*12*). In addition, hypoxic injury, metabolic inhibition, and lytic membrane disruption in perfused heart models alter net tissue extraction of Tc-MIBI independent of alterations in flow (*13,14*). In sum, the data are consistent with a membrane translocation mechanism for Tc-MIBI, like other lipophilic cations, involving diffusion and passive transmembrane distribution of the agent in proportion to an imposed transmembrane potential.

Other uptake models for hexakis (alkylisonitrile) technetium complexes have been previously proposed including simple lipid partitioning (*2*) and binding to an 8–10 kDalton cytosolic protein (*15*). In evaluating the relative merit of these models, a novel prediction of the potential-dependent uptake mechanism for Tc-MIBI is the augmentation of uptake kinetics by lipophilic anions. To test our proposed model of Tc-MIBI myocellular accumulation further and to provide insight into a rational approach for augmenting tissue extraction of Tc-MIBI in vivo, the response of myocellular kinetics of Tc-MIBI to permeant and impermeant anions as well as various $[K^+]_o$ were evaluated in cultured embryonic chick heart cells. Cultured cells have proven to be an efficacious and highly predictive cellular model for evaluating the biological properties and behavior in vivo of this class of imaging agents (*2,5,9,12*). We are positioned uniquely with this gamma-emitting agent such that understanding the biophysical mechanism of subcellular localization may direct experiments in cellular metabolism and furthermore enable this agent to be applied to the whole organism and to humans as a tracer of tissue energetics in vivo.

METHODS

Tissue Culture and Cellular Kinetic Studies

The techniques for obtaining monolayers of spontaneously contractile chick ventricular myocardial cells from 10-day-old

Received Jan. 9, 1991; revision accepted Apr. 23, 1991.

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chick embryo hearts disaggregated with trypsin have been described (*5,16*). Cells were incubated in 100 mm diameter plastic culture dishes containing seven coverslips (25 mm diameter) placed on the bottom of each dish to serve as substrates 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.

Radiotracer uptake methods have been described in detail (*5*). Briefly, uptake and retention experiments were initiated by immersion of preparations in 60 mm glass Pyrex dishes containing loading solution consisting of buffer with 0.1–0.6 n*M* [^{99m}Tc-MIBI] (0.1–0.4 Ci/nmole; 25–100 μCi/ml). Preparations were removed at various times and rinsed three times for 8 seconds each in 25 ml volumes of ice-cold (2°C) isotope-free buffer to clear extracellular spaces. Preparations and aliquots of the loading buffer and stock solutions were counted in a well-type sodium iodide gamma counter (Omega 1, Canberra, Meridan, CT) after which cell protein on each coverslip was extracted in 1% sodium dodecylsulfate with 10 m*M* Na-borate and assayed by the method of Lowry (*17*). Tc-MIBI binding to glass coverslips without cells was used as an estimate of nonspecific adhesion to the substrate (<7% of total activity obtained with cellular preparations); this value was subtracted from total uptake determinations to derive the cell-associated counts. Use of generator equilibrium equations (*18*) allowed calculation of absolute moles of Tc-MIBI in solutions and preparations. Results were therefore expressed as fmole cellular Tc-MIBI/mg protein · n*M* extracellular Tc-MIBI concentration. Division of this value by the cell water space (6.9 μl/mg protein) (*9*) yields a nominal intracellular/extracellular Tc-MIBI accumulation ratio neglecting subcellular compartmentation of the agent.

Experimental Solutions and Analytical Determinations

Control buffer was a modified Earle's balanced salt solution (MEBSS) with the following composition (m*M*): Na^+, 145; K^+, 5.4; Ca^{2+}, 1.2; Mg^{2+}, 0.8; Cl^-, 152; $H_2PO_4^-$, 0.8; SO_4^{2-}, 0.8; dextrose, 5.6; HEPES, 4.0; and bovine calf serum, 1% (v/v); pH 7.4 ± 0.05; 37°C. K-methanesulfonate was made by titration of methanesulfonic acid (Eastman Kodak, Rochester, NY) with KOH (*19*) and replaced NaCl in some solutions by equimolar substitution where indicated. Na-tetraphenylborate (1 n*M*–100 μ*M*), carbonyl cyanide-m-chloro phenylhydrazone (CCCP) (5 μ*M*), bumetanide (10 μ*M*) and valinomycin (1 μg/ml) (Sigma Chemical Co., St. Louis, MO) were dissolved into DMSO prior to addition to buffer (final DMSO concentration < 0.5%). These concentrations have been shown to have maximal effect on membrane transport properties in these preparations (*5,6*). DMSO alone has no significant affect on contractile activity, action potential configuration (*20*) or Tc-MIBI uptake kinetics (*5*). All solutions were clear with no evidence of precipitates.

