Hexakis(Carbomethoxyisopropylisonitrile) Technetium(I), A New Myocardial Perfusion Imaging Agent: Binding Characteristics in Cultured Chick Heart Cells

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Cellular kinetics and binding characteristics of hexakis(carbomethoxyisopropylisonitrile) technetium(I) (Tc-CPI), a new cationic, highly lipophilic myocardial perfusion imaging agent, were evaluated in chick embryo heart cells grown in monolayer culture. Myocytes showed uptake of Tc-CPI to a plateau level with a half-time ($t_{½}$) of 4.1 ± 0.7 min (mean ± s.e.m.; n = 6); $t_{½}$ appeared independent of extracellular Tc-CPI concentration. Plateau level Tc-CPI uptakes (10^{-16} to 10^{-11} mole Tc-CPI/mg cell protein) were a linear function of extracellular Tc-CPI concentration (range: $10^{-13}M$ to $10^{-8}M$, respectively). Tracer ^{99m}Tc-CPI uptake (binding) was not competitively displaced by carrier ^{99}Tc-CPI. Uptake was temperature-sensitive; however, several inhibitors of cationic membrane transport (ouabain, amiloride, bumetanide, and verapamil) showed no significant effect. Extreme alkalinization of external load solution (pH_o ~8.5) partially inhibited Tc-CPI uptake; however, intracellular pH changes showed no effect. Washout from contractile preparations could be described by a two component system: a fast component (myocytes) with a $t_{½}$ ~4.5 min and a slow component (fibroblasts) with a $t_{½}$ ~40 min. Cell fractionation experiments showed most of the activity to be associated with the cell membrane fraction. The data do not demonstrate a specific mechanism for uptake of Tc-CPI; however, results suggest binding to myocytes in a manner proportional to the delivery of the complex to the extracellular spaces. Such properties would be desirable for a myocardial perfusion imaging agent.

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Thallium-201 (^{201}Tl) is currently the most widely used myocardial perfusion imaging agent and has been generally accepted for the noninvasive evaluation of myocardial ischemia and infarction (*1*). Thallium-201, however, has several characteristics unfavorable for myocardial imaging including low photon flux, low energy of emission (69–83 keV) that contributes to poor spatial resolution and increased scatter in the image, long half-life (3 days), and high cost as well as inconvenience of cyclotron production (*1,2*). Technetium-99m (^{99m}Tc), on the other hand, has higher photon flux, nearly ideal gamma energy (140 keV), short half-life (6 hr), and is readily available by virtue of the ^{99}Mo/^{99m}Tc generator system. These properties have prompted the search for a ^{99m}Tc based radiopharmaceutical for use as a myocardial imaging agent (*3,4*).

Hexakis(alkylisonitrile) complexes of ^{99m}Tc have recently shown favorable characteristics for use as myocardial imaging agents in both animal studies and initial human trials (*4,5*). Recent experiments in rabbits with a more promising member of this class of complexes, hexakis(carbomethoxyisopropylisonitrile) technetium (I) (Tc-CPI), have shown cardiac perfusion images of diagnostic quality that correlate well with microsphere analysis of blood flow (*6*). Planar, gated, and tomographic images of high technical quality have also been obtained after exercise and at rest in the human and

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correlate well with ^{201}Tl scintigraphy for identification of transient ischemia (*7*). However, fundamental questions concerning the mechanism of localization, whether the Tc-CPI complex is accumulated in myocytes or localized to vascular and interstitial spaces, remain unanswered. Tc-CPI is a monovalent cation like ^{201}Tl and, therefore, transmembrane uptake of the complex mediated by a transport mechanism may exist in a manner analogous to sodium pump-mediated ^{201}Tl uptake (*8*). On the other hand, the lipophilicity of the complex may promote myocyte membrane binding. If so, is this binding nonspecific or specific (thus implying a receptor-mediated process)?

