

# The free cytoplasmic calcium concentration of tumorigenic and non-tumorigenic human somatic cell hybrids

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**Summary** The fluorescent indicator of  $\text{Ca}^{2+}$  concentration, quin-2, has been used to measure the concentration of free  $\text{Ca}^{2+}$  in the cytoplasm of tumorigenic and non-tumorigenic human somatic cell hybrids. The cell hybrids were derived from the fusion of a HeLa derivative (D98 AH2) and normal human fibroblasts. The calcium concentration of the tumorigenic cell lines was  $180 \pm 7 \text{ nM}$  and the level in the non-tumorigenic cells was  $136 \pm 6 \text{ nM}$ . This difference was statistically highly significant ( $P < 0.001$ ). Control experiments are reported which show that the level of  $3\text{a}^{2+}$  measured is not influenced by cell density or by the concentration of quin-2-tetra-(acetoxymethyl)ester used in these experiments. The possible implications of this elevated level of cytoplasmic calcium in tumorigenic cells are discussed.

Strong evidence suggests that  $\text{Ca}^{2+}$  plays an important role in the triggering of mitogen induced cell proliferation in a number of types of cells Tsien *et al.*, 1982a; Moolenaar *et al.*, 1984) and that calcium might be important in regulating cell differentiation (Bridges *et al.*, 1981). These observations are supported by other studies demonstrating that culture medium containing low  $\text{Ca}^{2+}$  ion concentrations retards the growth of normal cells while allowing the proliferation of transformed cells (Veigl *et al.* 1984).

In view of the involvement of  $\text{Ca}^{2+}$  in cell transformation and the lack of information concerning the relative cytosolic free calcium concentration ( $[\text{Ca}^{2+}]_i$ ) of normal and transformed cells, we have measured the level of  $[\text{Ca}^{2+}]_i$  in normal cells and somatic cell hybrids of tumorigenic and non-tumorigenic phenotypes (Stanbridge & Wilkinson, 1978). In this system, tumorigenicity of the cell lines is defined by the ability of the hybrids to grow progressively in nude mice and not by *in vitro* characteristics associated with transformed cells; like anchorage independent growth, lectin agglutination and dependence on serum growth factors (Stanbridge *et al.*, 1982). One of the few *in vitro* characteristics which does correlate with tumorigenicity in these cells is the disruption of the microfilaments (Gowing *et al.*, 1984), a complex process which may be influenced by the activity of calcium-dependent regulatory proteins. The magnitude of  $[\text{Ca}^{2+}]_i$  was measured by the non-disruptive use of the intracellularly trapped fluorescent  $\text{Ca}^{2+}$  indicator, quin-2 (Tsien *et al.*,

1982a; Moolenaar *et al.*, 1984). Since the studies of Swierenga *et al.*, (1984) have shown that the cellular organization of the microfilaments is sensitive to the calcium concentration of the growth medium, it is possible that there may be a direct connexion between  $[\text{Ca}^{2+}]_i$ , microfilament organization and the expression of tumorigenicity. The purpose of this study was to see if tumorigenic cells differ in their  $[\text{Ca}^{2+}]_i$  compared with normal or transformed cells.

## Materials and methods

All reagents were of analytical grade. Quin-2-tetra-(acetoxymethyl)ester (quin-2/AM) was purchased from Amersham (No. N.239) and Sigma Chemical Company (No Q-4875) and dissolved at a concentration of 10–20 mM in dimethyl sulphoxide (DMSO). The stock solution of quin-2/AM was stored as several aliquots at  $-20^\circ\text{C}$  in the dark. This solution was stable for many weeks. The fluorescence spectra of quin-2/AM from each source was identical.

## Cell culture

Cell lines were maintained in Dulbecco's Modified Eagle's Medium (Gibco No. 430.2100) supplemented with glutamine (2 mM), pyruvate (2 mM), 5% (v/v) foetal calf serum and 5% (v/v) newborn calf serum. The cells were regularly screened for mycoplasma according to the method of Chen (1977). Details regarding the routine cell culture methods have been published (Gowing *et al.*, 1984).

The cells were grown on rectangular glass coverslips for 48 h or more before each experiment. Routinely,  $4 \times 10^5$  cells were added to 5 cm

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diameter plastic tissue culture petri dishes containing 3 rectangular coverslips. Cells were cultured in 5% CO<sub>2</sub> in an air atmosphere at 37°C. The rectangular glass coverslips, 9 × 40 mm, were prepared from 40 × 24 mm Assistent Micro Cover glasses. Prior to tissue culture the coverslips were baked in a hot oven and washed with chloroform and then detergent (Pyroneg, Diversey, Sydney) and finally rinsed extensively in tap water and distilled water to remove any traces of grease.

