Thyrotropin-releasing Hormone-induced Changes in Intracellular [Ca^{2+}] Measured by Microspectrofluorometry on Individual quin2-loaded Cells

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ABSTRACT We have developed an accurate and practical method for measuring intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) in single cells in monolayer culture using the fluorescent Ca^{2+}-binding dye quin2. Quin2 was loaded into cells as a membrane-permeant ester which is hydrolyzed in the cytoplasm to the impermeant free acid, which is the indicator form (Tsien, R. Y., T. Pozzan, and T. J. Rink, 1982, *J. Cell Biol.*, 94:325–334). The method involves the measurement of fluorescence at 340-nm excitation (I_{340}), where dye fluorescence is dependent on Ca^{2+}, and at 360-nm excitation (I_{360}), where dye fluorescence is independent of Ca^{2+}. The ratio of these two values (I_{340}/I_{360}) is thus related to the concentration of Ca^{2+} but independent of dye concentration and can be used as a measure of $[Ca^{2+}]$.

To test the ratio method in the microscope, we measured $[Ca^{2+}]_i$ in GH_3 cells in monolayer culture. We found a resting $[Ca^{2+}]_i$ of 44 ± 28 nM (mean ± SD, n = 34), as compared with a suspension value (Gershengorn, M., and C. Thaw, 1983, *Endocrinology*, 113:1522–1524) of 118 ± 18 nM. We also measured $[Ca^{2+}]_i$ during stimulation of the cells with thyrotropin-releasing hormone (TRH) and found a 2.4-fold increase above resting levels within 20 s, a trough at 73% of resting at 90–100 s, and a peak slightly above resting at 3 min. Depolarization of the plasma membrane with KCl produced a sustained increase in $[Ca^{2+}]_i$. All of these data are in good agreement with the results of Gershengorn and Thaw on suspension cultures.

When measuring both resting $[Ca^{2+}]_i$ and the effects of TRH and KCl on small groups of cells, we found some variation among experiments. Using an image intensifier-video camera, we videotaped cells during TRH stimulation. Digital image analysis of these pictures demonstrated that there was a large variation in responsiveness from cell to cell. The microscope ratio method offers the possibility of resolving regions of differing $[Ca^{2+}]$ within the cytoplasm.

Ca^{2+} has been implicated as the mediator of numerous important cellular processes, acting as a second messenger for various hormones and growth factors (1, 10), as a modulator of differentiation (3), in stimulus-contraction coupling in muscle (8), and in stimulus-secretion coupling in neurons and secretory cells (4). It is thus important to have accurate and practical methods for measuring intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) and its changes in response to a variety of physiologic and pharmacologic stimuli. There are several methods for measuring $[Ca^{2+}]$ that are currently in use. These methods have provided valuable information, but each is subject to experimental limitations. For example, Ca^{2+}-sensitive microelectrodes can only be used in cells large enough to be impaled, and they only monitor $[Ca^{2+}]$ near the electrode tip. Also, impalement with a microelectrode may perturb the cell's calcium homeostasis (17). Photoproteins (e.g., aequorin) and bis-azo dyes (e.g., arsenazo III) share two disadvantages: (*a*) They can only be introduced into cells by disruptive methods (such as microinjection or osmotic lysis [7]). (*b*) They have limited specificity for Ca^{2+}, since they are affected by Mg^{2+}, ionic strength, temperature (photoproteins), and pH (bis-azo dyes) (17).

One of the best $[Ca^{2+}]_i$ measurement methods currently in use employs fluorescent tetracarboxylate dyes that bind Ca^{2+} with consequent changes in fluorescence emission intensity (15). The most widely used is quin2, which can be loaded

[1] *Abbreviations used in this paper*: quin2, methoxyquinoline derivative of bis (o-aminophenoxyl)ethane-N,N,N′,N′-tetraacetic acid; quin2/AM, tetraacetoxymethyl ester of quin2; BSS, balanced salt solution; TRH, thyrotropin-releasing hormone; I_{340}, intensity of fluorescence emission at 492 nm with 340-nm excitation; I_{360}, intensity of fluorescence emission at 492 nm with 360-nm excitation; R, the ratio I_{340}/I_{360}.

into cells nondisruptively as a membrane-permeant ester that is cleaved by cytoplasmic esterases. This traps the dye inside the cell (16). Quin2 is highly specific for Ca^{2+}, being unaffected by $[Mg^{2+}]$, pH and $[Na^+]$ within the physiologic range for cytoplasm (15–18).

