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IS CYTOPLASMIC Ca^{2+} IN LYMPHOCYTES ELEVATED IN CYSTIC FIBROSIS?

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An increased cytoplasmic Ca^{2+} concentration ($[Ca^{2+}]_i$) has been implicated in the pathogenesis of cystic fibrosis. We compared the $[Ca^{2+}]_i$ levels of normal and cystic fibrosis peripheral blood lymphocytes and Epstein-Barr virus-transformed lymphoblasts using quin 2, an internally trapped indicator. The $[Ca^{2+}]_i$ levels of normal and cystic fibrosis cells were not significantly different. The ionophore-releasable intracellular Ca^{2+} stores were also comparable in both types of individual.

Cystic fibrosis, an autosomal recessive exocrinopathy, is characterized by secretory and epithelial transport abnormalities. Because of the central role of Ca^{2+} in the control of transmembrane ion movements and in excitation-secretion coupling, alterations in the concentration of this cation have been suggested to underlie the anomalies observed in cystic fibrosis [1,2]. Indeed, a number of studies indicate that different aspects of Ca^{2+} homeostasis, such as transmembrane pumping [3–5] and mitochondrial accumulation [6] may be altered in cells of cystic fibrosis patients. Moreover, the overall Ca^{2+} content of cystic fibrosis cells has been reported to be significantly elevated [7,8]. However, the free cytoplasmic Ca^{2+} concentration ($[Ca^{2+}]_i$), which is presumably the parameter controlling transport and secretion, has not been compared in normal and cystic fibrosis cells. We here report measurements of $[Ca^{2+}]_i$ in cells from cystic fibrosis and normal subjects obtained using quin 2, a novel fluorescent quinoline Ca^{2+} indicator [9]. In addition, minimum estimates of intracellular Ca^{2+} stores were obtained by measuring the change in $[Ca^{2+}]_i$ induced by divalent cation ionophores in Ca^{2+}-free media.

Peripheral blood lymphocytes and cultured lymphoblasts were used for these studies. Peripheral blood lymphocytes are a convenient source of nucleated cells which can be maintained in suspension, facilitating the fluorescence measurements. However, the determinations are liable to interference arising from the drug treatments to which cystic fibrosis patients are subjected. For this reason, the data for peripheral blood lymphocytes were complemented by determinations in cultured lymphoblasts obtained by Epstein-Barr virus transformation of peripheral blood lymphocytes from normal and cystic fibrosis donors. The resulting cells have the advantage of an indefinite life span, thus avoiding the in vitro aging characteristic of human fibroblasts. Moreover, by maintaining the cells in controlled media, interference by therapeutic drugs is circumvented. A potential disadvantage is that transformation may alter cellular properties. However, the similarity of the results obtained in lymphoblasts and untransformed peripheral blood lymphocytes eliminates this concern.

Abbreviations: EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*′-tetraacetic acid; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid.

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Peripheral blood lymphocytes were isolated from heparinized blood as described [10]. To obtain lymphoblasts, peripheral blood lymphocytes from freshly drawn blood were incubated with a cell-free extract prepared by freeze-thawing a previously established lymphoblast line [11]. Growing clumps of cells were observed within 2 weeks which were expanded and stored frozen with 10% glycerol. Two weeks before $[Ca^{2+}]_i$ determination, lines from cystic fibrosis and normal subjects were thawed and cultured as clumps in flasks in minimum essential medium (α MEM) with 15% fetal calf serum. Cells were then transferred into culture tubes and grown at 37°C as single cell suspensions in a roller wheel for 3–4 days. One day before the measurement, exponentially growing cells were seeded in tubes at $4 \cdot 10^5$ cells/ml. At the time of the experiment the density had reached $(8–9) \cdot 10^5$ cells/ml. For the $[Ca^{+2}]_i$ measurements the peripheral blood lymphocytes or lymphoblasts were sedimented, resuspended at a concentration of $(15–35) \cdot 10^6$ cells/ml in either RPMI 1640 or α MEM with 10% serum and loaded with 10 μM quin 2 acetoxymethyl ester (kindly provided by Dr. T.J. Rink, Cambridge University) for 60 min at 37°C. The cells were sedimented again and resuspended in 140 mM NaCl, 3 mM KCl, 1 mM $MgCl_2$, 10 mM glucose, 10 mM Hepes (pH 7.3) with either 0.2 mM EGTA or 1.8 mM $CaCl_2$. Measurement of $[Ca^{2+}]_i$ from quin 2 fluorescence was essentially as described [9]. For calibration, the fluorescence from Ca^{2+}-saturated dye, F_{max},

