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Effects of ion channel modulators in the influx and efflux of Tc-99m-MIBI

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Possible involvement of cell membrane ion transport systems in the uptake and extrusion of Tc-99m-MIBI was investigated by using various buffers with or without Na^+ and Ca^{++}, and ion transport inhibitors in a tumor cell line. The ion transport modulators dimethyl amiloride (DMA), verapamil, flunarizine and monensin were used. The uptake of Tc-99m-MIBI was significantly increased in all buffers containing either Na^+ or Ca^{++} alone or none of them. There was significantly increased uptake of Tc-99m-MIBI especially in buffers without Na^+. Verapamil, a L-type Ca^{++} channel blocker, increased Tc-99m-MIBI uptake in all buffers. Flunarizine, which inhibits Na^+/Ca^{++} channels, caused significantly increased accumulation of Tc-99m-MIBI only in buffer containing both Na^+ and Ca^{++}. Monensin, a sodium ionophore, significantly increased uptake of Tc-99m-MIBI. DMA, a potent Na^+/H^+ antiport inhibitor, significantly inhibited the uptake of Tc-99m-MIBI in all buffers. In conclusion, Tc-99m-MIBI behaves like Na^+ during its uptake and extrusion. Extrusion of Tc-99m-MIBI may involve both verapamil- and flunarizine-sensitive pathways.

Key words: Tc-99m-MIBI, tumor cells, Na^+/Ca^{++} channels, verapamil, flunarizine

INTRODUCTION

TECHNETIUM-99m-hexakis-methoxy-isobutyl-isonitrile (Tc-99m-MIBI) is a monovalent cation that is widely used as myocardial perfusion and tumor imaging agents.[1–4] Accumulation of Tc-99m-MIBI in the cells is related to cell membrane potential, and passage through this membrane is thought to involve passive diffusion,[5–7] although involvement of ion transport systems of the cell membrane has been identified in our previous experiments.[8,9] Furthermore, Tc-99m-MIBI is localized mostly inside mitochondria due to negative mitochondrial membrane potential.[5,10] In recent reports Tc-99m-MIBI has been identified as a substrate of p-glycoprotein (Pgp), a protein which is overexpressed in cells with a multi-drug-resistant (MDR) gene.[11] Pgp extrudes various chemotherapeutic drugs as well as Tc-99m-MIBI out of cells by an energy dependent active transport system.[12,13] Both clinical and basic experiments showed decreased uptake of Tc-99m-MIBI in cells which contain a higher level of Pgp.[11,14,15] Various agents including verapamil, which enhanced the cytotoxic effect of various chemotherapeutic drugs, caused increased uptake of Tc-99m-MIBI in cells with a higher Pgp level.[11,16,17] Our previous studies pointed out that Tc-99m-MIBI behaves like Na^+ and utilizes Na^+/H^+ antiport during its uptake, and its uptake is related to the intracellular Na^+ concentration.[9,18] If Tc-99m-MIBI behaves like Na^+ and utilizes Na^+/H^+ antiport, it may involve the Na^+/Ca^{++} exchange pathway to maintain the equilibrium which is one of the pathways for maintaining the intracellular Na^+ and Ca^{++} concentrations.[19] But it is not yet determined whether Na^+/Ca^{++} exchange pathway is involved during the influx or efflux of Tc-99m-MIBI in the cells.

The aim of this study was to determine whether cell membrane Na^+ or Ca^{++} ion transport systems are involved in the influx and efflux of Tc-99m-MIBI by using various buffer solutions with or without Na^+ and/or Ca^{++}, and various ion channel modulators, such as dimethyl amiloride (DMA), a selective and potent Na^+/H^+ antiport inhibitor,[20,21] verapamil, an L-type Ca^{++} channel blocker,[17,22] flunarizine, a blocker of Na^+/Ca^{++} channels,[23] and monensin, a Na^+ ionophore that increases Na^+ uptake into the cells.[24]