To begin to address these questions and to assist in the further development of this class of compounds, cultured chick heart cells were used as a model system to characterize binding and cellular uptake and washout kinetics of Tc-CPI. Heart cells in culture provide a unique system to study myocyte interaction with this compound unencumbered by the complexity of whole organ preparations (*9*). Correlation of radiochemical and in vivo studies with an understanding of the cellular mechanisms of Tc-CPI uptake should aid in the clinical application and interpretation of examinations performed with these agents. Evidence is presented that indicates preferential binding of Tc-CPI to myocyte membranes.

METHODS

Tissue Culture

The techniques for producing monolayer cultures of spontaneously contracting chick heart cells from 10-day-old chick embryos disaggregated with trypsin have been described (*10–12*). Circular glass coverslips (25 mm) served as the substrate for preparations used in binding and kinetic studies. Cells were grown for 3 days in media containing L-[4,5-^{3}H(N)] leucine which allowed normalization of each preparation to cell protein content as described (*12*). Noncontractile (fibroblast) preparations were produced from cells adhering to the culture dish surface following a differential cell enrichment procedure (*10*).

Synthesis of Hexakis(Carbomethoxyisopropylisonitrile) Technetium(I)

Tc-CPI preparation using the free ligand and sodium dithionite as the reducing agent has been described elsewhere (*13*). To prevent possible toxic effects due to the presence of excess isonitrile in this reaction mixture, the Tc-CPI was purified by loading onto a pre-wetted Millipore SEP-PAK C-18 cartridge, and subsequently washing with normal saline 2 × 5 ml followed by ethanol/water (40% v/v), 4 × 2 ml. The remaining absorbed Tc-CPI was then eluted with an ethanol/saline mixture (90% v/v), 2 ml. This eluate was buffered with ammonium acetate (0.5*M*) 0.5 ml and tested for radiochemical purity by reverse phase high performance liquid chromatography (HPLC) and found to be >98% in all cases. The eluate was tested for free isonitrile using a colormetric test adapted from Crabtree, et al. (*14*).

Experimental Solutions

Kinetic and binding studies were performed in control HEPES-buffered solution with the following composition (in m*M*): NaCl, 137; KCl, 4.5; MgCl, 0.5; $CaCl_2$, 0.9; HEPES, 4.0; dextrose, 5.6; pH 7.35 in air; 37°C. Ammonia solution also contained 20 m*M* NH_4Cl. Where appropriate, buffered solutions contained the following (TRIS/HEPES/pH, respectively): 20 m*M*/5 m*M*/8.5; 10 m*M*/18 m*M*/7.5; 4 m*M*/25 m*M*/6.5. Ouabain, verapamil, amiloride, and bumetanide were dissolved directly from solid powder into solutions.

Cellular Kinetic Studies

Monolayers of heart cells attached to glass coverslips were preincubated in HEPES-buffered solution for 1 min, and then incubated in 40 mm Pyrex dishes with solution containing Tc-CPI. Total Tc-CPI solvent volume was in general kept at ~0.5%. After the desired uptake time period, coverslips were rapidly removed and rinsed for 8 sec each in three 30-ml volumes of ice-cold HEPES-buffered control solution (2–4°C). Control experiments confirmed clearance of >95% of extracellular radioactivity with this protocol. At the time of removal of the coverslips, a 200-μl aliquot of the Tc-CPI loading solution was obtained for normalization of Tc-CPI cellular uptake to extracellular isotope concentration. Some preparations were then returned to Tc-CPI-free solution (37°C) for washout experiments. All cells were scraped off the coverslips and dissolved into 1.4 ml of a solution containing 1% sodium dodecyl sulfate and 10 m*M* sodium borate in glass scintillation vials. These were immediately assayed for ^{99m}Tc activity in a well-type NaI gamma counter. Radioactive samples were then allowed to decay for 5–6 days, after which liquid scintillation techniques were used for detecting ^{3}H activity. Control experiments showed no detectable interference from ^{99m}Tc decay product emissions in the ^{3}H window. Blank glass coverslips showed that plateau level Tc-CPI uptake was no more than 9% of the total cell-associated counts.