#### Principle of the quin-2 method

The validity of the quin-2 method as a monitor of [Ca<sup>2+</sup>]<sub>i</sub> has been documented for lymphocytes (Tsien *et al.*, 1982b). The method has also been applied to many other cell types including myocytes (Powell *et al.*, 1984), fibroblasts (Moolenaar *et al.*, 1984), neutrophils (Nakagawara *et al.*, 1984), platelets (Rink *et al.*, 1982) and adrenal glomerulosa cells (Capponi *et al.*, 1984). In brief, cells are incubated with quin-2/AM which crosses the cell membrane and intracellular esterases hydrolyze quin-2/AM to quin-2 which is then trapped within the cells. The binding of Ca<sup>2+</sup> to quin-2 results in a marked change in the quin-2 fluorescence spectrum. By measuring the fluorescence of the cells, the magnitude of [Ca<sup>2+</sup>]<sub>i</sub> (nM) can then be determined from equation 1 (Tsien *et al.*, 1982a, b);

$$[\text{Ca}^{2+}]_i = Kd[F - 0.16F_{\text{max}}]/[F_{\text{max}} - F] \quad (1)$$

where  $F$  is the fluorescence intensity of quin-2 within the cells,  $F_{\text{max}}$  is the fluorescence intensity of the quin-2 saturated with Ca<sup>2+</sup>,  $0.16F_{\text{max}}$  is the fluorescence intensity of Ca<sup>2+</sup>-free quin-2 (or the Mg<sup>2+</sup> complex) and  $Kd$  (115 nM) is the dissociation constant for the Ca<sup>2+</sup>-quin-2 complex at cytoplasmic pH and ionic conditions.  $F_{\text{max}}$  is typically obtained by making the cells permeable to Ca<sup>2+</sup> from the external supporting buffer (Ca<sup>2+</sup> = 1.8 mM) which saturates the cytoplasmic quin-2 with Ca<sup>2+</sup>. This is usually achieved by treatment of the cells with a detergent such as digitonin.

#### Loading of cells with quin-2

The cells, attached to their rectangular coverslips, were rinsed 9 times in PBS (7 mM Na<sub>2</sub>HPO<sub>4</sub>·2H<sub>2</sub>O, 3 mM NaH<sub>2</sub>PO<sub>4</sub>·2H<sub>2</sub>O, 137 mM NaCl) and transferred to DMEM-10 mM HEPES, pH 7.4 containing the quin-2/AM. As a control, equivalent dishes of cells were prepared and transferred to media containing DMSO in the same proportion (always <0.25%, v/v). The cells were then incubated for 1 h at 37°C in an air atmosphere, in the dark. The cells were rinsed again 9 times with PBS and transferred to HBS buffer (140 mM NaCl,

5 mM KCl, 1.8 mM CaCl<sub>2</sub>, 1.0 mM MgCl<sub>2</sub>, 10 mM glucose, 10 mM HEPES, pH 7.4) at 37°C. The cells in the HBS buffer were used as soon as possible. The quin-2 fluorescence signal was found to be constant over a period of at least 2 h.