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MATERIALS AND METHODS

Preparation of cells

RPMI-1640 (contained in mM, $Ca(NO_3)_2 = 0.42$, $MgSO_4 = 0.4$, KCl = 5.36, $NaHCO_3 = 11.9$, NaCl = 102.67, $Na_2HPO_4 = 5.36$, pH = 7.3–7.4, Nissui Seiyaku, Tokyo, Japan) supplemented with heat-inactivated 10% fetal bovine serum (FBS), and antibiotics (penicillin 10 IU/m*l* and streptomycin 10 μg/m*l*) was used as a growth medium for the small-cell carcinoma of the adrenal cortex cell line, SW-13.[25] The cells were grown as previously described.[8,9] In brief, after being harvested with 0.25% trypsin and washed with fresh medium, the cells were transferred to 22 mm cell culture wells (Corning Glass Works, NY) at 2×10^5 cells per well in 2 m*l* of medium and kept at 37°C in a humidified 5% CO_2/95% air atmosphere for 48 hours. The old medium was then discarded, the cells were washed once, and then 0.5 m*l* of buffered solution was added as medium and kept at 37°C for 30 minutes for equilibration. There were 4 buffered solutions of different compositions (Table 1) and in each batch there were 4 groups of wells containing cells (one group for each buffered solution).

Preparation of radiotracers

A lyophilized MIBI kit was purchased from Daiichi Radiopharmaceuticals Co. Ltd., Japan. Each vial contains 1 mg of MIBI. After labeling with 185–370 MBq of Tc-99m, Tc-99m-MIBI was diluted with Na^+ and Ca^{++}-free solution (buffer B) to 0.5 μg/10 μl and added to the wells.

Preparation of chemicals

All chemicals were dissolved in their respective solvents [DMA, in DMSO; monensin, verapamil and flunarizine in methanol (Sigma Chemicals Co., St. Louis, MO)], at 1000-fold concentration and diluted with Na^+ and Ca^{++}-free solution (buffer B) to obtain the final concentration. The final concentration of DMSO or methanol in each preparation was < 0.1%. The control study revealed no significant effect of DMSO or methanol on the uptake of the tracers.

Uptake studies

After incubation with the radiotracers, wells containing cells were quickly washed once with their respective ice cold buffered solutions, and the cells were harvested with 0.25% trypsin, transferred into tubes, and counted by an auto gamma-well counter (ARC-380, Aloka, Japan). The uptake in control cells (in their buffered solution) after 15 minutes of incubation with the radiotracers was taken as the control uptake for that group in each batch.

Effects of Na^+ and Ca^{++} on the uptake

Calcium is one of the essential ion for growth of tumor cells.[26,27] Though Na^+/Ca^{++} exchange is very active in myocardial, muscle and neuronal cells, some other cells also show the presence of the Na^+/Ca^{++} exchange,[28,29] especially when there was manipulation of intra- or extracellular Na^+ concentrations. To see the effect of Na^+ or Ca^{++} on the uptake of Tc-99m-MIBI in SW-13 cells, buffers were made with or without Na^+ and/or Ca^{++}. Na^+ was substituted for equimolar impermeant cation N-methyl-D-glucamine (NMDG)-Cl. Uptake in each buffer was compared with that in buffer containing both Na^+ and Ca^{++} (control buffer). For cells after equilibration with different buffers at basal conditions, Tc-99m-MIBI was added to each wells and kept for 15 minutes. Then the cells were washed and counted, and the results were expressed as a percentage of the uptake values obtained for cells in the control buffer.