Biodistribution in Neonatal Chickens

New born, 5–11-day-old male chicks, ranging in weight from 45–86 g, were injected without anesthesia through the central wing vein with 50 μCi of Tc-CPI in 50 μl (25% ethanol/saline). After 5 min, chicks were killed, organs immediately dissected, weighed and counted in a gamma counter.

Cellular Fractionation

Five-day-old male chicks were injected as above and killed at 15 min postinjection. The hearts were removed and buffer solution immediately chilled to 4°C in a 0.01*M* PO_4^{-3}/0.15*M* NaCl (pH 7.0). Each heart, weighing 0.20 g, was minced on a tray over ice and homogenized in 3 ml of ice-cold buffer in a Teflon pestle homogenizer for 2 min at 3,600 rpm. The homogenate was separated by differential centrifugation into "cell membrane fragments" (10 min @ 1K G), "mitochondria" (10 min @ 3K G), "microsomes" (3 hr @ 30K G), and "cytosol" using an ultracentrifuge (SW 50.1 Rotor) and a superspeed centrifuge (SS34 Rotor).

Statistics

Values are presented as the mean ± s.e.m. unless indicated. Statistical significance was evaluated by the two-tailed Student's t-test (*15*) or one-way analysis of variance (*16*) as indicated in text. Some washout curves were fit by a least squares fitting procedure. Equilibrium calculations were used to estimate total ^{99m}Tc (*17*).

TABLE 1
Biodistribution of Tc-CPI in 5–11-Day-Old Male Chicks*

	% ID/organ	% ID/g	Heart/organ ratio
Heart	1.3 ± 0.2	4.0 ± 1.3	—
Blood	5.4 ± 1.5	1.6 ± 0.6	2.5
Lung	1.1 ± 0.4	2.3 ± 0.8	1.7
Liver	22.0 ± 2.0	11.0 ± 3.0	0.4
Intestines	11.9 ± 2.3	—	—
Kidneys	5.4 ± 1.4	12.0 ± 4.0	0.3
Muscle	13.0 ± 3.0	0.6 ± 0.2	7.0
Gallbladder	4.3 ± 2.2	—	—

Results are expressed as the mean ± s.d. (n = 7) of the percent injected dose (ID) per organ and per gram 5 min postinjection. Total recovery of activity in all cases was between 78% and 89%. Heart/organ ratio is derived per g.

RESULTS

Tc-CPI has demonstrated promise as a myocardial perfusion imaging agent with favorable biodistribution characteristics in several mammalian species including guinea pig, rabbit, and swine (*6*) as well as man (*7*). To assure the applicability of a chicken model, biodistribution experiments were performed in newborn, 5–11-day-old male chicks (Table 1). Results showed that chick biodistribution compared favorably to those previously demonstrated in mammalian species. Cardiac localization of 4% of injected dose/g tissue was well above that observed in lung and blood. These studies suggested that chick heart cells in culture would be a suitable model system.

Tc-CPI cellular uptake in contractile cultured chick heart cells reached a plateau level after 20 min (Fig. 1) with a half-time ($t_{1/2}$) for the uptake process of 4.1 ± 0.7 min (n = 6). Half-time appeared to be independent of extracellular Tc-CPI concentration.

Following uptake to plateau levels in contractile cultures (30 min), Tc-CPI washout into isotope-free solution showed at least two components (Fig. 2). A slow component comprising ~33 ± 9% (n = 4) of total Tc-CPI content/mg cell protein had a $t_{1/2}$ of ~40 min and a fast component comprising the remaining Tc-CPI content had a $t_{1/2}$ of ~4.5 min.