We have sought to develop a method using quin2 based on microscope spectrofluorometry and digital image analysis that would allow rapid, accurate measurements of $[Ca^{2+}]$ within individual cells. Ideally, such a method should provide high temporal and spatial resolution, as compared with existing methods. This microscopic method could be used for several studies that cannot be performed on large populations of cells. For example, the response of a specific cell type (e.g., neurons) in a mixed cell population could be studied, or calcium levels at different stages of mitosis could be measured. The cell-to-cell variation in response to a stimulus could be evaluated. Also, microscopic observation of the cells should increase the accuracy of measurements since dye bound to dead cells can be excluded from the analysis. Finally, high resolution video microscopy may be useful in identifying the intracellular sites of calcium release.

Fluorescence emission intensity from a quin2-loaded cell depends on both calcium concentration and dye concentration within the cell. To use quin2 to measure $[Ca^{2+}]_i$, one must be able to factor out the dye concentration. There are two published methods for this. One method, intended for use on cells in suspension in a cuvette, involves lysing the cells in saturating $[Ca^{2+}]$ after measurements on them have been performed. Then, by addition of excess EGTA to obtain a very low free $[Ca^{2+}]_i$, the minimal fluorescence is measured (18). Quin2 fluorescence values in intact cells are converted to free calcium concentrations by reference to a calibration curve and the fluorescence values obtained with saturating and very low calcium after lysis. This method is not applicable to microscopic methods, as lysing the cells disperses the quin2 from the measurement field. A second method works on the same principle, but instead of lysing the cells to manipulate the $[Ca^{2+}]$ accessible to dye, a calcium ionophore is added to achieve saturation. The Ca^{2+}-free fluorescence is measured by adding Mn^{2+} to the cells, which quenches the Ca^{2+}-dependent quin2 fluorescence (9). Although such a method can be used for microscopic measurements (9), two limitations have been noted. First, A23187, the most readily available calcium ionophore, is itself fluorescent (9). Second, the calibration can only be made once, at the end of an experiment. This means that only one field of cells can be examined on each dish, and leakage of quin2 during long-term experiments could lead to errors in calibration.

The published fluorescence excitation spectrum of quin2 (18) shows an isosbestic point at 360 nm (i.e., quin2 fluorescence is independent of $[Ca^{2+}]$ at this wavelength.) The maximum dependence on $[Ca^{2+}]$ occurs at 340-nm excitation. Thus, the ratio of quin2 emission intensity at 340-nm excitation to that at 360-nm excitation should provide a measure which is related to calcium concentration and independent of quin2 concentration. We show here that the I_{340}/I_{360} intensity ratio can be used as the basis for measuring $[Ca^{2+}]_i$ by microscope spectrofluorometry and by digital image analysis. As an initial application, we show cell-to-cell variation in the response of GH_3 cells to thyrotropin-releasing hormone (TRH).

MATERIALS AND METHODS

Cell Culture: Experiments were performed in GH_3 cells (14) which were a gift of Dr. M. Gershengorn (Cornell University Medical College). The cells were grown in Ham's F-10 medium (Gibco Laboratories, Grand Island, NY) with 15% (vol/vol) horse serum and 2.5 percent (vol/vol) fetal calf serum (both from Gibco Laboratories) at 37°C in a 95% air–5% CO_2 humidified atmosphere. Cells were plated at low density on polylysine-coated glass number 1 coverslips attached to the bottom of 35-mm tissue culture dishes which had holes drilled out of the bottom. Cells were used 1–5 d after plating. Before use, cells were rinsed three times with a balanced salt solution (135 mM NaCl, 4.5 mM KCl, 1.5 mM $CaCl_2$, 0.5 mM $MgCl_2$, 5.6 mM glucose, 10 mM HEPES).

Microscopy: We used a Leitz Diavert microscope with 100× and 40× Nikon UV-Fluor fluorite objectives; epifluorescence illumination was provided by a 100 W mercury vapor lamp. All illumination optics (except the objective) were quartz. Excitation filters were 10-nm bandpass quartz filters centered at 340 and 360 nm (Oriel Corp. of America, Stamford, CT). The 360-nm filter was always paired with a 25% transmission glass neutral density filter (E. Leitz, Rockleigh, NJ) to give approximately equal quin2 fluorescence intensities at the two excitation wavelengths. Emission intensities were measured with a Leitz MPV microscope spectrofluorometer with a 40-nm bandpass emission filter centered at 500 nm. For experiments with cells, illumination intensity was set to reduce dye bleaching to <1.5% per 0.1-s exposure.