Synthesis of the radiolabeled compound Tc-MIBI was performed using a one-step kit formulation (kindly provided by T.R. Carroll, Cardiolite, E.I. DuPont, Medical Products Division, Billerica, MA); excess starting materials and reducing agent were removed by reverse-phase chromatography and radiochemical purity was documented by thin-layer chromatography (*5*).

The K concentration of selected buffers was determined by atomic absorption spectrophotometry (Model 3030, Perkin-Elmer, Norwalk, CT) as described (*5*).

Cell viability was assessed qualitatively by Trypan blue exclusion. Preparations were incubated in serum-free MEBSS of identical composition as the loading buffers. After the times indicated in the text, preparations were stained by addition to the buffer of 0.40% Trypan blue solution (1:0.75 v/v) for 5–6 min and overall viability determined by observation of cells in 3–6 fields. Control experiments showed >99% viability in 5.4 m*M* K_o buffer and <10% viability after 2 hours of metabolic inhibition in iodoacetate (1 m*M*) and rotenone (10 μ*M*), a condition known to inhibit 90% of Tc-MIBI initial uptake rates (*6*).

Statistics

Values are presented as mean ± s.e.m. of triplicate determinations unless indicated. Statistical significance was determined by one-way analysis of variance or the two-tailed unpaired Student's t-test (*21*). All data in each figure or panel were obtained from the same culture; statistical analysis of experimental points were always evaluated relative to paired control preparations from the same culture. Data similar or identical to that illustrated in Figures 2–6 and Table 1 were obtained with preparations from at least one additional culture.

RESULTS

Myocytes incubated in MEBSS containing Tc-MIBI (0.5 n*M*) accumulated the lipophilic cation to an apparent equilibrium or steady state. A low concentration of tetraphenylborate (TPB; 10^{-9} *M*) minimally increased the rate of Tc-MIBI accumulation, but did not significantly affect final content ($p > 0.5$). However, at concentrations equal to or greater than 5×10^{-7} *M*, TPB increased both maximal accumulation and the uptake kinetics of Tc-MIBI (Fig. 1). In 10^{-5}M TPB, peak accumulation of Tc-MIBI was 4-fold greater than control ($p < 0.001$) and occurred within 10–20 min, 3-fold faster than control. At these high concentrations of TPB, accumulation of Tc-MIBI was not constant over time, but rather declined after achieving a transient maximum value. Further evaluation of the TPB-induced acceleration of Tc-MIBI kinetics was performed with 2-min uptakes in various concentrations of TPB. A concentration-effect curve for TPB enhancement of Tc-MIBI uptake rates is shown in Figure 2. TPB increased

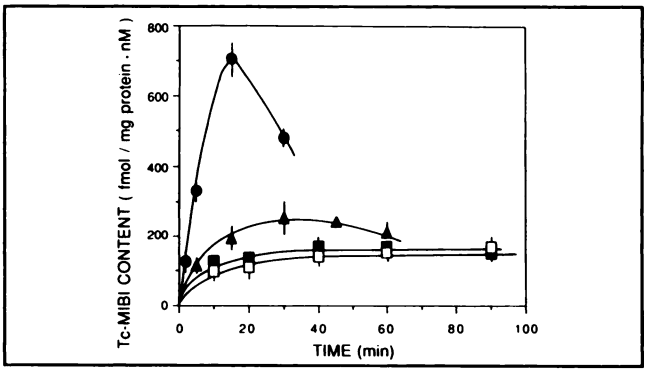

FIGURE 1. Effect of TPB on net accumulation of Tc-MIBI in cultured chick heart cells. Preparations were incubated for the times indicated in control MEBSS buffer containing tracer Tc-MIBI alone (□) and with various concentrations of TPB: (■) 10^{-9} *M*; (▲) 5×10^{-7} *M*; (●) 10^{-5} *M*. Results are expressed as fmole cellular Tc-MIBI/mg protein · n*M* extracellular Tc-MIBI. Each point represents the mean ± s.e.m. of three determinations. Solid lines were drawn by eye.