The two components demonstrated on Tc-CPI washout experiments from contractile preparations could reflect either two subcellular compartments in the preparations or represent the two cell types known to be present in contractile preparations; i.e., contractile myocytes and noncontractile fibroblasts that may account for 10–50% of the total cells in the preparation (*9,12*). Thus, noncontractile (fibroblast) preparations were grown in culture and evaluated using identical conditions for Tc-CPI uptake and washout as that used for contractile cultures. Noncontractile preparations demonstrated uptake kinetics similar to that shown for contractile preparations and also achieved plateau loading by 20 min. These preparations yielded a washout curve best approximated by a single component with rate constant graphically matching the slow component of washout from contractile preparations (Fig. 2). Thus, it was concluded that the slow washout component from contractile preparations probably represented washout of Tc-CPI from contaminating fibroblasts in the preparations. Similar conclusions regarding compartmental analysis of other tracers in cultured heart preparations have been previously published (*9*).

Since Tc-CPI is a monovalent cation (*6*), several known inhibitors of cationic membrane transport in cardiac muscle were tested for their effect on 1-min Tc-CPI uptake (Table 2). Amiloride, an inhibitor of Na/H

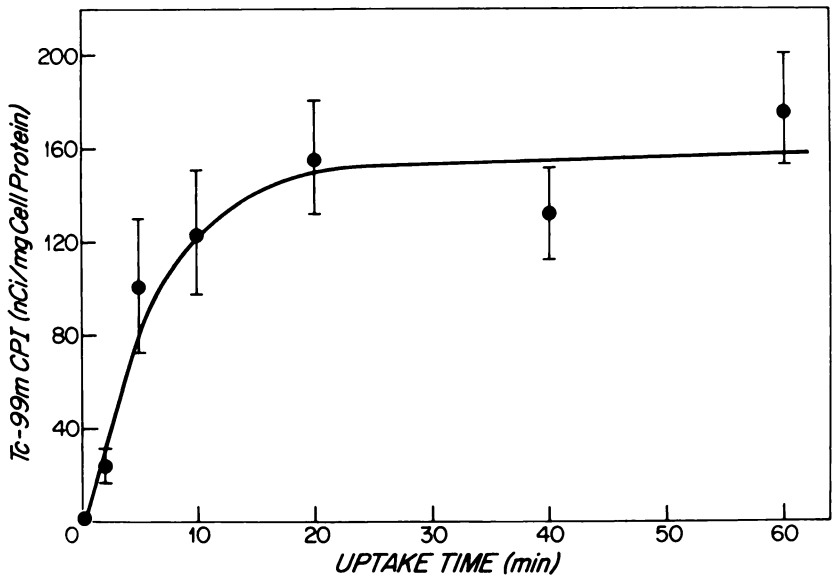

FIGURE 1
Tc-CPI uptake in contractile cultured chick heart cells. Coverslips with cells were incubated in HEPES-buffered solution containing 13 μCi/ml of Tc-CPI for various times before washing in ice-cold isotope-free solution. Each point represents the mean ± s.e.m. of three determinations each. Solid line was drawn by eye assuming a saturable process with $t_{1/2}$ of 4.5 min.