Table 1 Details composition of different buffers

Compositions	Control buffer	Buffer A	Buffer B	Buffer C
NaCl	140 mM	140 mM	—	—
NMDG-Cl	—	—	140 mM	140 mM
KCl	5 mM	5 mM	5 mM	5 mM
Ca_2Cl	1.26 mM	—	—	1.26 mM
HEPES	5 mM	5 mM	5 mM	5 mM
pH	7.4	7.4	7.4	7.4

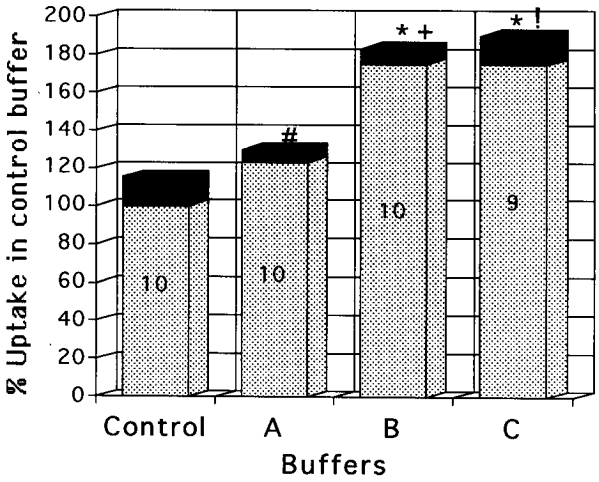

Fig. 1 Shows the uptake of Tc-99m-MIBI in different buffers. Note the higher uptake in buffers without Na^+ (buffer B, C) despite the presence of Ca^{++} (buffer C). The number shown in each column represents the number of measurements. The black area at the top of each column represents the SEM.
* = $p < 0.001$, # = $p < 0.05$, compared to uptake in control buffer.
+ = $p < 0.001$, ! = $p < 0.05$, compared to uptake in buffer A.

Evaluation of effects of ion transport modulators

Cells were pre-incubated with either (final concentration) 100 μmol/*l* of DMA, verapamil, flunarizine or 10 mmol/*l* of monensin alone, or in various combinations and then incubated with Tc-99m-MIBI for 15 minutes. All chemicals were added to the cells 15 minutes before incubation with Tc-99m-MIBI except monensin which was added at

Table 2 Uptake of Tc-99m-MIBI in different buffers after preincubation with various ion channels modulators

Ion channel modulators	Control butter	Buffer A	Buffer B	Buffer C
Verapamil	154.75 ± 11.16* (7)	113.4 ± 4.41§ (6)	126.88 ± 21.37 (7)	155.24 ± 18.44§ (6)
Flunarizine	131.83 ± 4.12* (5)	90.65 ± 6.77 (6)	85.38 ± 14.04 (6)	81.27 ± 5.63* (6)
Monensin	144.56 ± 7.86* (7)	88.59 ± 2.28§ (7)	118.58 ± 10.06‡ (7)	132.03 ± 11.53§ (6)
DMA	72.29 ± 4.52* (7)	50.23 ± 9.83* (7)	62.70 ± 5.88* (7)	84.36 ± 4.06* (6)
Verapamil + DMA	83.89 ± 5.32† (7)	73.97 ± 3.23† (7)	58.15 ± 2.40# (7)	79.37 ± 6.62# (6)
Flunarizine + DMA	72.56 ± 4.83† (6)	49.86 ± 3.14† (6)	43.63 ± 2.63∞ (6)	49.95 ± 2.62† (6)
Monensin + DMA	115.98 ± 6.92∞ (6)	65.98 ± 5.99† (7)	60.02 ± 2.84† (7)	80.88 ± 6.08# (6)

Data are expressed as mean ± SEM. Number in the parenthesis is the number of measurements.
DMA = dimethyl amiloride.
* = $p < 0.001$, § = $p < 0.01$, ‡ = $p < 0.05$, compared to control uptake in respective buffer.
† = $p < 0.001$, # = $p < 0.01$, ∞ = $p < 0.05$, compared to corresponding agent without DMA.

the time of incubation with Tc-99m-MIBI. The dose of each of these agents used was sufficient to cause its greatest effects.[21–24,30]

Assessment of viability
After preincubation with chemical agents, cell viability was checked by the trypan blue dye exclusion technique and compared with that of control cells in each experiment.