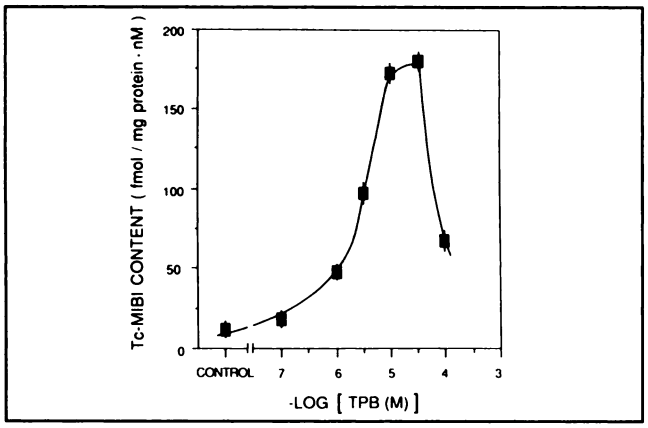

FIGURE 2. Concentration-effect curve for TPB enhancement of 2-min uptake of Tc-MIBI. Preparations were pre-incubated for 1 min in control MEBSS buffer containing TPB at the indicated concentrations prior to determination of tracer Tc-MIBI uptake rates in the continued presence of TPB. Each point represents the mean ± s.e.m. of three determinations.

myocellular uptake rates of Tc-MIBI up to 3×10^{-5} *M* (half-maximal concentration ~3×10^{-6} *M*); higher concentrations of the lipophilic anion markedly reduced cellular kinetics of Tc-MIBI. Cell viability after 20 min in 10^{-4} *M* TPB was reduced correspondingly to 60%–70%.

As opposed to TPB, relatively high concentrations of the impermeant anion methanesulfonate (MSA; 5.4 m*M*) (*19*) did not significantly alter uptake kinetics or plateau content of Tc-MIBI (Fig. 3). Furthermore, 5.4 m*M* MSA did not interfere with the TPB-induced enhancement of Tc-MIBI accumulation (Fig. 3). Even 130 m*M* MSA had little effect on Tc-MIBI net uptake in the presence of TPB (10^{-5} *M*) and 130 m*M* K_o (130 m*M* KCl buffer + bumetanide [10^{-5} *M*]: 63.7 ± 3.2 fmole Tc-MIBI/mg protein · n*M* versus 130 m*M* K-MSA buffer: 79.3 ± 1.1; n = 3; p = 0.01).

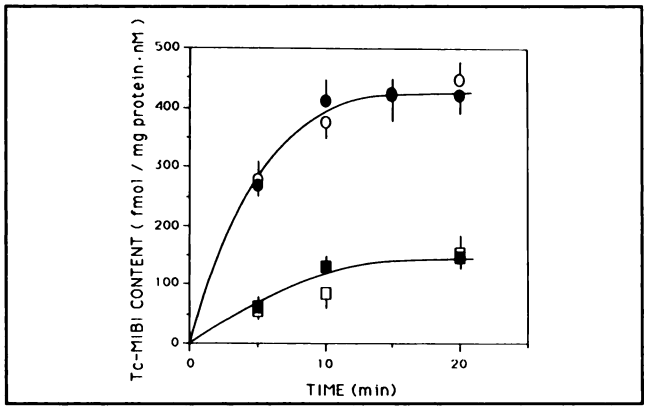

FIGURE 3. Effect of MSA on Tc-MIBI net accumulation. Preparations were incubated for the times indicated in Tc-MIBI loading buffer containing 5.4 m*M* KCl (□, ○) or 5.4 m*M* K-MSA (■, ●; prepared by equimolar substitution of K-MSA for KCl) in the absence (□, ■) or presence (○, ●) of TPB (10^{-5} *M*). Each point represents the mean ± s.e.m. of three determinations.

The myocellular plasma membrane potential is primarily a K diffusion potential for $K_o > 10$ m*M* (*22*); consistent with this, we observed a strong dependence of Tc-MIBI *net uptake* on K_o (Fig. 4). TPB increased Tc-MIBI net accumulation at all K_o concentrations and the K_o-dependence of Tc-MIBI content was preserved. Possible K_o-induced cell volume changes could not have significantly contributed to net uptake of Tc-MIBI: low $[Cl]_o$ buffer (K-MSA substitution for NaCl) or use of bumetanide (10^{-5} *M*) (an inhibitor of the volume-responsive Na+K+2Cl cotransporter (*23*) during KCl substitution for NaCl) were equally effective in maintaining cell volume in various K_o buffers (data not shown). Nominal Tc-MIBI intracellular/extracellular ratios, based on a distribution with no organelle binding or uptake and calculated by dividing the Tc-MIBI content values in Figure 4 by the previously determined cell water of 6.9 μl/mg protein (*9*), were greater than those expected from the K_i/K_o ratios in the absence of TPB; this effect was even more pronounced in the presence of TPB. This provided evidence for enhanced subcellular compartmentation or membrane adsorption of Tc-MIBI by TPB.