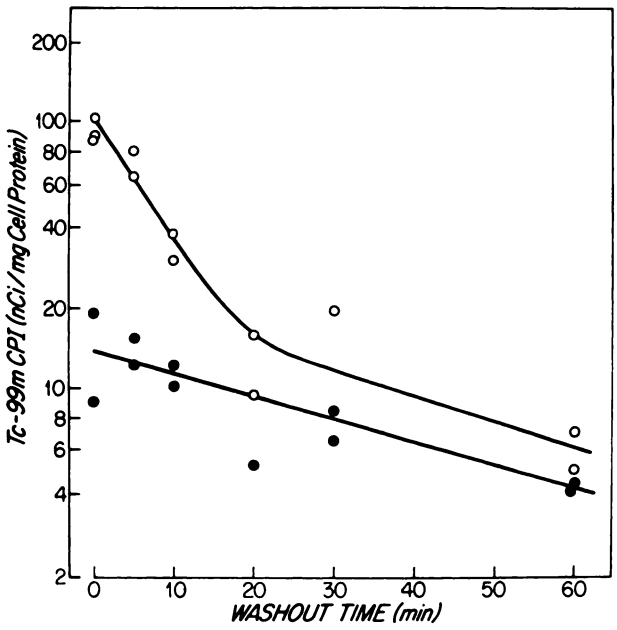

FIGURE 2
Tc-CPI washout in contractile (O) and noncontractile (●) preparations. All preparations were incubated for 20 min in loading solution containing 63 μCi/ml of Tc-CPI of identical specific activity and switched to Tc-CPI-free control solution for washout. Each point represents a different preparation. Solid lines were fit by a least squares curve fitting procedure and graphic curve peeling that yielded the following equations: fibroblast preparations $y = 14e^{-0.017t}$; contractile preparations $y = 79e^{-0.16t} + 22e^{-0.017t}$. A second series of experiments confirmed the two component washout from contractile preparations.

exchange (*18,19*), showed a marginal inhibitory effect on Tc-CPI uptake which was statistically insignificant by one-way analysis of variance. Ouabain which inhibits the Na/K ATPase (*9*), bumetanide which inhibits Na/K/2Cl co-transport (*20*), and verapamil which blocks slow Ca^{2+} channels (*21*) also showed no significant effect on Tc-CPI uptake. In sum, these results suggested that Tc-CPI cellular uptake was unlikely to be through known cationic membrane transport mechanisms.

TABLE 2
Effect of Various Drugs on Tc-CPI Uptake in Cultured Heart Cells*

Drug	1 min Tc-CPI uptake (% control)
Amiloride [$10^{-4}M$]	76 ± 4% (N = 4)
Amiloride [$10^{-3}M$]	79 ± 13% (4)
Ouabain [$10^{-4}M$]	90 ± 8% (4)
Ouabain [$10^{-3}M$]	98 ± 10% (4)
Bumetanide [$10^{-5}M$]	101 ± 31% (4)
Verapamil [$10^{-6}M$]	106 ± 27% (3)

*Values are mean ± s.e.m. (number of determinations) expressed as percent of control 1-min uptake. Comparison between all values for Tc-CPI uptake showed no significant differences based on one-way analysis of variance (*15*).

One-minute Tc-CPI uptake was temperature sensitive. Compared to control uptake at 37°C ($1.4 \times 10^{-14} \pm 1.3 \times 10^{-15}$ mole Tc-CPI (mg cell protein)$^{-1}$ (min)$^{-1}$; n = 3), 22°C and 4°C uptakes were $4.4 \times 10^{-15} \pm 9.8 \times 10^{-16}$ (n = 3) and $3.9 \times 10^{-15} \pm 2.3 \times 10^{-15}$ (n = 3), respectively. Comparing experiments performed at 37°C to those at 22°C yielded a Q_{10} (*22*) of 2.16 suggesting that a highly complex biologic process was unlikely to be mediating Tc-CPI uptake.

Since tissue pH changes are known to occur during myocardial ischemia (*23*) and may effect uptake of a myocardial imaging agent, the effects of intracellular pH changes on Tc-CPI uptake were tested using the NH_4Cl "pre-pulse" technique. Incubation of heart cells in NH_4Cl solution causes an intracellular alkalinization during the first few minutes of exposure and subsequent return to control solution produces an intracellular acidification (*19,24*). Tc-CPI uptake performed during the first minute of NH_4Cl exposure (alkaline pH_i) and during the first minute of return to control solution (acid pH_i) showed no significant difference from control Tc-CPI uptake (Fig. 3A). In addition to demonstrating the lack of pH_i effect on Tc-CPI uptake, these data confirmed that Tc-CPI was not transported into the cell on the Na site of Na/H exchange since this exchanger has been shown to be strongly stimulated by intracellular acidification (*19*). Large variations in external pH (pH_o), however, produced inhibition of Tc-CPI uptake particularly with alkalinization (pH_o = 8.5) (Fig. 3B). No alkaline hydrolysis of the complex was observed below pH_o 9.0 in control in vitro studies (22°C). This may suggest that pH_o could affect binding of Tc-CPI to the external membrane surface.