Fluorometry: Fluorescence spectra of quin2 solutions were obtained using the microscope spectrofluorometer or with 1-cm quartz cuvettes using a Perkin-Elmer 650-10S spectrofluorometer (Perkin-Elmer Corp., Eden Prairie, MN) with excitation slit width of 4 nm and an emission slit width of 10 nm.

Calibration Curve: To convert I_{340}/I_{360} values (the ratio of fluorescence emission intensity at 340-nm to that at 360-nm excitation) to free calcium concentrations, we constructed a calibration curve of I_{340}/I_{360} as a function of $[Ca^{2+}]$ by using the spectrofluorometer or the microscope photometer to measure the I_{340}/I_{360} ratios of a series of quin2-containing solutions buffered to various $[Ca^{2+}]$ values. The solutions were prepared similarly to Tsien et al. (18). We prepared stocks of ~1 M $CaCl_2$ and ~1 M potassium EGTA (free EGTA brought to pH 8 with KOH). Hundred-fold dilutions of these stocks were made, each into 50 mM MOPS, pH 8, and the diluted EGTA solution was titrated with the diluted $CaCl_2$ solution to Ca-EGTA equimolarity, indicated by a pH minimum (6). We then mixed the original stocks in the same proportions, yielding an equimolar Ca-EGTA stock. We measured the calcium content of this stock by the oxalate method (20) and confirmed the value by atomic absorption spectrometry (U. S. Testing Company, Hoboken, NJ). EGTA concentrations for preparation of buffers were determined by titration against the calibrated $CaCl_2$ stock. Calculations for the preparation of standard solutions were based on a dissociation constant for Ca-EGTA of 400 nM at 23°C based on an ionic strength of 160 mM (5) and corrected to pH 7.05 (21). Each standard solution was prepared by the addition of 0.118 μl of 0.35 M Ca-EGTA stock per nM $[Ca^{2+}]$ desired into 10 ml of the calibration buffer: 20 μM quin2, 115 mM KCl, 20 mM NaCl, 10 mM MOPS, 1.56 mM EGTA, and 1.2 mM $MgCl_2$, titrated to pH 7.05 with KOH (except for the saturating Ca^{2+} solution, which was prepared by addition of 1 M $CaCl_2$ to the buffer, to yield a final concentration of 100 μM).

An untreated cover-slip bottom dish was placed on the microscope stage. 1 ml of a standard solution was placed in the dish, and intensity readings were made at each excitation wavelength using the microscope photometer and filters as described above (see *Microscopy*).

$[Ca^{2+}]_i$ Measurements: A dish of cells was rinsed free of medium and placed in balanced salt solution (BSS). A group of 1 to 10 cells was chosen and centered in the measuring field of the microscope photometer, and cellular autofluorescence (fluorescence of cells before dye addition) was measured at both 340- and 360-nm excitation. Quin2/AM was then added to a final concentration of 20 μM, and the cells were incubated for 20 min on the microscope stage at room temperature. Cells (still centered in the measuring field) were rinsed three times with BSS in situ and then placed in 1 ml BSS for measurement of resting $[Ca^{2+}]_i$. Cells were then stimulated with either TRH (final concentration 1 μM) or KCl (BSS with 50 mM additional KCl). For static measurements (e.g., autofluorescence or resting $[Ca^{2+}]_i$), at least two 0.1-s measurements were made of each field at each wavelength and averaged. For dynamic measurements (stimulation time courses), only one measurement at one wavelength could be taken at each time point. (Our apparatus does not allow excitation filters to be changed quickly enough to make measurements at both wavelengths at one time point.) After subtraction of cellular autofluorescence at each wavelength, the I_{340}/I_{360} ratio was determined. The ratio was converted to free calcium concentration using the calibration curve.

The time course for TRH stimulation at early times (up to 10 s) was recorded in some experiments using a chart recorder connected to the photometer output while illuminating the cells continuously from time of TRH addition. This is only practical for early times, as the quin2 bleaches rapidly even at the lowest intensity sufficient to yield precise measurements.

Image Analysis: Video images were obtained using a Zeiss-Venus TV3 image intensifier-vidicon camera mounted on the microscope and were video taped using a Panasonic NV 8030 videotape recorder. Both the camera and the

recorder were set in the manual mode. Illumination time for each image was 0.25 s.

Images were digitized using a CAT-800 image digitizer as described previously (19). Four video frames from each exposure were averaged to reduce the noise.

Materials: Quin2 and quin2/AM were from Lancaster Synthesis (Eastgate, England) and were stored as 50 mM stock solutions in DMSO at −20°C. TRH was a gift from Dr. M. Gershengorn (Cornell University Medical College, NY). HEPES was from Research Organics (Cleveland, OH). Sodium oxalate (primary oxidimetric standard), sulfuric acid, and 0.1 N potassium permanganate solution (National Bureau of Standards) were from Fisher Scientific (Springfield, NJ). All other chemicals were from Sigma Chemical Co. (St. Louis, MO), unless otherwise specified.