Data analysis
All experiments were done at least twice to see the reproducibility and all samples were at least in triplicate. Uptake by the cells in the respective groups, both under basal conditions and after the chemical challenges, was expressed as a percentage of the control uptake values obtained for that group in each batch. Data are expressed as the mean and standard error of mean (SEM), and tests of significance were performed with one-way analysis of variance (ANOVA) with a p-value of less than 0.05 considered statistically significant.

RESULTS

Since the number of cells was the same in all wells for a single batch of experiments and uptake after various chemical challenges was expressed as a percent of the control uptake values obtained for each group in every batch, there would be no significant inter-culture variation in the results.

Effects of Na^+ and Ca^{++} in the uptake
Uptake of Tc-99m-MIBI was significantly increased in all buffers containing either Na^+ or Ca^{++} alone or none of them (buffers A, B and C). There was significantly increased uptake of Tc-99m-MIBI especially in buffers without Na^+ (buffers B and C) when compared with the buffer containing Na^+ (buffer A) (Fig. 1).

Effects of ion transport modulators
Table 2 shows the results of the effects of ion-transport modulators and their significant differences. Verapamil significantly increased Tc-99m-MIBI uptake in all buffers except in buffer B, in which no significant difference was observed. Flunarizine, which inhibits Na^+/Ca^{++} channels, caused significant increased accumulation of Tc-99m-MIBI only in the buffer containing both Na^+ and Ca^{++} (control buffer). Monensin, a sodium ionophore, significantly increased uptake of Tc-99m-MIBI in all buffers except in buffer A. DMA significantly inhibited the uptake of Tc-99m-MIBI in all buffers and the inhibitory effects were observed even when DMA was incubated with verapamil, flunarizine or monensin despite the stimulatory effects of these agents.

Viability of the cells
Cell viability was similar in the control and cells after chemical challenges. More than 95% of cells in all samples were viable.

DISCUSSION

Effects of Na^+ and Ca^{++} in the uptake
Our previous studies[9,18] on both SW-13 and myocardial cells revealed the involvement of the Na^+/H^+ antiport system in the uptake of Tc-MIBI, and we pointed out that Tc-99m-MIBI behaves like Na^+ during its uptake into myocardial cells. To determine the possible involvement of cell membrane Na^+ or Ca^{++} ion transport systems in the influx and efflux of Tc-99m-MIBI, various buffer solu-

tions with or without Na^+ and/or Ca^{++}, and various ion channel modulators were used. As expected, Tc-99m-MIBI uptake is significantly increased in buffers without Na^+, which is in agreement with our previous study with myocardial cells.[18] Unexpectedly, Tc-99m-MIBI uptake is also significantly increased in buffers without Ca^{++}. The increased uptake of Tc-99m-MIBI in Na^+ free buffer might be due to its behavior similar to Na^+ during uptake, which was also suggested by the results of our previous study.[18] But the increased uptake of Tc-99m-MIBI in Ca^{++} free buffers might be due to both or either of the two mechanisms. (a) Deprivation of extracellular Ca^{++} might initiate the exchange of intracellular Ca^{++} with extracellular Na^+ and/or Tc-99m-MIBI through an Na^+/Ca^{++} exchange pathway, which was demonstrated in various investigations.[30,31] Similarly, when there was no Na^+ in extracellular fluid, Tc-99m-MIBI went into the cells instead of Na^+ and caused more significant uptake in Na^+ free buffers; (b) when there was no extracellular Ca^{++}, the reverse Na^+/Ca^{++} exchange (exchange of intracellular Na^+ and/or Tc-99m-MIBI with extracellular Ca^{++}) did not take place and Tc-99m-MIBI could not be extruded through the exchanger and caused increased accumulation of Tc-99m-MIBI in Ca^{++} free buffers. There is a report showing increased acid induced uptake of Na^+ in Ca^{++} free solution, and the authors suggested that it could be due to stimulation of Na^+/H^+ exchange rather than exchange of extracellular Na^+ with intracellular Ca^{++}.[32] Because part of Tc-99m-MIBI involves Na^+/H^+ antiport during its uptake, the increased uptake of Tc-99m-MIBI in Ca^{++} free solution also might be due to stimulation of Na^+ and/or Tc-99m-MIBI/H^+ exchange.