To determine whether TPB was increasing membrane partitioning (adsorption) of Tc-MIBI or enhancing a potential-dependent component of net uptake of Tc-MIBI, cells were exposed to various sequential combinations of Tc-MIBI, TPB and the protonophore CCCP. As shown in Figure 5, TPB (10^{-5} *M*) increased myocyte content of Tc-MIBI to similar values above control whether added to the buffer at time zero or subsequent to the attainment of a Tc-MIBI plateau. The time of onset for TPB enhancement of Tc-MIBI was rapid (Fig. 5). Augmentation of Tc-MIBI

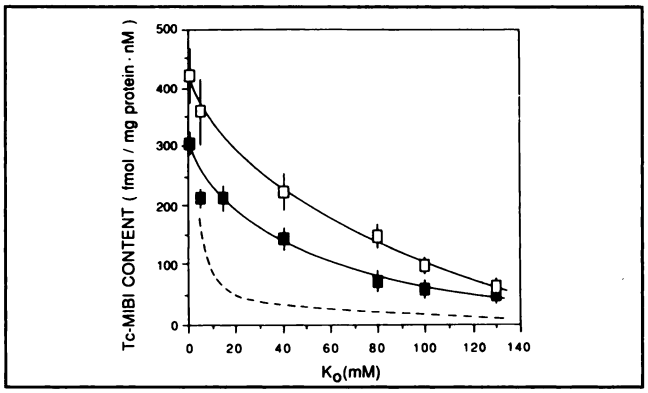

FIGURE 4. K_o-dependence of Tc-MIBI net uptake in the absence (■) and presence (□) of TPB (10^{-5} *M*). Peak accumulation of Tc-MIBI was determined in preparations incubated in loading buffers containing the indicated K_o (equimolar replacement of NaCl by KCl) for 40 minutes (control) or for 20 min (+TPB). K_o-induced cell volume changes were prevented by addition of bumetanide (10^{-5} *M*) to each buffer. Each point is the mean ± s.e.m. of three determinations. Solid lines have no theoretical significance. The dashed line represents the Tc-MIBI content expected at each K_o assuming an intracellular/extracellular Tc-MIBI concentration ratio equal to the K_i/K_o ratio. K_i = 130 m*M* (*5*).

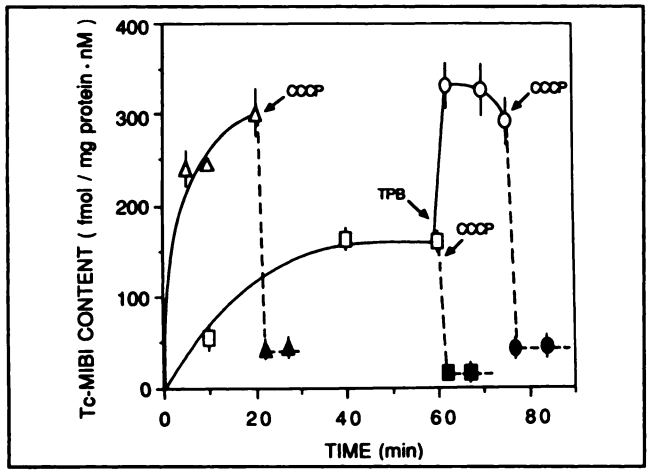

FIGURE 5. Effect of the mitochondrial uncoupler CCCP (5×10^{-6} *M*) on enhancement of Tc-MIBI accumulation by TPB (10^{-5} *M*). Preparations were incubated for the times indicated in 5.4 m*M* K_o control loading buffer containing tracer Tc-MIBI and TPB together (△) or in loading buffer containing Tc-MIBI alone (□). For the latter incubation, TPB was added to the loading buffer at the arrow and cell content of Tc-MIBI subsequently determined (○). Under each condition, CCCP was added to the loading buffers as indicated by the arrow and cell content of Tc-MIBI determined (▲, ■, ●). Each point is the mean ± s.e.m. of three determinations.

myocellular kinetics could be detected as early as 5 sec into a pre-incubation period with TPB (control: 1.23 ± 0.15 fmole Tc-MIBI/mg protein · nM_o; + 10^{-7} *M* TPB: 3.37 ± 0.57; + 5×10^{-6} *M* TPB: 20.6 ± 0.43; 2-min uptakes with n = 3–4 each). Depolarizing mitochondrial membrane potentials with CCCP (5 μ*M*) caused the rapid and near complete release of myocellular Tc-MIBI within 2 min both in the presence and absence of TPB ($p < 0.001$). Despite the depletion of Tc-MIBI, exclusion of Trypan blue by >95% of cells was observed under these conditions. The CCCP-insensitive retention of Tc-MIBI was slightly higher in TPB-treated cells. This could reflect either increased lipid partitioning of Tc-MIBI in the presence of TPB or differential effects of TPB on membrane potentials under these conditions.