RESULTS

I_{340}/I_{360} as a Specific Indicator of Calcium Concentration

The quin2 excitation spectrum of Tsien et al. (18) shows clearly that quin2 fluorescence is independent of $[Ca^{2+}]$ at 360-nm excitation, whereas quin2 fluorescence increases more than fivefold between zero and saturating $[Ca^{2+}]$ at 340-nm excitation. Consequently, the ratio of fluorescence at 340-nm excitation to that at 360-nm excitation (denoted here as I_{340}/I_{360}) is a measure of $[Ca^{2+}]$ that is independent of quin2 concentration.

To show that I_{340}/I_{360} is a specific measure of $[Ca^{2+}]$ and is not affected by other cations in their physiological concentration ranges, we obtained excitation spectra of quin2 in solutions of varying ionic composition. We measured the excitation spectra of quin2 in solutions of various pH, $[Mg^{2+}]$, and $[K^+]$:$[Na^+]$. The data are summarized in Table I. Varying $[Mg^{2+}]$ between 0 and 10 mM resulted in a 50% increase in I_{340}/I_{360} at zero calcium but had no effect at saturating $[Ca^{2+}]$. There were no significant changes in the I_{340}/I_{360} ratio in the physiological pH range, between pH 6.8 and 7.4. In solutions having $[K^+]$:$[Na^+]$ between 155:0 and 115:40 mM, we found no significant change in the ratio. These results show that changes in I_{340}/I_{360} are highly selective for Ca^{2+} in the physiological intracellular ionic environment.

Calibration Curve

To use quin2 fluorescence as a measure of absolute $[Ca^{2+}]$, we must relate values of I_{340}/I_{360} to the corresponding $[Ca^{2+}]$.

TABLE I

Effect of Ions Other Than Calcium on quin2 Fluorescence

			I_{340}/I_{360}	
pH	K^+:Na^+	Mg^{2+}	No Ca^{2+}	Saturating Ca^{2+}
	mM	*mM*		
6.8				
↕	150:5	1	Mean 0.87*	Mean 4.07*
7.4			Range 0.82–0.92	Range 3.73–4.34
	155:0			
7.0	↕	1	Mean 0.93*	Mean 4.21*
	115:40		Range 0.92–0.93	Range 3.88–4.38
		0	0.83	4.08
		1	0.89	4.30
7.0	150:5	5	1.16	4.15
		10	1.25	4.15

All solutions contained 2 μM quin2 and 10 mM morpholinopropanesulfonic acid. "No Ca^{2+}" solutions were 5 mM in EGTA; "saturating Ca^{2+}" solutions were 10 μM in $CaCl_2$. Excitation spectra were obtained with a spectrofluorometer, monitoring emission at 492 nm.

* No statistically significant differences in I_{340}/I_{360} were found within this range of ionic conditions.

We prepared a series of quin2 solutions with calcium concentrations in the range of interest and measured their excitation spectra. In Fig. 1, we show the I_{340}/I_{360} ratio as a function of $[Ca^{2+}]$. The curve obtained shows the expected behavior for saturable binding of Ca^{2+} to quin2. The relation between I_{340}/I_{360} and Ca^{2+} may be expressed as:

$$R = I_{340}/I_{360} = \frac{R_0 + R_{sat}([Ca^{2+}]/K_d)}{1 + ([Ca^{2+}]/K_d)}, \quad (1)$$

where $R_0 = I_{340}/I_{360}$ at zero Ca^{2+}, $R_{sat} = I_{340}/I_{360}$ at saturating Ca^{2+}, and K_d = the dissociation constant for Ca^{2+} binding to quin2. We linearized this function in a form analogous to that of Scatchard (11) in order to estimate K_d. We plotted $(R - R_0)/[Ca^{2+}]$ as a function of $R - R_0$, using $R - R_0$ as a measure of bound $[Ca^{2+}]$. "Scatchard-like" plots of calibration curves obtained with the fluorometer and with the microscope photometer are shown in Fig. 2. Both the microscope and the fluorometer yield linear plots which have the same slope. The dissociation constant, derived from these plots as the negative reciprocal of the slope, is ~180 nM—somewhat higher than the value of 115 nM at 37°C reported by Tsien et al. (18). We fit our data to equation 1 using our experimental values of R_0