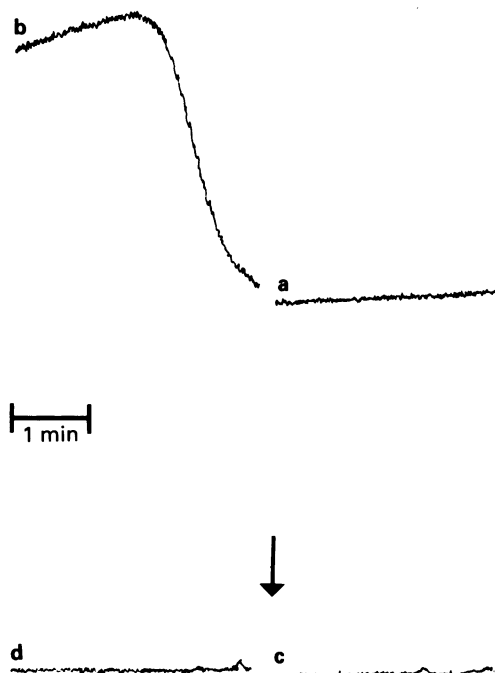
#### Fluorescence measurements

Quin-2/AM-treated cells grown on glass slides were inserted into 3 mL fluorescence cuvettes (1 cm × 1 cm) which contained 2.2 mL of HBS buffer equilibrated to 37°C. Each cuvette contained within it a plastic base and lid each with slots cut so as to retain the rectangular glass coverslip in a central and vertical position. This arrangement allowed reproducible positioning of the glass slide. Neither the base nor the lid interfered with the fluorescence measurements. It was important to use slides which were close to the internal width of the fluorescence cuvette (1 cm) to minimize fluorescence artefacts arising from the edge of the slide. A Perkin Elmer LS-5 luminescence spectrometer with a thermostated cell holder was used for fluorescence measurements. The fluorescence intensity was recorded at an emission wavelength of 492 nm (10 nm slit width) with excitation at 339 nm (5 nm slit width).

## Results

#### Measurement of [Ca<sup>2+</sup>]<sub>i</sub>

The free cytosolic Ca<sup>2+</sup> ion concentration has been measured in a number of cell lines by the use of the intracellularly trapped fluorescent Ca<sup>2+</sup> indicator, quin-2. The majority of previous studies have been performed with cell suspensions of blood cells or cells derived from a number of solid tissues. In the latter case the mechanics of tissue disruption and cell suspension may alter the magnitude of [Ca<sup>2+</sup>]<sub>i</sub>. To avoid these problems we have used a technique recently described by Moolenaar *et al.* (1984) which allows the *in situ* measurement of [Ca<sup>2+</sup>]<sub>i</sub> of cells grown on glass coverslips. Figure 1 shows a typical set of experimental data. The top tracings follow the fluorescence change that occurs when digitonin (10 μM final concentration) is added to cells which have been loaded with quin-2/AM (50 μM). The lower tracings measure the equivalent result for control cells. Before the addition of digitonin, the fluorescence intensity of quin-2/AM treated cells is constant at a level ~5-fold greater than the signal of control cells. Addition of digitonin to quin-2/AM treated cells results in a fluorescence increase following a small lag period of 0.5–1.0 min. A maximum fluorescence intensity is reached 1–3 min after the addition of 10 μM digitonin. Presumably



**Figure 1** A typical quin-2 fluorescence profile showing the experimental data which allows the determination of the values of  $F$  and  $F_{\max}$ . Cells (5E) were grown on glass coverslips and loaded with quin-2/AM as described in **Materials and methods**. The slides were positioned within a 3 ml fluorescence cuvette containing 2.2 ml of HBS buffer at 37°C. The fluorescence intensity before the addition of digitonin (a) was measured and then the contents of the cuvette were made 10  $\mu\text{M}$  in digitonin. The ensuing time course was monitored until a final maximum fluorescence intensity was obtained (b). Corresponding control experiments (c and d) were also performed with cells treated in the same way but without the addition of quin-2/AM. The arrow shows the time at which digitonin was added. Time progresses from right to left.

digitonin permeabilizes the cell to  $\text{Ca}^{2+}$ , from the HBS buffer, which contains 1.8 mM  $\text{Ca}^{2+}$ , and this saturates the intracellular quin-2 with  $\text{Ca}^{2+}$  thereby leading to the fluorescence increase. The concentration of digitonin used in these studies was chosen to selectively permeabilize the cells to extracellular  $\text{Ca}^{2+}$  but to prevent the rapid outflow of cytoplasmic quin-2. The slow fluorescence decay that is observed after  $\sim 3$  min probably represents the leakage of quin-2 from the cells. Higher concentrations of digitonin decreased the initial lag period, accelerated the approach to the maximum fluorescence intensity and increased the rate of

fluorescence decay following this maximum. At these higher concentrations of digitonin the selective permeabilization, to extracellular  $\text{Ca}^{2+}$ , is overtaken by the leakage of quin-2 from the cells. Thus, the concentration of digitonin chosen is important for the reliable measurement of  $F_{\max}$ . Experiments were done to maximize this selective permeabilization of the cells to extracellular  $\text{Ca}^{2+}$ . The optimal concentration of digitonin was 10  $\mu\text{M}$ . Digitonin (10  $\mu\text{M}$ ) increased the background fluorescence intensity of control cells by  $\sim 5-7\%$ . This increase occurred within the time taken to mix the solution and no time course was observed. The fluorescence of the control slides was probably due to light scattering and autofluorescence. The value for  $F$  was calculated by subtracting the appropriate control value from the initial fluorescence intensity.  $F_{\max}$  was calculated by subtracting the background fluorescence of the digitonin-treated control from the maximum fluorescence intensity of the quin-2/AM treated cells after the addition of digitonin (Figure 1). These values were used in conjunction with equation 1 to calculate the  $[\text{Ca}^{2+}]_i$  value. It should be noted that small changes in the absolute fluorescence intensity can be translated into large changes in the magnitude of  $[\text{Ca}^{2+}]_i$ . If the initial fluorescence intensity shown in Figure 1 had been 10% higher, the magnitude of the increase in  $[\text{Ca}^{2+}]_i$  would be 73%.