To explore further the influence of TPB on any potential-independent accumulation of Tc-MIBI in myocytes, preparations were exposed to 130 m*M* K_o 20 m*M* Cl_o buffer (Table 1). Intracellular potassium (K_i) in these preparations has been previously determined to be 130 m*M* (*5*), therefore collapsing the transmembrane K gradient with this buffer produces an isovolumic depolarization of the plasma membrane potential to nearly zero millivolts (*22*). The residual accumulation of Tc-MIBI in high K_o buffer could be attributed to intact mitochondrial membrane potentials since collapsing mitochondrial potentials with a high concentration of valinomycin (1 μg/ml) eliminated net accumulation of Tc-MIBI (Table 1). TPB (10^{-5} *M*) increased myocellular content of Tc-MIBI in high K_o buffer, but again, this augmentation was completely valinomycin-sensitive. Cell viability was 90–95% in high K_o buffer with or without TPB and >98% when

TABLE 1
Effect of Valinomycin on Tetraphenylborate Enhancement of Tc-MIBI Accumulation in High K_o Buffer

Buffer	Tc-MIBI net uptake (fmole/mg protein · nM_o)	Tc-MIBI$_i$/Tc-MIBI$_o$
130 K_o	65.3 ± 5.3 (n = 3)	9.5
130 K_o + TPB	93.9* ± 4.4 (n = 3)	13.6
130 K_o + val	6.2* ± 0.3 (n = 3)	0.9
130 K_o + TPB + val	7.6* ± 0.8 (n = 3)	1.1

Preparations were incubated in 130 m*M* K_o 20 m*M* Cl_o loading buffer ± valinomycin (1 μg/ml) for 20 min in the presence of TPB (10^{-5} *M*) or for 40 min in its absence to determine peak accumulation of Tc-MIBI under each condition. Values are the mean ± s.e.m. Tc-MIBI in/out ratios were obtained by dividing net uptake values by the cell water space of 6.9 μl/mg protein (9). The F-test statistic was 474 by one-way analysis of variance.

* $p < 0.001$ compared to net uptake in 130 m*M* K_o buffer by the modified t-test.

valinomycin was also present. Equilibration of intracellular and extracellular spaces occurred in the presence of high K buffer plus valinomycin under all conditions (Tc-MIBI in/out ratio ~1; p = n.s.) indicating the lack of any detectable potential-independent membrane binding of Tc-MIBI.

Unidirectional *uptake rates* of Tc-MIBI into heart cells determined by 2-min uptake values was also strongly K_o-dependent (Fig. 6A). As with net uptake, TPB enhancement of Tc-MIBI unidirectional uptake rates were also K_o-dependent (Fig. 6B). Control experiments indicated that complete onset of the valinomycin-induced depolarization of mitochondrial potentials required approximately a 3-min pre-incubation period (data not shown). Therefore, preparations were pre-treated in valinomycin (1 μg/ml) for 5 min prior to determination of Tc-MIBI uptake rates; use of the Goldman flux equation (*24*) allowed estimation of the plasma membrane potential as a function of K_o (Fig. 6C). As can be seen, Tc-MIBI approximated a Nernstian probe of plasma membrane potential under these conditions (slope = −67 mV/decade; r = −0.99; p = 0.02 compared to ideal slope of −60 mV/decade). Whereas Tc-MIBI uptake rates in the absence of TPB were only modestly valinomycin-sensitive (Fig. 6A), Tc-MIBI uptake rates in its presence were highly valinomycin-sensitive (Fig. 6B), implying enhanced mitochondrial compartmentation of Tc-MIBI by TPB.

DISCUSSION

The fundamental cellular uptake mechanism of Tc-MIBI, a lipophilic cation, appears to involve the transmembrane distribution of the probe in response to membrane potential. Unlike other proposed mechanisms of Tc-MIBI localization such as lipid partitioning and binding to a cytosolic protein, a novel prediction of the membrane potential model is augmentation of the kinetics of Tc-MIBI uptake by lipophilic anions such as tetraphenylbor-