Plateau level Tc-CPI uptake (binding) depended on external Tc-CPI concentration ($[Tc\text{-}CPI]_o$) as shown in Figure 4. From $[Tc\text{-}CPI]_o$ of 10^{-13} *M* to 10^{-8} *M*, plateau level Tc-CPI binding was linearly proportional to [Tc-CPI] in the extracellular solution. In contrast to what might have been expected for an uptake mechanism requiring specific binding (i.e., a receptor), no concentration saturation was demonstrated within this range. Assuming 4.04×10^6 cells/mg cell protein (*25*), the range of plateau level binding demonstrated in this experiment represented 16 to 3.3×10^5 molecules of Tc-CPI bound per cell.

In addition, Tc-CPI uptake was allowed to occur to plateau levels and then washout experiments performed for 30 min to estimate slow compartment binding. This demonstrated that the compartment loaded in a manner also directly proportional to the initial extracellular Tc-CPI loading concentration (data not shown). Therefore, fibroblasts in the preparation also showed no evidence of concentration saturation.

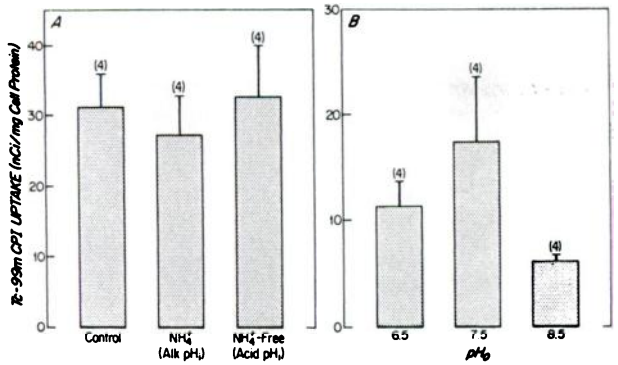

FIGURE 3
Effect of intracellular pH (pH_i) changes (A) and extracellular pH (pH_o) changes (B) on 1-min Tc-CPI uptake in cultured heart cells. Alkaline pH_i changes: Tc-CPI uptakes were performed concurrently upon switching preparations to control solution containing 20 m*M* NH_4Cl which typically alkalines pH_i by ~0.4 pH units (*23*). Acid pH_i changes: Tc-CPI uptakes were performed concurrently upon switching preparations into control solution following a 15-min preincubation period in 20 m*M* NH_4Cl solution. This typically acidifies pH_i by ~0.4 units. External pH changes: preparations were pre-rinsed in HEPES/TRIS buffered solutions of the indicated pH_o for one minute to clear extracellular solution spaces prior to Tc-CPI uptake. Values are mean ± s.e.m. of four determinations each. Only pH_o 8.5 was significantly different from the appropriate control value.

Equilibrium cell binding experiments were performed to confirm the nonspecific nature of Tc-CPI uptake. Contractile preparations were incubated in solutions containing equal amounts of tracer (^{99m}Tc-CPI) with increasing amounts of carrier (^{99}Tc-CPI) (Fig. 5). No significant competitive displacement of ^{99m}Tc-CPI by carrier ^{99}Tc-CPI could be demonstrated.

Independent confirmation of the myocardial subcellular localization of Tc-CPI was obtained by cell fractionation techniques. Following exposure to Tc-CPI for 15 min, fractionated cells showed localization of 61.2 ± 3.4% of the activity within the cell membrane fragment, 8.7 ± 3.1% within the mitochondria, 2.4 ± 0.4% within the microsomes, and 24.9 ± 3.2% in the cytosol (n = 4). Examination of the membrane fraction under the light microscope confirmed the absence of intact cells. Subcellular distribution was not altered by addition of SDS, sucrose of triton X-100 to the buffer solutions prior to homogenizing.

DISCUSSION

Cultured chick heart cells are a physiologically stable myocardial preparation with a loose extracellular matrix which minimizes extracellular diffusion delays. This property in combination with the lack of vascular endothelial or neural elements provides a simple system advantageous for the evaluation of cellular kinetics of tracers without the complexities of whole tissue preparations (*9*).