#### *The effect of cell density on the measured level of $[\text{Ca}^{2+}]_i$*

Cell density could be a variable which in itself may influence  $[\text{Ca}^{2+}]_i$ . In view of this, experiments were done to determine the possible influence of cell density on  $[\text{Ca}^{2+}]_i$ . Table I shows no significant alteration in the magnitude of  $[\text{Ca}^{2+}]_i$  for cell densities ranging between  $1.6 \times 10^4$  and  $4.4 \times 10^4$  cells  $\text{cm}^{-2}$ . The range of cell densities shown in Table I encompasses those that were used in all subsequent experiments.

**Table I** Effect of cell density on intracellular free calcium concentration

Cell density <sup>a</sup>		
Plated (cells/dish)	Coverslip (cells $\text{cm}^{-2}$ )	$[\text{Ca}^{2+}]_i$ (nM)
$8 \times 10^5$	$4.4 \times 10^4$	126 ( $\pm 29$ ; n = 8)
$4 \times 10^5$	$3.3 \times 10^4$	136 ( $\pm 23$ ; n = 8)
$2 \times 10^5$	$1.6 \times 10^4$	125 ( $\pm 16$ ; n = 5)

<sup>a</sup>5E cells plated as described in **Materials and methods**.

*The effect of the concentration of quin-2/AM on the measured level of  $[Ca^{2+}]_i$*

Quin-2, at sufficiently high concentrations (2–20 mM), may have toxic effects upon cells (Spray *et al.*, 1984). In addition, quin-2 may act as an intracellular  $Ca^{2+}$  buffer dampening  $Ca^{2+}$  transients and also possibly influencing the magnitude of  $[Ca^{2+}]_i$ . As a consequence we have measured  $[Ca^{2+}]_i$  as a function of quin-2/AM concentration (Table II). At low quin-2/AM concentrations the ratio of the fluorescence intensity to the background control signal decreased leading to greater variability in the magnitude of the measured  $[Ca^{2+}]_i$ . Nonetheless, it is clear that quin-2/AM concentrations between 10–100  $\mu$ M have no significant effect on the measured value of  $[Ca^{2+}]_i$ . Examination of the cells at the end of the period of experiment by phase contrast microscopy revealed no obvious abnormality.

**Table II** Effect of quin-2/AM concentration on intracellular free calcium concentration

Quin-2/AM concentration ( $\mu$ M)	$[Ca^{2+}]_i$ (nM)
10	118 ( $\pm 27$ ; n=3)
50	117 ( $\pm 17$ ; n=3)
100	157 ( $\pm 36$ ; n=3)

\*5E cells plated at  $4 \times 10^5$  cells/dish.

#### *Comparison of $[Ca^{2+}]_i$ in tumorigenic and non-tumorigenic cells*

The magnitude of  $[Ca^{2+}]_i$  was measured in 10 human cell lines (Table III). Eight of the cell lines examined are somatic cell hybrids four of which are tumorigenic in nude mice and four of which are non-tumorigenic. In addition, the tumorigenic parental cell line D98-AH2 was studied. The parental non-tumorigenic fibroblast used in the original cell fusion (Stanbridge *et al.*, 1982) was difficult to obtain so a non-tumorigenic foetal fibroblast cell line of similar type (MRC-5) was also included in the study. The tumorigenic status of these cells was reported by Stanbridge *et al.* (1982) and has been confirmed in this laboratory (Gowing *et al.*, 1984).