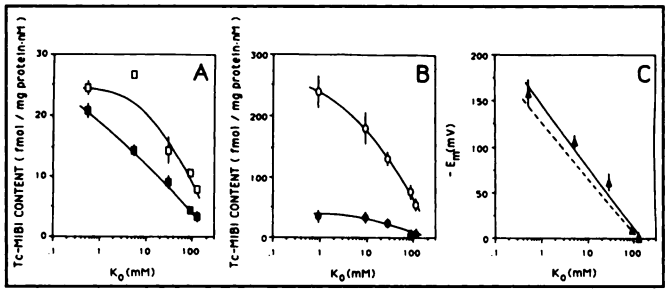

FIGURE 6. Effect of TPB on the K_o-dependence of Tc-MIBI uptake rates. (A) Preparations were pre-incubated in MEBSS containing the indicated K_o (K-MSA substitution for NaCl) for 1 min in the absence (□) and for 5 min in the presence (■) of valinomycin (1 μg/ml) prior to determination of 2-min Tc-MIBI uptakes in buffer of the same composition. (B) Same conditions as A with a 5-min pre-incubation period in buffers that also contained TPB (10^{-5} *M*) in the absence (○) or presence (●) of valinomycin. (C) Resting membrane potential (E_m) as a function of K_o. E_m was calculated from the Tc-MIBI unidirectional uptake data shown in A in the presence of valinomycin using the Goldman flux equation (*27*):

$$J/J_o = -x/(1-e^x)$$

where $x = E_mF/RT$ and J/J_o is the ratio of Tc-MIBI uptake rates at E_m and zero membrane potential (K_o = 130 m*M*), respectively. RT/F equals 26 mV at 37°C. The solid lines in A and B have no theoretical significance. The solid line in C represents a linear regression of the data: $-E_m = -67 \log K_o + 147$mV ($r = -0.99$). The dashed line in C represents the calculated K diffusion potential (Nernst slope equal to −60 mV/decade) for K_i = 130 m*M*. Points with error bars are the mean ± s.e.m. of three determinations; points without error bars are the average of two determinations.

ate. This can be understood in terms of the transmembrane potential profile derived from thermodynamic measurements of tetraphenylphosphonium and tetraphenylborate interactions with neutral membranes (*25–27*). The free energy of a hydrophobic ion as a function of position within a membrane can be quantified in terms of non-electrical energy terms (hydrophobic, van der Waals and steric factors) plus electrical interactions represented by the effects of bulk dielectrics, dielectric interfaces and intrinsic dipole potentials. These combined factors result in negative potential wells located near the membrane surface (probably adjacent to the ester groups of the glycerol backbone) and broad positive energy barriers within the middle of the membrane. Thermodynamic considerations estimate this barrier as several hundred millivolts (200–250 mV), inside positive, for hydrophobic cations, thus representing a substantial energy barrier for their translocation across bilayers (*26*). Adsorption of hydrophobic anions such as TPB into the membrane causes the electrostatic potential within the membrane to change significantly in the negative direction (*25*), thereby increasing the conductance of positively charged probes. Since translocation rates vary exponentially with the free energy of this barrier, the effect can be substantial (*27*).

Selected hydrophobic neutral compounds such as phloretin are also capable of reducing the intramembranous energy barrier thereby enhancing cation permeability, but decreasing anion permeability in bilayer lipid membranes and red blood cells (*28,29*). However, the biophysical complexities of this process are demonstrated by the variable results obtained with other organic molecules possessing dipole moments similar to phloretin such as phloroacetophenone, 2,6-dihydroxyacetophenone, p-nitrophenol, and m-nitrophenol. These compounds increase cation conductances in lipid bilayers (*29*), but have no effect on K^+ conductance in squid giant axons (*30*) which may be related to differences in membrane solubility, possible receptor binding, and net charge at various buffer pH values. In addition, co-ion (pairing) transport cannot be excluded. Nonetheless, enhanced kinetics of myocellular accumulation of tracer Tc-MIBI produced by low concentrations ($<10^{-8}$ *M*) of TPB are consistent with this general scheme of hydrophobic ion transport and further support our model of myocellular uptake of Tc-MIBI.