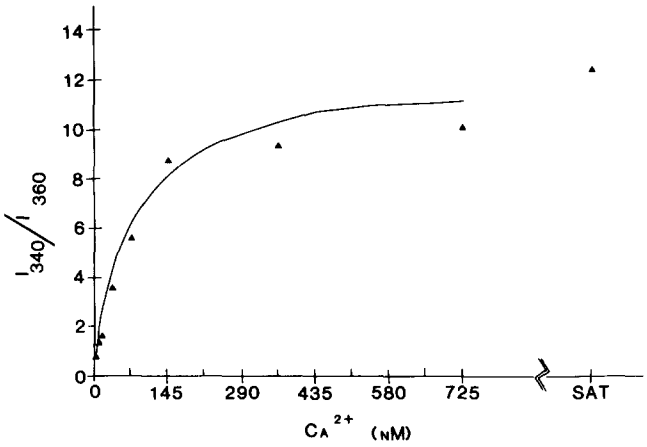

FIGURE 1 Calibration curve for I_{340}/I_{360} vs. $[Ca^{2+}]$. Fluorescence of a series of quin2-containing solutions buffered to precise Ca^{2+} from 0 to 725 nM was measured in the microscope photometer at 340- and 360-nm excitation. Points are the averages of at least three measurements in two separate experiments. The solid line is the curve calculated using Eq. 1, with K_d = 180 nM.

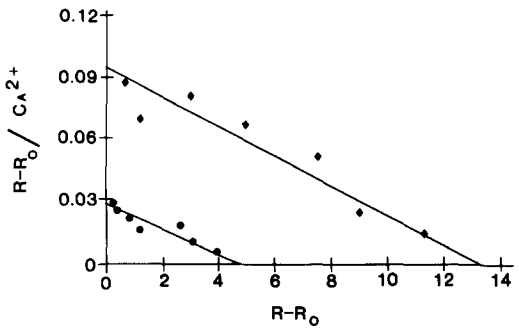

FIGURE 2 Scatchard plot. Calibration curves obtained from measurements with the fluorometer (circles) or with the microscope photometer (diamonds) were linearized in a Scatchard plot as described in the text. Solid lines are least-squares fits to each set of data. R is the I_{340}/I_{360} ratio, and R_0 is the ratio obtained in the absence of Ca^{2+}.

and R_{sat} and the K_d from the "Scatchard plot." The best-fit curve is represented by the solid curve in Fig. 1.

Application of the I_{340}/I_{360} Method to Measure $[Ca^{2+}]$ in Cells

To test the method on live cells, we needed a cell line which could grow in monolayer or suspension, and for which both resting $[Ca^{2+}]_i$ and changes in $[Ca^{2+}]_i$ with pharmacologic treatment had been measured in suspension. Such a cell line was available in the GH_3 clonal rat anterior pituitary tumour line, in which hormone-stimulated calcium fluxes are currently under intensive investigation (12, 13). Gershengorn and Thaw (1) recently published measurements of resting $[Ca^{2+}]_i$ and changes in $[Ca^{2+}]_i$ induced by TRH or membrane depolarization in these cells.

We determined resting $[Ca^{2+}]_i$ by selecting a field of cells within a dish and measuring the I_{340}/I_{360} ratio of quin2 loaded cells as described in Materials and Methods. The ratios were converted to free $[Ca^{2+}]$ using equation 1 with $R_0 = 0.776$, $R_{sat} = 12.8$, and $K_d = 180$ nM.

We found an average $[Ca^{2+}]_i$ in resting GH_3 cells of 44 ± 28 nM (mean ± SD, $n = 34$). This is lower than the 118 ± 18 nM reported by Gershengorn and Thaw (1). Our values of resting $[Ca^{2+}]_i$ are lower than those generally reported for suspensions of cells measured in a cuvette. Possible explanations of this difference will be considered in the Discussion.

Stimulation of Cells with TRH and KCl and Detection of Changes in $[Ca^{2+}]_i$

One of the important uses of a system for measuring $[Ca^{2+}]_i$ is to measure changes in Ca^{2+} concentrations during various physiological processes or when cells are treated with a variety of drugs, hormones, etc. We therefore wished to determine whether our microscope method could be used to measure the time course of changes in $[Ca^{2+}]_i$. We again employed GH_3 cells, which are known to increase $[Ca^{2+}]_i$ in response to TRH and to depolarization of the plasma membrane with KCl (1).