The data obtained in these experiments were analysed statistically by pooling the data according to the tumorigenic potential of the cell types. The mean  $[Ca^{2+}]_i$  of the tumorigenic cells was 180 ( $\pm 7$ ) nM compared with 136 ( $\pm 6$ ) nM in the non-

**Table III** Comparison of the intracellular free calcium concentration in tumorigenic and non-tumorigenic human hybrid cells

Cell name	$[Ca^{2+}]_i$ (nM)	
	Tumorigenic	Non-tumorigenic
5L	185 ( $\pm 34$ ; n=9) <sup>a</sup>	
5E		137 ( $\pm 67$ ; n=24)
D98AH2	196 ( $\pm 68$ ; n=14)	
MRC-5		147 ( $\pm 41$ ; n=8)
ESH39	155 ( $\pm 45$ ; n=16)	
39EC13		139 ( $\pm 36$ ; n=21)
IA3CNTG	203 ( $\pm 29$ ; n=9)	
IA3CN2.1		119 ( $\pm 22$ ; n=8)
5A7MP26.15	175 ( $\pm 56$ ; n=9)	
CN2 B1 COL1		138 ( $\pm 32$ ; n=5)

*Statistical comparison of tumorigenic and non-tumorigenic cells*

Mean	180 nM	136 nM
Number	57	67
s.e.	6.9 nM	5.8 nM
	$t = 4.88$ ; $P < 0.001$	

<sup>a</sup>  $\pm$  s.d.; n = number of determinations.

tumorigenic cells (Table III). The difference in the means is highly significant at  $P < 0.001$ . The activity of a number of calcium-dependent regulatory proteins may be influenced by change in  $[Ca^{2+}]_i$  of the magnitude observed between the tumorigenic and non-tumorigenic cells. The observed difference in  $[Ca^{2+}]_i$  should be due to the tumorigenic potential of the cells and not related to a difference between epithelial and mesothelial cells, since the non-tumorigenic hybrids show an epithelial morphology similar to the tumorigenic hybrids and the parent tumour cell line.

#### **Discussion**

$Ca^{2+}$  is an important step in the course of events which commits a cell to divide (Klee *et al.*, 1980; Michell, 1982; Berridge, 1984) and is also important in the regulation of differentiation (Bridges *et al.*, 1981). Our results show clearly that the tumorigenic cell lines have a higher level of  $[Ca^{2+}]_i$  than the non-tumorigenic cell lines.

It is difficult to suggest the mechanism whereby this elevation of  $[Ca^{2+}]_i$  in tumorigenic cells is induced and regulated. The studies of Smith & Tupper (1984) demonstrate that the control of  $[Ca^{2+}]_i$  in SV-40 transformed human fibroblasts is different from that in the non-transformed counterpart. They suggest that the use of ATP,

derived from glycolysis, is important in regulating the efflux of  $\text{Ca}^{2+}$  from the normal cell but that the mechanism of regulation might be different in the SV-40 transformed cell. Another pathway for the regulation of  $[\text{Ca}^{2+}]_i$  is the release of inositol-1,4,5-triphosphate from the breakdown of phosphatidyl inositol (Berridge, 1984). Inositol-1,4,5-triphosphate has been shown to be active in mobilizing intracellular calcium stores (Streb *et al.*, 1983). The rate of release of inositol-1,4,5-triphosphate is accelerated by the action of growth factors on specific cell surface receptors. A number of these growth factors are related to oncogene products, in particular *sis* with platelet derived growth factor and *v-erb-B* with the epidermal growth factor receptor. Which of these pathways is the crucial one for the regulation of  $[\text{Ca}^{2+}]_i$  in tumorigenic cells remains to be established.

Interestingly, Mitchell *et al.* (1976) have argued that cell division is initiated by a threshold level of cytoplasmic  $\text{Ca}^{2+}$ . Further, they suggest that neoplastic cells divide uncontrollably because of an excessive influx of  $\text{Ca}^{2+}$  through the cytoplasmic membrane and/or failure of cell mitochondria to maintain a low cytoplasmic calcium ion concentration. Our results directly support this contention. Close examination of our present data shows that

the normal, non-transformed cell line MRC-5 has a  $[\text{Ca}^{2+}]_i$  equivalent to the transformed, but not tumorigenic, somatic cell hybrids. This suggests that there is no increase in the levels of  $[\text{Ca}^{2+}]_i$  in the transformed but non-tumorigenic cells. This means that a permanently elevated  $[\text{Ca}^{2+}]_i$  is a characteristic of tumorigenic cells. It will be important to extend these observations to other systems before any general conclusions can be drawn from this important point.

The consequence of the observed elevation of  $[\text{Ca}^{2+}]_i$  in tumorigenic cells is difficult to predict because of the multifarious roles that  $\text{Ca}^{2+}$  plays in cell structure (Korn, 1982; Schliwa, 1981) and metabolism (Hume *et al.*, 1978). However, an elevation of  $[\text{Ca}^{2+}]_i$  may contribute to the disruption of microfilaments which is seen in tumorigenic cells, through the activity of a number of calcium-dependent microfilament regulatory proteins.

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