Physiologic Effects at Modest TPB Concentrations

Moderate concentrations of TPB, however, produced a significant increase in final myocellular content of tracer Tc-MIBI in addition to an increase in Tc-MIBI kinetics. This augmented content of tracer Tc-MIBI appeared to be localized to the mitochondria. Evidence in support of this included:

1. The nominal myocellular accumulation ratio for plateau uptake of Tc-MIBI in the presence of TPB exceeded expectations for the plasma-membrane potential (based upon a Nerstian distribution of the transmembrane K concentrations), a finding consistent with, but not proof of enhanced compartmentation of Tc-MIBI within mitochondria.
2. The majority of the TPB-enhanced net uptake of Tc-MIBI was releasable by the mitochondrial uncoupler CCCP. However, the residual CCCP-insensitive Tc-MIBI content, representing only 13% of the TPB-enhanced Tc-MIBI content, was slightly higher than the control residual. This might imply an increase in a component of potential-independent binding of tracer Tc-MIBI in the presence of TPB or TPB- and CCCP-induced changes in plasma-membrane potentials.
3. However, virtually eliminating any contribution from the plasma-membrane potential by incubating cells in 130 m*M* K_o buffer (*5,31*) and collapsing mitochondrial potentials with valinomycin caused equilibration of intracellular and extracellular Tc-MIBI in both the presence and absence of TPB without loss of cell viability. Thus, there was no evidence of significant TPB-induced potential-independent membrane partitioning under these conditions with moderate concentrations of TPB. (The membrane binding of carrier-added Tc-MIBI discussed below occurs at 10^6-fold higher concentra-

tions.) Therefore, the plateau data indicated enhancement of mitochondrial localization of tracer Tc-MIBI in the presence of TPB.

This stands in partial contrast to data reported from human lymphocytes (*32*). TPB (10^{-5} *M*) significantly enhances tetraphenylphosphonium (TPP) kinetics in human lymphocytes, but only slightly increases equilibrium uptake of the cation. However, lymphocytes contain few mitochondria relative to myocardial cells which may explain the failure of TPB to increase TPP content.

It therefore follows that in the absence of TPB, Tc-MIBI may not completely equilibrate with the mitochondrial membrane potential and final myocyte Tc-MIBI content under control conditions may have represented a quasi-equilibrium in which another undetectably slow component was still loading. In accord with the general model of hydrophobic ion transport discussed earlier, this may suggest that control mitochondrial intramembranous energy barriers are sufficiently positive to slow the kinetics of a component of Tc-MIBI loading thereby preventing maximum distribution ratios. In addition, we cannot exclude that Tc-MIBI plateau uptake is a steady-state, not an equilibrium process, thereby implying that Tc-MIBI is actively extruded from the cell by some TPB-sensitive mechanism. Two alternative mechanisms could be dismissed:

1. TPB may actually be increasing mitochondrial membrane potential and Tc-MIBI simply responding to the induced hyperpolarization. This could occur, for example, if the protonated form of tetraphenylborate crosses the inner mitochondrial membrane and subsequently releases a proton within the alkaline inner matrix which in turn is electrogenically extruded by respiratory chain cytochromes. However, the pK_a of TPB is <3 (*33*), thus making this possibility unlikely.
2. Alternatively, TPB binding to mitochondrial membranes could directly influence mitochondrial function and induce a hyperpolarization to which Tc-MIBI passively responds. However, the data do not support the presence of an induced hyperpolarization. For example, TPB increases both the uptake kinetics and final content of the hydrophobic cation dibenzyldimethylammonium (DDA) in respiring membrane vesicles of *E. coli* (*34*). Membrane potentials somewhat less negative than physiologic (−125 mV) were calculated from peak DDA uptake in vesicles in the presence of TPB, but calculated potentials were approximately −75 mV in its absence. Similarly, assuming a time-averaged plasma-membrane potential of −41 mV in spontaneously beating cultured chick heart cells (*22*), intracellular mitochondrial space of 20%–30% of cell volume (*35*), and inner matrix space equal to 50% of mitochondrial volume (*36*), mean mitochondrial membrane potentials of −128 mV to −138 mV can be calculated from the Tc-MIBI data in Figure 1 in the presence of 10^{-5} *M* TPB, but only −84 mV to −94 mV in its absence; changes in activity coefficients, volumes, or any potential-independent binding were neglected for these estimations. The calculated values for respiring mitochondria within chick heart cells or bacterial vesicles in the presence of TPB appear somewhat less than physiologic rather than hyperpolarized, supporting the hypothesis that TPB facilitates the passive responses of hydrophobic cations such as DDA and Tc-MIBI to pre-existing membrane potentials.

Toxicity

High TPB concentrations (10^{-4} *M*) produced relatively less enhancement of Tc-MIBI kinetics compared to moderate TPB concentrations suggesting cellular toxicity at high concentrations of the lipophilic anion. This was supported by the observation of increased Trypan blue staining of cells at high TPB concentrations. These data are compatible with the previously reported competitive displacement of tracer ^{99m}Tc-MIBI by carrier ^{99}Tc-MIBI at a half-maximum molar ratio (apparent K_D) of 7×10^{-5} (*6*), a result consistent with cellular toxicity at very high carrier-added concentrations of the lipophilic cation. Therefore, results from cultured heart cells may have demonstrated onset of binding saturation (and membrane disruption) for very high concentrations of these hydrophobic ions and are consistent with reports of electrostatic saturation of both TPP binding to egg phosphatidylcholine vesicles at 6×10^{-4} *M* (*36*) and TPB binding to bacterial phosphatidylethanolamine bilayers at concentrations exceeding 10^{-6} *M* (*25*).