Cultured heart cell preparations have provided much information regarding mechanisms of uptake of ^{201}Tl, currently the most widely used myocardial perfusion imaging agent. Sixty percent of Tl, a monovalent cation, has been shown to be rapidly transported into the cell by a ouabain-sensitive mechanism, i.e., the Na/K ATPase (*2,8*). A component of ouabain-insensitive Tl uptake may gain access to the cell through other transport mechanisms or channels (*8*). Thus, cellular uptake of ^{201}Tl is in large part dependent on cellular metabolism and ATP content and could also vary depending on the availability of sodium pump binding sites.

The current study has characterized cellular kinetics and binding of Tc-CPI, a promising compound emerging from a new class of myocardial imaging agents, the ^{99m}Tc-hexakis(alkylisonitrile) complexes. Tc-CPI is a highly lipophilic complex with overall monovalent cation charge (*6*). Unknown was whether the lipophilicity or monovalent charge of the complex would dominate in any mechanism of cellular uptake or binding.

Tc-CPI cellular uptake was found to reach a plateau with $t_{½}$ of ~4.5 min. Several known inhibitors of cationic membrane transport in heart tissue were tested and shown to have no significant effect on Tc-CPI uptake. Increasing extracellular Tc-CPI concentration linearly increased plateau levels of Tc-CPI uptake (binding). Carrier Tc-CPI did not compete for tracer Tc-CPI binding. Furthermore, cell fractionation experiments demonstrated Tc-CPI to be closely associated with the cell membrane. In sum, these data suggest that Tc-CPI uptake is dominated by its lipophilic properties and probably involves nonspecific partitioning into the cell membrane. This would make Tc-CPI an ideal myocardial perfusion imaging agent since cellular binding would depend on perfusion alone, i.e., on delivery of the complex to the extracellular spaces of the myocardial tissue. Thallium-201 imaging, on the other hand, depends on perfusion as well as cellular uptake (extraction) which in turn depends in part on cell metabolism and theoretically on available cell binding (sodium pump) sites.

Two other hexakis(alkylisonitrile) agents, t-butylisonitrile (Tc-TBI) and isopropylisonitrile (Tc-IPI), were also reported to show high affinity for membranes, in this case, rat neonatal heart cells in culture and human erythrocytes (*26*). As with Tc-CPI, Tc-TBI uptake reached a plateau after ~20 min, although none was demonstrated with Tc-IPI. Ouabain also did not affect uptake of these agents.

The concentrations of extracellular Tc-CPI used in this study on cellular binding appear to be within the clinically relevant range. In current clinical imaging protocols, 5 mCi Tc-CPI (specific activity ~1 × 10^{-7} mole of total ^{99m}Tc-CPI and ^{99}Tc-CPI/Ci) are injected