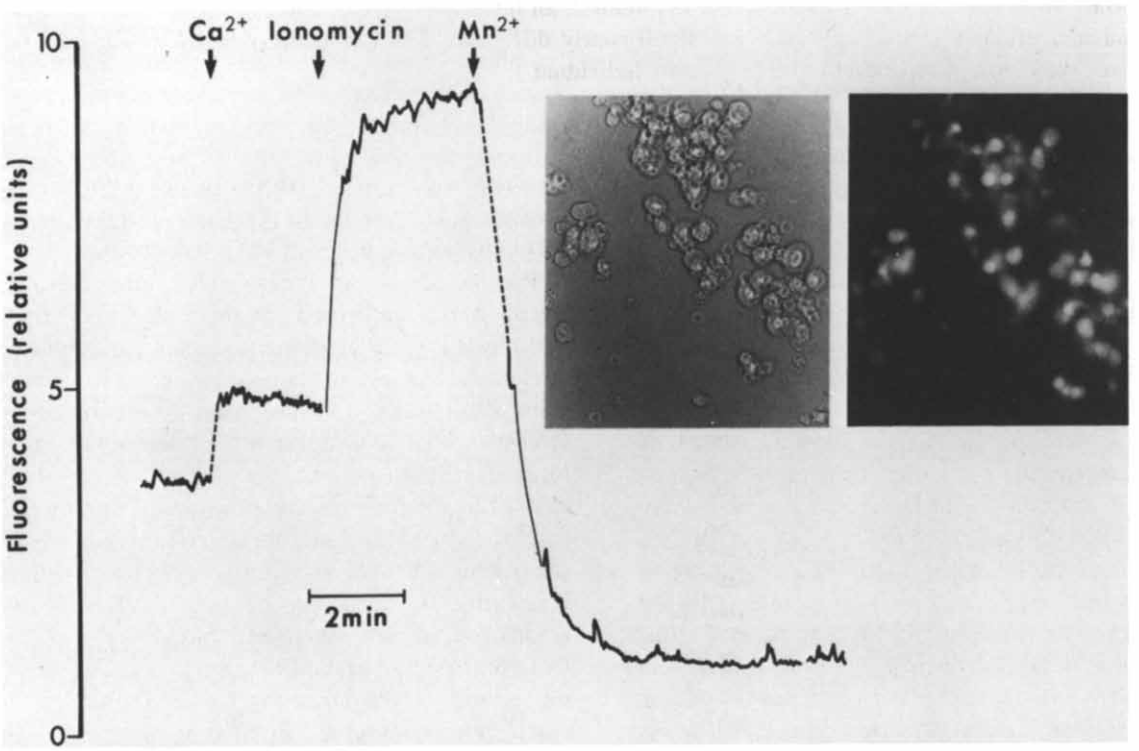

Fig. 1. Determination of $[Ca^{2+}]_i$ in lymphoblasts using quin 2. Fluorescence (excitation: 339 nm with 2 nm slits and emission: 495 nm with 15 nm slits) was recorded in a Perkin Elmer 650-40 spectrofluorimeter attached to an LKB Bromma 2210 plotter. Samples ($3 \cdot 10^6$ cells/ml) were maintained in thermostatted 1-cm square quartz cuvettes and stirred only after each addition. Recording was started 8 min after resuspension of the cells in Ca^{2+}-free medium (left end of trace). Where indicated, Ca^{2+} (final 1.8 mM) was added, followed by 500 nM ionomycin. Finally, 2 mM Mn^{2+} were stirred into the suspension. The dotted lines join the trace where recording was stopped because the sample compartment was opened for additions and stirring. Inset: Phase contrast (left) and fluorescence (right) micrographs of quin 2 loaded cells. Microscopy was performed using a Nikon DIAPHOT epifluorescence microscope with a Nikon MD-12 camera and recorded on Ilford XP1400 film. The filter system had a 395 nm excitation maximum and a cut off at 420. The cells were suspended in Ca^{2+}-containing medium.

