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Effects of Extracellular Calcium on Calcium Transport during Hyperthermia of Tumor Cells

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Abstract—*The effects of different concentrations of extracellular ion calcium on the transport of calcium by tumor cells have been studied by means of the uptake of radiocalcium. Tumor cells incubated at 45°C take up 4-10 times the amount of radioactivity incorporated by cells incubated at 37°C. The difference is still greater (up to 100 times) for the intracellular incorporation as assessed by elimination of the membrane-bound calcium by EGTA treatment. The possible mechanisms involved in this differential behavior are discussed.*

INTRODUCTION

A CHARACTERISTIC of tumor cells is a changed plasma membrane permeability to small ions and molecules. Differences in the concentration of various cations in comparison to normal cells have been reported by several authors [1-3]. The observed increase of intracellular calcium during carcinogenesis has been proposed as the event triggering the neoplastic transformation [4-6]. Recent experimental observations concerning the effects of hyperthermia on tumor cells indicate that an increased permeability of tumor cells to extracellular calcium [7, 8] may play an important role in their susceptibility to heat. Modifications of the ATP-dependent calcium extrusion mechanism and an increased transport of calcium have been observed during hyperthermia [9], suggesting that the cell toxic death by calcium overloading [10] is the most probable cause of hyperthermia lethality.

MATERIALS AND METHODS

Experiments were performed with Ehrlich ascites tumor cells kept in the ascitic form by weekly inoculation into Swiss mice. Eight days after inoculation, a pool of cells from at least three animals was used for each experiment. Care was taken to avoid ascites contamination with blood.

Ehrlich ascites cells were suspended in Tyrode medium (4×10^7 cells/ml) containing the amount of calcium chloride, plus 0.5 μCi of ^{45}Ca, to give the concentration to be assayed.

After 1 hr incubation at 37 or 45°C under an atmosphere of 95% oxygen plus 5% carbon dioxide, triplicate series of incubation tubes were cooled to 4°C and the viability of the cells was assessed by the standard trypan blue technique on aliquots of cell suspensions. After centrifugation the sedimented cells from one triplicate series of tubes were washed three times with ice-cold 0.1 M EGTA, pH 7.2. The other series were washed three times with normal saline. Finally, the cells were dissolved in Protosol (New England Nuclear, Boston, MA, USA) and ^{45}Ca radioactivity counted in a scintillation counter. The radioactivity values of EGTA-washed cells correspond to intracellular calcium while total calcium was counted on saline-washed cells.

RESULTS

The trypan blue exclusion test used to demonstrate plasma membrane alterations in the tumor cells indicated that cells incubated in the presence of relatively low concentrations of extracellular calcium (1.1-2.6 mM) at 37 and 45°C showed no significant differences. Contrary to this, the difference becomes more significant for higher concentrations (5.7-8.6 mM) (Fig. 1).

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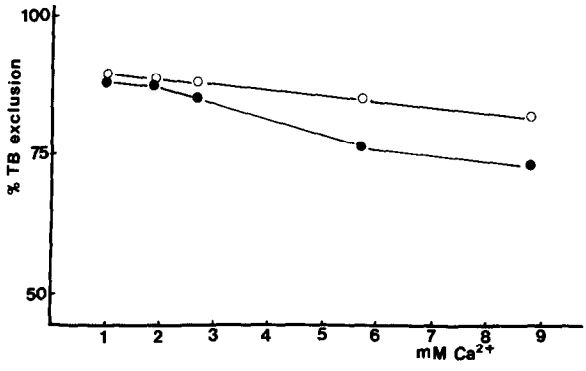

Fig. 1. Trypan blue exclusion by Ehrlich ascites cells as a function of extracellular calcium concentration: ● incubated at 45°C and ○ incubated at 37°C.