The effect of stimulations was observed on a group of cells whose resting $[Ca^{2+}]_i$ had just been measured. Cells in BSS were then stimulated by addition of either TRH (final concentration 1 μM) or KCl (BSS containing 50 mM additional KCl). Measurements of fluorescence were performed at various times after addition. Generally, I_{360} was measured at the beginning and end of each stimulation time course, and a series of I_{340} measurements were made at various times. By making a series of measurements of I_{360} in separate experiments, we verified that I_{360} does not change upon TRH or KCl stimulation. We also determined, in experiments on cells not loaded with quin2, that cellular autofluorescence is not changed significantly by TRH or KCl treatment (not shown).

We found some experiment-to-experiment variation in responsiveness to TRH. Some cells were totally unresponsive, while others responded with different magnitudes or time courses. When measuring from a small number of cells or single cells, we were able to obtain a fairly reproducible time course, a typical one of which is shown in Fig. 3. Based on five experiments, the average time course shows a 2.4 ± 0.7-fold increase in $[Ca^{2+}]_i$ within 20 s or less, a decline to 73% of resting levels at ~70–120 s, and a plateau slightly above resting levels around 3 min. Eventually, $[Ca^{2+}]_i$ returns to approximately resting levels. In some cases, we used continuous recording to determine the time to reach maximum $[Ca^{2+}]_i$. In four experiments, we found that the maximum was reached in an average of 3 s. These results are in agreement with the time course obtained by Gershengorn and Thaw (1) on suspensions of GH_3 cells, which shows an immediate peak followed by a steep decline, with a slight increase beginning again at ~2.5 min.

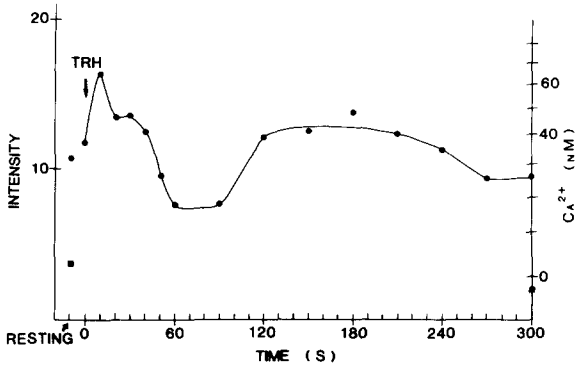

FIGURE 3 Time course of $[Ca^{2+}]_i$ following TRH stimulation. A group of cells was selected and centered in the microscope spectrofluorometer measuring field. Cellular autofluorescence (i.e., cellular fluorescence in the absence of quin2) was measured at 340- and 360-nm excitation. The cells were then loaded in situ with quin2 by incubation with 20 μM quin2/AM for 20 min at room temperature. Free quin2/AM was washed away, and resting Ca^{2+}_i was measured. The cells were then treated with 1 μM TRH and the I_{340} (circles) was measured at various times. I_{360} (squares) was measured at the beginning and the end of the time course. Both I_{340} and I_{360} have been corrected for autofluorescence at their respective wavelengths. The $[Ca^{2+}]$ scale on the right hand side was derived from the calibration curve, Fig. 1.

To examine further cell-to-cell variation in response, we videotaped fluorescence images of cells being stimulated by TRH and analyzed them by image digitization. Fig. 4 shows the 340-nm fluorescence of a group of cells just before TRH addition (Fig. 4*a*) and 3 seconds after addition (Fig. 4*b*). In Fig. 4*c*, we show an image which represents the ratio of the fluorescence after TRH stimulation to that in resting cells (i.e., Fig. 4, *b* "divided by" *a*). Fig. 4*c* clearly demonstrates the cell-to-cell variation in TRH response. One cell (arrow) in Fig. 4*c* is very bright, compared to the cells around it, indicating that it is responding to TRH with a larger increase in $[Ca^{2+}]_i$ than the other cells in the field.

Depolarization of the plasma membrane by increasing $[K^+]$ in the medium by 50 mM yielded a 1.59 ± 0.2-fold increase in $[Ca^{2+}]_i$ which was maintained for at least 10 min, similar to the results of Gershengorn and Thaw (1). A typical time course for KCl stimulation is shown in Fig. 5.

From these data, and especially from the images, it is clear that the microscope method for $[Ca^{2+}]_i$ measurement has the capability of resolving cell-to-cell differences in both resting $[Ca^{2+}]_i$ and the time course of $[Ca^{2+}]_i$ changes.