was taken as the emission from cells treated with 500 nM of either ionomycin or Br-A23187, a non-fluorescent analog of A23187 [12] in Ca^{2+}-containing medium (Fig. 1). $MnCl_2$ (2–5 mM), which totally quenches quin 2 and is also transported by the ionophores was then added (Fig. 1), leaving only the signal from the cells (autofluorescence). F_{min}, the fluorescence from Ca^{2+}-free dye, was then estimated as:

$$F_{min} = [1/6 \cdot (F_{max} - \text{autofluorescence}) + \text{autofluorescence}]$$

and $[Ca^{2+}]_i$ was calculated from the observed fluorescence, F, as

$$[Ca^{2+}]_i = 115 \text{ nM } (F - F_{min})/(F_{max} - F)$$

115 nM being the apparent K_d for Ca^{2+}/quin 2 at 37°C.

As shown in the inset of Fig. 1, all the cells accumulated the dye, which was found to be distributed homogeneously throughout the cytoplasm, in agreement with the fractionation experiments of Tsien et al. [9] in pig lymphocytes. No fluorescence was detected microscopically in unloaded cells. A typical fluorescence recording is illustrated in Fig. 1. Quin 2-loaded cells were suspended in Ca^{2+}-free medium with EGTA and the fluorescence recorded after 6–8 min. At this time the rate of decline of the signal, indicative of a gradual reduction in $[Ca^{2+}]_i$, is rather slow and reliable measurements can be accomplished. Addition of extracellular Ca^{2+} rapidly restores normal and steady $[Ca^{2+}]_i$ levels. Maximum fluorescence is obtained by increasing $[Ca^{2+}]_i$ with ionomycin or BrA-23187, but only in the presence of extracellular Ca^{2+}. Finally, quin 2 was quenched by allowing Mn^{2+} into the cells through the ionophore. Fluorescence microscopy confirmed that uniform quenching had occurred (not illustrated). Using this procedure, we then compared the $[Ca^{2+}]_i$ levels of peripheral blood lymphocytes from cystic fibrosis patients and age and/or sex matched control subjects. The results are summarized in Table I. The mean $[Ca^{+2}]_i$ in normal peripheral blood lymphocytes was 160 ± 9 nM (mean ± S.E.). This value is similar to those reported for pig mesenteric and mouse spleen lymphocytes [9]. $[Ca^{+2}]_i$ in peripheral blood lymphocytes from cystic fibrosis patients was 156 ± 13 nM (mean ± S.E.). This value is not significantly different ($P > 0.05$) from that of normal cells.

TABLE I

$[Ca^{2+}]_i$ AND RELEASABLE Ca^{2+} STORES IN CYSTIC FIBROSIS AND NORMAL PERIPHERAL BLOOD LYMPHOCYTES

Subject	Sex	Age (years)	$[Ca^{2+}]_i$ (nM)	Ca^{2+} stores (pmol/10^6 cells)
Normal subjects				
B.E.	M	21	130	110
S.G.	M	33	173	178
A.S.	F	9	127	35
B.B.	F	47	175	90
L.A.	F	22	189	123
J.G.	F	22	185	74
B.P.	M	23	128	74
O.L.	M	9	172	44
Mean ± S.E.			160 ± 9	91 ± 16
Cystic fibrosis patients				
J.D.	M	16	167	237
H.G.	M	32	98	153
R.T.	M	9	153	83
B.K.	F	42	172	122
M.A.	M	15	195	79
P.N.	M	24	124	98
S.B.	M	12	186	106
Mean ± S.E.			156 ± 13	125 ± 21