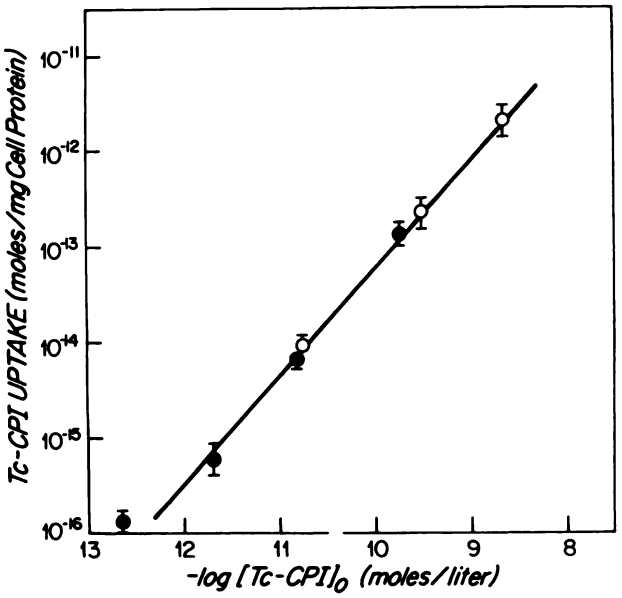

FIGURE 4
Plateau level Tc-CPI uptake (binding) dependence on extracellular Tc-CPI concentration at constant specific activity. Loading solutions were prepared by addition of increasing volumes of the same Tc-CPI stock solution to control solution. Solvent volume was adjusted to maintain constant total volume. Each point represents the mean ± s.e.m. of three preparations each incubated for 20 min at the indicated total Tc-CPI extracellular concentration. Closed and open symbols represent different cultures. Solid line is described by: $y = (3.7 \times 10^{-3})x^{1.09}$.

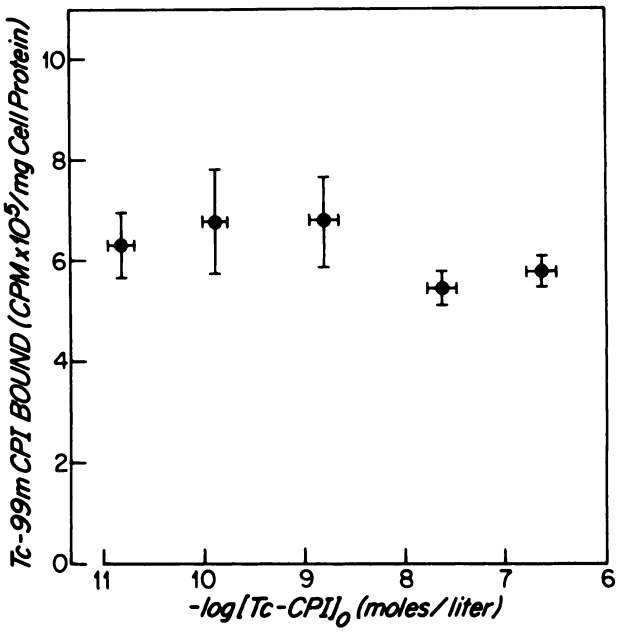

FIGURE 5
Equilibrium binding of ^{99m}Tc-CPI (20-min incubation) as a function of carrier ^{99}Tc-CPI concentration. Load solutions were prepared by addition of equal aliquots of ^{99m}Tc-CPI with increasing aliquots of ^{99}Tc-CPI. Total extracellular Tc-CPI is indicated on abscissa. Each point represents the mean ± s.e.m. of three preparations.

into patients (*7*). Assuming 5 l of blood volume for initial volume-of-distribution, Tc-CPI extracellular concentration would be typically 10^{-10} mole/l. Of course, if Tc-CPI were distributed additionally into the extracellular spaces, Tc-CPI concentration would be several fold lower. Nevertheless, these estimates are well within the linear bounds of the experimental series illustrated in Figure 4.

Despite the apparent nonspecific partitioning of Tc-CPI into heart cell membranes, some selectivity for heart tissue was suggested by these experiments: (a) when both contractile and noncontractile fibroblast preparations were equilibriated with Tc-CPI loading solution at the identical specific activity, contractile preparations showed a sevenfold increase in binding (This analysis assumed the same mg protein per ^{3}H counts for heart and fibroblast cells and may need further testing in additional cell types), (b) assuming similar compartment sizes, analysis normalized to cell protein indicated that the myocyte Tc-CPI compartment concentrated activity three to four times over that found in the fibroblast compartment (Fig. 2). (However, for myocyte compartment sizes >50%, the calculated concentration of activity would be less than three- to fourfold), and (c) since myocyte preparations contain half the number of cells per mg protein as fibroblast preparations (3.7×10^{6} versus 6.2×10^{6} cells/mg cell protein), but twice the surface area (2,000 versus 1,000 μm^2/cell) (*25*), normalizing to cell membrane area would still yield the same conclusion. We can only speculate that certain lipid components of myocyte cell membranes may impart favorable characteristics for lipid partition of this hexakis(isonitrile) complex compared to other cell types.

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