DISCUSSION

Single cell measurements of intracellular calcium concentrations have been made for several years with Ca^{2+}-sensitive microelectrodes and more recently using microinjected photoproteins and bis-azo dyes. These methods hae been used to provide important information about $[Ca^{2+}]_i$, but there are

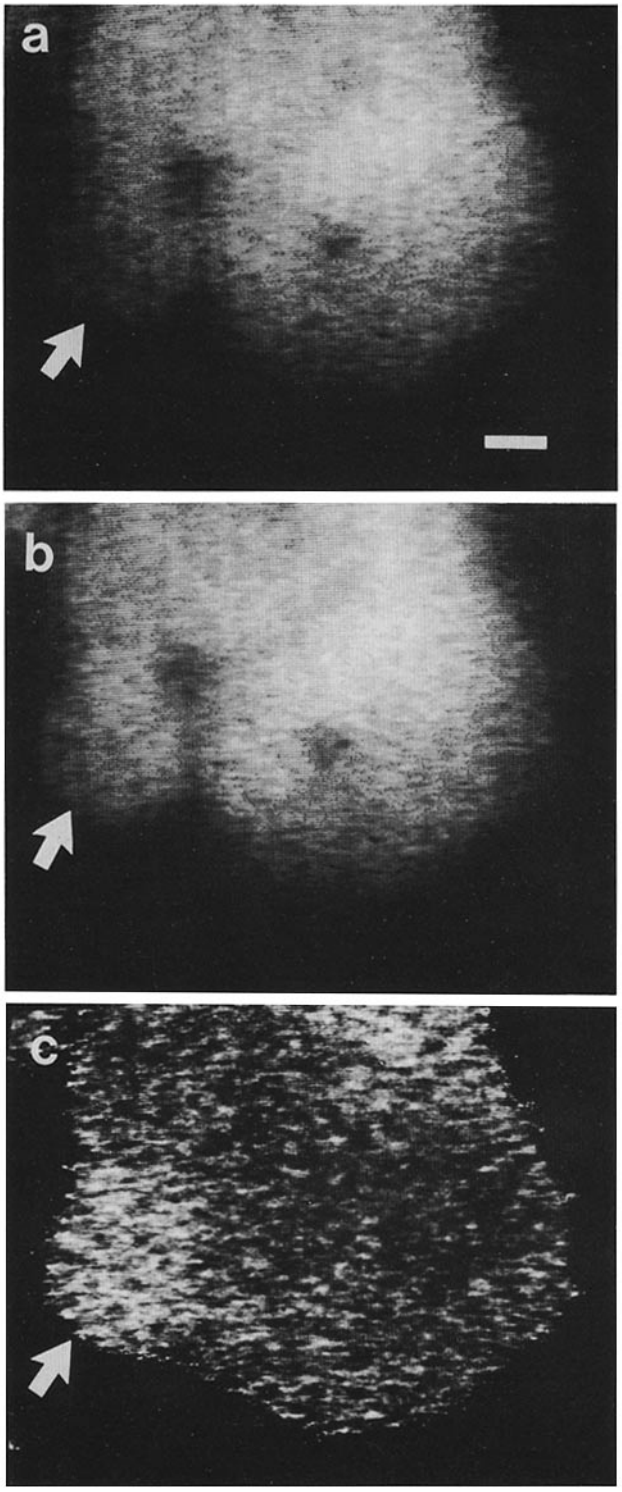

FIGURE 4 Images of cells following TRH stimulation. (a) Digitized image of 340-nm fluorescence of cells just before TRH addition. (b) Digitized image of 340-nm fluorescence of the cells 3 s after TRH addition. (c) Ratio of *b* to *a*, i.e., ratio of peak fluorescence to unstimulated fluorescence. In the ratio image (c) an intensity ratio of 2.5 was set to white, and a ratio of 0 was set to black. The arrow indicates a cell which is strongly TRH-responsive (i.e., brightest in c). Bar, 5 μm. × 1,700.

technical difficulties which limit their usefulness (17). The availability of quin2 has allowed a major advance in measuring intracellular $[Ca^{2+}]$.

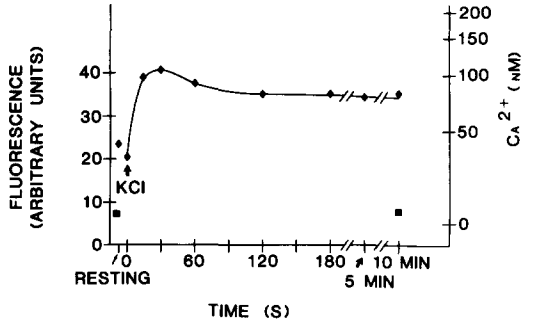

FIGURE 5 Time course of $[Ca^{2+}]_i$ following KCl stimulation. A group of cells was selected and centered in the photometer measuring field. Cellular autofluorescence (i.e., cellular fluorescence in the absence of quin2) was measured at 340- and 360-nm excitation. The cells were then loaded in situ with quin2 by incubation with 20 μM quin2/AM for 20 min at room temperature. Free quin2/AM was washed away and resting $Ca^{2+}{}_i$ was measured. The cells were then treated with BSS containing 50 mM additional KCl and the I_{340} (diamonds) was measured at various times. I_{360} (squares) was measured at the beginning and the end of the time course. Both I_{340} and I_{360} have been corrected for autofluorescence at their respective wavelengths. The Ca^{2+} scale on the right hand side was derived from the calibration curve, Fig. 1.