We also compared the magnitude of the intracellular Ca^{2+} stores in cystic fibrosis and normal peripheral blood lymphocytes. An estimate of the unbound Ca^{2+} stores inside the cells was obtained using ionophores. Cells were suspended in Ca^{2+}-free medium with EGTA and after 6–8 min, 500 nM of either ionomycin or Br-A23187 were added. An increased signal generated by release of Ca^{2+} from internal stores was recorded. Knowing the cellular content of quin 2 (by calibration against a standard) and the dissociation constant of the quin 2-Ca^{2+} complex, the amount of Ca^{2+} released by the ionophores can be calculated from the recorded fluorescence change. These values will be lower limits for the Ca^{2+} stores, since: (a) certain Ca^{2+} pools may not be released by the ionophores and (b) rapid Ca^{2+} efflux from the cells during the course of the measurements can result in underestimation of the stores. Nevertheless, if carried out under identical conditions, this type of measurement provides valid and valuable comparisons between cystic fibrosis and normal cells. The data are summarized in Table I. Normal cells contained 91 ± 16 pmol $Ca^{2+}/10^6$ cells (mean ± S.E.; $n = 8$); this value, as expected, is somewhat lower than the reported estimates of total Ca^{2+} in other lymphoid cells [13,14]. In cystic fibrosis cells, the ionophore-releasable Ca^{2+} stores averaged 125 ± 21 pmol $Ca^{2+}/10^6$ cells, which is not significantly different from the control values ($P > 0.05$).

Table II summarizes experiments with lymphoblast lines obtained by viral transformation of peripheral blood lymphocytes from cystic fibrosis and normal subjects. $[Ca^{2+}]_i$ in normal lymphoblasts was 104 nM, which is in the same range as the values of peripheral blood lymphocytes and other lymphocytes [9]. No significant difference was noted between cystic fibrosis and normal cells. Similarly, the ionophore releasable stores are not different in cells derived from cystic fibrosis and normal donors. The larger Ca^{2+} stores of the lymphoblasts compared to peripheral blood lymphocytes can probably be accounted for by the larger size of the former.

Because of earlier reports of mitochondrial abnormalities in cystic fibrosis, attempts were also made to measure the Ca^{2+} stored in the mitochondrial compartment. For this purpose, cells suspended in Ca^{2+}-free media were treated with FCCP, a mitochondrial uncoupler. Only a very small change in fluorescence was elicited by the protonophore so that no accurate estimates of mitochondrial Ca^{2+} stores could be made. This is similar to the results obtained in other types of lymphocytes [9] and can be explained by the very small fractional volume (approx. 4%) occupied by

TABLE II

$[Ca^{2+}]_i$ AND RELEASABLE Ca^{2+} STORES IN CYSTIC FIBROSIS AND NORMAL LYMPHOBLASTS

Cell strain numbers	Sex	Age (years)	$[Ca^{2+}]_i$ (nM)	Ca^{2+} stores (pmol/10^6 cells)
Normal subjects				
93	F	4	110	114
92	F	12	95	69
50	M	9	102	152
55	M	11	99	291
15	M	3	116	245
Mean ± S.E.			104 ± 4	174 ± 41
Cystic fibrosis patients				
115	F	4	98	128
202	F	15	105	173
135	M	9	124	121
139	M	9	109	180
132	M	18	125	138
192	F	3.5	113	218
Mean ± S.E.			112 ± 4	160 ± 15

mitochondria in lymphoid cells.

In summary, no significant differences were found between the $[Ca^{2+}]_i$ levels of cystic fibrosis and normal peripheral blood lymphocytes and lymphoblasts. Moreover, the ionophore-sensitive Ca^{2+}-stores in these cells were also found to be equivalent. The latter is in disagreement with the results of Shapiro et al. [15] in cultured fibroblasts. These authors have attributed the observed differences to a mitochondrial abnormality [6], so that the apparent discrepancy could be accounted for by the higher mitochondrial content of fibroblasts. Alternatively, aging, which is a critical factor in the expression of the cystic fibrosis anomaly in fibroblasts [15,16], may explain the difference in the results.

Although it is possible that alterations in Ca^{2+}-metabolism in cystic fibrosis occur only in certain cell types, e.g. in secretory tissues, the present evidence suggests that generalized changes in $[Ca^{2+}]_i$ do not occur. Considering the critical involvement of $[Ca^{2+}]_i$ in a number of essential cell functions, it is unlikely that mutations resulting in drastic changes of this parameter would be viable.

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