When the transport of calcium as a function of its extracellular concentration was calculated considering the uptake of ^{45}Ca after isotopic dilution, very significant differences were observed for all the assayed concentrations of calcium. For the total or the intracellular fraction of calcium ion (total $^{45}Ca^{2+}$ minus EGTA-soluble ^{45}Ca) a fairly similar pattern was observed at 37°C: increased ^{45}Ca uptake with increasing extracellular calcium. Cells incubated at 45°C presented a quite different pattern of total and intracellular uptake of ^{45}Ca. The total value showed a similar but sharper-sloped curve than the corresponding cells incubated at 37°C. At 45°C intracellular ^{45}Ca exhibited a slight decrease as a function of increasing extracellular calcium (Fig. 2). The total uptake of ^{45}Ca was higher by a factor of 4–10 for the cells incubated at 45°C, but the most striking differences were observed for the intracellular uptake at 45 and 37°C. In the presence of 1.1–2.6 mM extracellular calcium the uptake of ^{45}Ca was 100 times lower at 37°C, and this difference diminished as the extracellular concentration of calcium increased (Fig. 2).

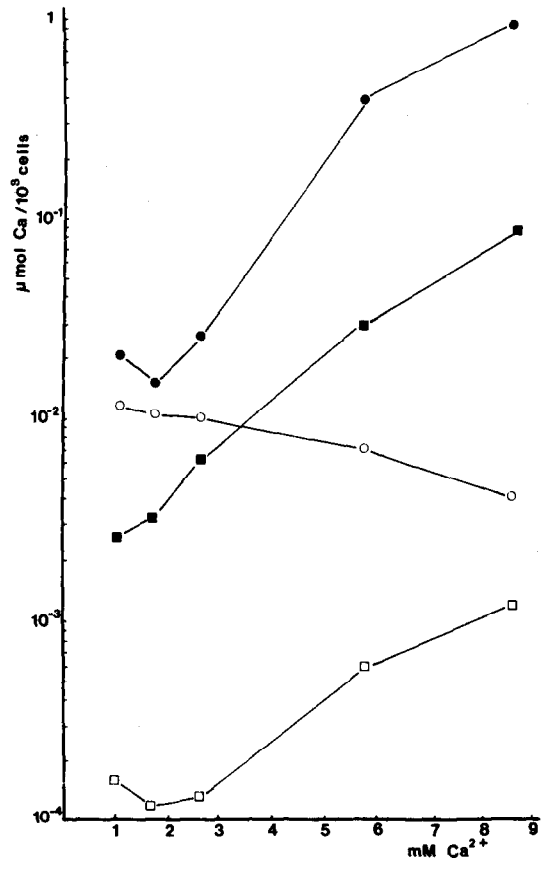

Fig. 2. Uptake of calcium calculated on the basis of ^{45}Ca incorporation and as a function of extracellular calcium concentration: ● as total calcium at 45°C; ■ as total calcium at 37°C; ○ as intracellular calcium at 45°C; and □ as intracellular calcium at 37°C. The values are the mean of a three-tube series under the experimental conditions described in Materials and Methods.

DISCUSSION

The choice of ascitic tumor cells instead of cultured cells was made disregarding their relative resistance to heat and considering that their resistance to mechanical injury minimizes the membrane damage due to experimental handling.

The intracellular concentration of Ca^{2+} is 10^4-fold lower than the extracytoplasmic environment, and is maintained at a value lower than 10^{-7} M by energy-dependent mechanisms at the level of the plasma membrane, endoplasmic reticulum and mitochondria [11]. If we consider the intra-extracellular difference of concentration we can assume that after isotopic dilution of ^{45}Ca in the extracellular environment with a Ca^{2+} concentration of 1.1–8.6 mM the subsequent isotopic exchange at the cell level does not count significantly in the total uptake of ^{45}Ca by the cells. For this reason this technique, which does not give a measure of total calcium, is widely used to measure the uptake and efflux of Ca^{2+} in biological systems [12]. On these bases we chose it to determine Ca^{2+} transport and uptake during hyperthermia of tumor cells. On the other hand, under our experimental conditions we can consider that the contribution to ^{45}Ca uptake by an increased calcium inflow into the cells induced by an increase in the extracellular concentration of Ca^{2+} by a process of Ca^{2+}-Ca^{2+} exchange is minimized with respect to total uptake by the isotopic dilution.