In this paper, we have demonstrated that a ratio of quin2 fluorescence intensities (I_{340}/I_{360}) can provide a reliable measure of intracellular calcium. The ratio I_{340}/I_{360} is a specific indicator of $[Ca^{2+}]$ under ionic conditions that approximate normal cytoplasmic conditions. The time courses of TRH or KCl-induced changes in $[Ca^{2+}]_i$ in GH_3 cells agree well with previously published results obtained on cell suspensions (1). When coupled with microscope spectrofluorometry and digital image analysis, the ratio method described here should allow several types of studies to be made on individual cells in culture. We note that the ratio method could also be used to measure $[Ca^{2+}]_i$ in individual cells with a two-wavelength fluorescence activated cell sorter.

An alternative method for measuring $[Ca^{2+}]_i$ in the microscope has been described recently by Rogers et al. (9). In their method, the quin2 fluorescence with 340-nm excitation is measured, and calibration is provided by exposure to an ionophore in the presence of either high extracellular Ca^{2+} or Mn^{2+}, which suppresses the Ca^{2+}-dependent quin2 fluorescence. The ionophore method should be capable of providing accurate measurements of $[Ca^{2+}]_i$, but it can only provide calibration at a single time on one field of cells. Thus photobleaching of quin2 or changes in quin2 content (e.g., as a result of leakage from the cell) could lead to errors in the calibration. (In studies on mitotic cells, it was found that loss of quin2 fluorescence was significant during the time required for a cell to pass through mitosis (22).

Although our results from the time course of $[Ca^{2+}]_i$ changes upon TRH stimulation are in good agreement with a previous report on the same cells in suspension (1), our absolute values of $[Ca^{2+}]_i$ are lower than those found in suspension. The reasons for this difference are not clear, but we feel that the microscope method described here may avoid certain experimental problems that could lead to overestimates of $[Ca^{2+}]_i$. First, every cell that we use is examined microscopically before measurements are made. Thus, fields which contain obviously unhealthy cells or debris are excluded from the analysis. Second, since we measure $[Ca^{2+}]_i$ of individual cells or small groups of cells, unusual values can be excluded from the

analysis. In determining the average resting value for $[Ca^{2+}]_i$, we excluded one group of cells which had $[Ca^{2+}]_i$ of 1 μM compared to the average for the other 34 fields of 44 nM. Third, measurement using the microscope method should minimize the effects of extracellular quin2 on determination of $[Ca^{2+}]_i$. In measurements in a cuvette, quin2 which has leaked out of cells or is incompletely rinsed away will report the extracellular $[Ca^{2+}]$, and this quin2 fluorescence is averaged with the intracellular quin2 fluorescence. In microscope measurements, the cells are centered in the measuring area, and the relative contribution from extracellular buffer is small. We do not know whether these differences can account for the lower $[Ca^{2+}]_i$ values obtained in our measurements.

There are some inherent limitations to the use of microscopic measurements of $[Ca^{2+}]_i$ with quin2. Of course, our measurements are subject to general problems which affect all quin2 measurements such as possible side effects of quin2 (2, 18) or possible interference by untested components of the cytoplasm. Since our measurements are made from only one or a few cells, the signal is relatively weak. From repeat measurements on single fields, we estimate that noise could led to errors of $\pm 5\%$ in resting values of $[Ca^{2+}]_i$. When we used high light levels to increase the signal, the bleach rate increased to $>1.5\%$ per measurement (0.1-s exposure). With our present equipment, we could not continuously record fluorescence without unacceptable bleaching or noise.

When coupled with digital image analysis, the ratio method described here should allow us to simultaneously obtain single cell values of $[Ca^{2+}]_i$ from a large number of cells. This will give us the capability to assess cell-to-cell variation in response to various stimuli and to examine certain types of cells in a mixed cell population. As an example, data have been obtained by the 2 methods described here which demonstrate that mitotic PtK_2 cells have a lower value of $[Ca^{2+}]_i$ than interphase cells (22). When used at high magnification, it should also be possible to detect sites of intracellular Ca^{2+} release by analysis of sequential video frames.

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