Quantitative Evaluation of Membrane Potential

The data are most consistent with a three-compartment model involving extracellular space, the cytosol and mitochondria in series. The dependence of *net* cellular accumulation of Tc-MIBI predominantly on mitochondrial membrane potential stands in contrast to unidirectional cellular uptake rates of Tc-MIBI: estimates of initial uptake rates of Tc-MIBI were dominated by the outer plasma membrane potential (Fig. 6A and 6C). Use of the Goldman flux equation to analyze the K_o-dependence of uptakes rates in control buffer resulted in slight overestimation of an ideal Nernstian slope. From the perspective of evaluating Tc-MIBI as a quantitative probe of plasma-membrane potential in cells, the valinomycin sensitivity of this overestimation indicated the small, but detectable, contribution of mitochondria to the control unidirectional uptake data. However, in the presence of valinomycin, uptake rates of Tc-MIBI demonstrated close to ideal Nernstian behavior (Fig. 6C).

Clinical Consequences

These data have implications for the initial biodistribution in vivo of intravenous bolus injections of Tc-MIBI.

Initial tissue uptake in vivo, simulated in part by the unidirectional influx experiments in vitro, may be relatively more influenced by plasma membrane potentials compared to mitochondrial potentials. Therefore, Tc-MIBI, although a flow tracer, would also be a viability agent responsive to tissue energetics reflected primarily in the plasma-membrane potentials. Although severe myocardial injury during ischemic events in clinical circumstances would often go hand-in-hand with loss of the plasma-membrane potential, it is important to emphasize that membrane potential per se appears to be the primary driving force for accumulation of Tc-MIBI.

The TPB data have other implications for the clinical use of Tc-isonitriles in general and Tc-MIBI in particular as myocardial imaging agents. Compared with thallous chloride (^{201}Tl) perfusion imaging, a limitation of Tc-MIBI is the relatively low myocardial extraction fraction on first pass (*37*). This has been postulated to reduce myocardial localization of the agent and hinder clinical imaging. The results from this study imply that co-injection of a biologically suitable hydrophobic anion or neutral dipolar compound could significantly increase tissue extraction of Tc-MIBI and improve the diagnostic usefulness of this class of metallopharmaceuticals. An appropriate companion compound for clinical use remains to be found. Alternatively, ligand modification of Tc-isonitrile complexes to maximize transmembrane kinetics across the intramembranous potential barrier may prove fruitful.

In summary, the hydrophobic anion TPB markedly enhances Tc-MIBI uptake kinetics and accumulation levels in cultured chick heart cells. These results further support the membrane potential-dependent mechanism for Tc-MIBI cell uptake and indicate a rational approach for improving image quality as well as directing new applications for this agent.

ACKNOWLEDGMENTS

The authors thank Dr. Gary Strichartz for his critical review of this manuscript and Georgia Washington for her secretarial assistance. This work was supported by a grant from the National Institutes of Health (HL 42966). DP-W is a Squibb Diagnostics/Radiological Society of North America Research Fund Scholar.

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ERRATA

In the July issue of the *Journal*, the reprint address for Giovanni Di Chiro's editorial, "Which PET Radiopharmaceutical for Brain Tumors?", was printed incorrectly. The correct address is: Giovanni Di Chiro, MD, Neuroimaging Branch, Building 10, Room 1C451, National Institutes of Health, Bethesda, MD 20892.

In the June issue of the *Journal*, sentences regarding feces collection in the Materials and Methods and Results sections of the article, "In Vivo Use of a Radioiodinated Somatostatin Analogue: Dynamics, Metabolism, and Binding to Somatostatin Receptor-Positive Tumors in Man" by Willem H. Bakker et al., were printed incorrectly. Under the heading Measurements of Radioactivity in Blood, Urine, and Feces in the Materials and Methods section, the sentence stating If feasible, feces were not collected until 48 hr after injection should read If feasible feces were collected until 48 hr after injection. In the Results section, the sentence stating In four patients with a normal intestinal function, feces were not collected until 48 hr after injection of ^{123}I-Tyr-3-octreotide contained less than 2% of the administered radioactivity should read In four patients with a normal intestinal function, feces were collected until 48 hr after injection .....