Effects of Ca^{++} channel blocker

Though expression of Pgp in SW-13 was not measured, increased accumulation of Tc-99m-MIBI in all buffers after verapamil preincubation was according to our expectation, because previously researchers also found increased accumulation of Tc-99m-MIBI in fibroblastic cells after verapamil where Pgp expression was negligible.[11] Previous experiments showed that Pgp mediated efflux of Tc-99m-MIBI is an active transport system[11] but involvement of Na^+/Ca^{++} exchange was not predicted because L-type Ca^{++} channel does not take part in Na^+/Ca^{++} exchange.[30] Greenberger et al.[12] has demonstrated a common domain of Pgp for both L-type Ca^{++} channel blocker and other anti tumor agents. In view of this finding, we thought that not only the L-type channel of Ca^{++} current but other Ca^{++} pathways might be involved in the extrusion of Tc-99m-MIBI. We therefore used an inhibitor of Na^+/Ca^{++} channels, flunarizine, to exploit the channel to find out the other mechanisms of efflux of Tc-99m-MIBI. There are reports showing the least effect of flunarizine in the reversal of chemoresistance whereas verapamil had the strongest effect.[17,33] The increased uptake of Tc-99m-MIBI only in buffer containing both Na^+ and Ca^{++} (control buffer) after flunarizine preincubation indicated that Tc-99m-MIBI also might involve another Na^+/Ca^{++} channel for efflux out of the cells, but the presence of both Na^+ and Ca^{++} is essential to activate the channel[34,35] or to have the effect of flunarizine on Na^+/Ca^{++} channels because no increase in the uptake of Tc-99m-MIBI was seen in other buffers preincubated with flunarizine.

Our previous study[18] and this present one confirm the behavior of Tc-99m-MIBI to be similar to Na^+, because incubation with monensin resulted in significantly increased uptake of Tc-99m-MIBI in buffers with or without Na^+, and DMA significantly inhibited the uptake of Tc-99m-MIBI in all buffers even in the presence of stimulatory effects of verapamil, flunarizine and monensin. Increased uptake of Tc-99m-MIBI after monensin might be explained in the following ways. (a) The primary effect of monensin is depolarization of cell membrane potential due to increased accumulation of intracellular Na^+. Because uptake of Tc-99m-MIBI depends on the cell membrane potential[7] it should not enter the cell when the cell membrane potential is depolarized. But as there is increased accumulation of Tc-99m-MIBI after monensin, Tc-99m-MIBI must have entered the cells along with Na^+ after monensin incubation. (b) There are reports showing that increased intracellular Na^+ might stimulate the electrogenic sodium pump to hyperpolarize membrane potentials[5] and may cause increased uptake of Tc-99m-MIBI as its uptake is also dependent on the cell membrane potential.[7] Increased uptake of Tc-99m-MIBI in Na^+ free buffer might be due to hyperpolarization of membrane potential.[36]

Calcium is one of the essential elements for the growth of tumor cells.[26,27] Although clinical implication of our findings in the tumor cells might not be very important in tumor imaging, there are reports showing the importance of reverse Na^+/Ca^{++} exchange in the contraction of ventricular myocytes from a failing human heart.[37] A similar study to explore the relationship of Na^+ and Ca^{++} to the uptake and extrusion of Tc-99m-MIBI in isolated ventricular myocytes is under way.

CONCLUSION

Influx and efflux of Tc-99m-MIBI in SW-13 might depend on both Na^+ and Ca^{++} ions. The efflux of Tc-99m-MIBI involves verapamil- and flunarizine-sensitive pathways.

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