The minimal difference of trypan blue exclusion observed with extracellular Ca^{2+} concentrations between 1.1 and 2.6 mM might be related to a protective effect of calcium. It is known that Ca^{2+} decreases membrane fluidity by a concentration-dependent saturable process that reaches a plateau at about 2.2 mM [13, 14]. It seems likely that in the presence of those concentrations the physicochemical change due to cation interaction may counteract the effects of heat on the plasma membrane fluidity.

Considering the values of ^{45}Ca uptake, we can infer the implication in that process of a major compartment at the tumor cell membrane level. On the other hand, the observed differences in this uptake that are not coincident with a diminution of trypan blue exclusion appear to indicate that the increase of intracellular Ca^{2+} is an event preceding any important change of the physicochemical characteristics of the tumor cell plasma membrane [6]. Nevertheless, the 100-fold increase of intracellular uptake of ^{45}Ca in the presence of 1.1–2.6 mM extracellular Ca^{2+} reflects a drastic change in the transport of Ca^{2+}. This phenomenon, which seems mainly temperature-dependent, supports already published observations on the inhibitory effects of hyperthermia on the ATPase activity of tumor cells [9].

The inhibition of the calcium pump as the major cause of increased Ca^{2+} during hyperthermia is supported by previous observations on $^{42}K^+$, $^{86}Rb^+$ and $^{45}Ca^{2+}$ fluxes during hyperthermia [15–17] that indicate a relationship between damage at the plasma membrane level and important changes in the transport of electrolytes. One of the most interesting results on hyperthermia potentiation by membrane-active agents has been obtained *in vitro* and *in vivo* with La^{3+} [16, 17]. In this case a drastic change of Ca^{2+} influx is accompanied by inhibition of Ca^{2+} ATPase and K^+, Na^+ ATPase. This phenomenon is in agreement with the reported observation that La^{3+} inhibits K^+, Na^+ ATPase [18], and that the calcium pump is also inhibited by arresting the protein in the cell membrane Ca^{2+}-pump ATPase in a phosphorylated form [19]. These observations confirm early reports on La^{3+} action on Ehrlich ascites tumor cells that indicated alteration in the membrane potential and in electrolyte fluxes resulting from La^{3+} interaction with plasma membrane protein, and that causes alterations in membrane permeability and capacity to active transport of ions [20]. In addition to this, the presence of a high-affinity Ca^{2+}-ATPase pump has been demonstrated in ascitic tumor cells [21].

In the absence of La^{3+} an increase of Ca^{2+} concentration has been reported to inhibit ATPase activity [22, 23] as well as ATP synthesis [24]. Coincidentally, during hyperthermia there is a drastic decrease in ATP concentration [25]. In this respect it is interesting to note that whatever is the factor provoking an increased intracellular Ca^{2+}, if it is not counteracted by the cell buffering systems (mainly sarcoplasmic reticulum and mitochondria) this electrolyte change triggers an increased inflow of Ca^{2+} by Ca^{2+}-pump failure, and an energetic impoverishing by ATP synthesis inhibition; both phenomena leading to calcium-dependent toxic cell death [10].

The higher total uptake of ^{45}Ca at 45°C while the intracellular incorporation remains almost steady could be explained as the result of an augmentation of available calcium-binding sites at the plasma membrane level produced by changes in the fluidity of the lipid double layer. In the light of the available literature and our own results it seems reasonable to suggest that three main factors increase the uptake of ^{45}Ca at 45°C: (1) more available calcium-binding sites as a result of lipid double layer fluidity changes; (2) augmented passive diffusion; and (3) inhibition of calcium-pump. It is likely that simultaneously, the efflux due to augmented diffusion and to the death of cells provokes a transport of calcium capable of counterbalancing the intracellular incorporation and results in the almost steady uptake observed in Fig. 2. On the other hand, when the cells are heated to 37°C the curve of the intracellular uptake suggests an increased passive diffusion, concentration-dependence being the only factor affecting ^{45}Ca uptake.

These experimental observations point out that in the tumor cells there is an important passive diffusion of calcium that is greatly increased by heat. Work is in progress to evaluate the therapeutic possibilities of this phenomenon to potentiate the hyperthermic treatment of tumor lesions.

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