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THE EFFECTS OF PH AND TEMPERATURE ON FLUORESCENT CALCIUM INDICATORS AS DETERMINED WITH CHELEX-100 AND EDTA BUFFER SYSTEMS

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Summary: A novel method of determining the apparent dissociation constants of fluorescent calcium indicators is described which utilizes Chelex-100 ion exchange resin and ^{45}Ca. The affinity for calcium of indicators fluo-3, fura-2 and indo-1 measured at either 22° or 37°C decreases as pH is decreased from 7.4 to 5.5. These measurements agree with determinations made using EDTA-calcium buffers. The 1:1 calcium:indicator complex is maintained under all conditions. The necessity to correct dissociation constants during intracellular acidification to properly interpret fluorescence measurements is illustrated by indo-1 measurements in the ischemic rat heart.

Fluorescent calcium indicators such as fura-2, indo-1 and fluo-3 have become a popular method of qualitatively or quantitatively determining calcium levels in many cell types (1). Despite the diverse conditions in which these indicators have been used, their K_ds have been determined only at relatively few conditions of ionic strength and pH (2,3). These measurements are difficult because they are generally made with EDTA or EGTA-calcium buffers, which are very sensitive to slight changes in pH, and whose critical values must be extrapolated from a limited set of empirical data (4). Compounding this problem, the necessity for K_d corrections may not be readily apparent to experimenters making qualitative comparisons of changes in fluorescence ratio measurements.

In the present study, a method for the determination of K_ds of fluorescent calcium indicators is described which utilizes Chelex-100 ion exchange resin to buffer a $^{40}Ca/^{45}Ca$ admixture. The effects of changes in temperature and pH on the K_ds of fluo-3, indo-1 and fura-2 are determined and compared to results achieved with calcium-EDTA buffers. This information is then utilized to correct measurements of intracellular calcium activity in the ischemic heart. Some preliminary results have been reported (5).

Abbreviations: EDTA, ethylenediaminetetraacetic acid; EGTA, ethylene glycol bis-(b-aminoethyl ether)N,N,N',N'-tetraacetic acid: K_d, apparent dissociation constant.

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Materials and Methods

Preparation of Chelex-100. Water was obtained from a Nanopure still (Barnstead, Dubuque, IA) with initial resistance of >16 MΩ. 100 gm of Na^+-Chelex-100 (Biorad, Richmond, CA) was placed in a glass column and washed with 250 ml water, followed by 300 ml 1N HCl, 250 ml water, 300 ml 1N KOH, 600 ml water, 400 ml 0.4M MES (pH 6.0, neutralized with KOH) and 250 ml water with a final eluent pH of 6.0. The resin was dried overnight at 22°C and then stored in a sealed container. To determine residual calcium in the K^+-Chelex-100, four 0.1 gm portions of resin were each eluted with 3ml of 0.6M perchloric acid with 0.01% $LaCl_3$ in a glass tulip column (Radnoti Glass Technologies, Monrovia, CA) and the eluent measured in an atomic absorption spectrophotometer. Ca^{++}-Chelex-100 was prepared and approximately 1 mg was used to remove Sc from ^{45}Ca (ICN, Irvine, CA) (6). Total calcium in the ^{45}Ca was verified by the use of arsenazo III (Sigma, St. Louis, MO) and fluo-3 (Molecular Probes, Eugene, OR).

Determination of K_ds. Acrylic cuvettes were used as reaction vessels and their contents mixed with 2x7 mm stirbars. Solutions composed of 0.1M KCL, 30 mM KOH with either 40 mM HEPES or 40 mM MES were then backtitrated with HCL to adjust pH to 7.40 or 7.00 (HEPES) or pH 6.50, 6.00 or 5.50 (MES). Total adventitious calcium buffer levels were <0.5 μM as determined by atomic absorption and fluo-3 measurements.

Calcium standards were prepared from calcium carbonate and HCL : dilutions were compared to NBS traceable standards (Orion, Cambridge, MA) with atomic absorption spectroscopy and arsenazo III absorbance measurements. To start the assay, additions of calcium (5-90 μM final concentration) were made to 3ml buffer, followed by the addition of 1-2 μCi of ^{45}Ca, which increased total calcium by <1 μM. The solutions were stirred except during fluorescence determinations. An aliquot was removed for scintillation counting, then 0.1 or 0.2 gm K^+-Chelex-100 was added and stirring was maintained for 30 minutes at the selected temperature. Fluorescence measurements were made with a spectrofluorometer (Aminco-Bowman, Silver Springs, MD) with a stirred, water-jacketed cuvette chamber. Excitation slit width was 5.5nm and emission slit width was 11 nm. Excitation and emission wavelengths in nm were excitation 340 and 380 and emission 500 (fura-2); excitation 350 and emission 410 and 490, or excitation 320, emission 400 (indo-1); excitation 506 and emission 530 (fluo-3). Initial measurements of buffer autofluorescence were made and then fluorescent indicators were added at 125 or 250 nM final concentration.

The indicators were obtained from Molecular Probes as the acetoxymethyl esters, dissolved in dry DMSO and stored at -20°C until used. Aliquots of dye were thawed and saponified with 0.1 N KOH which was diluted 1/1000 for use. After the indicator was added, fluorescence measurements were taken and then a second aliquot removed for scintillation counting. R_{min}, the ratio of the fluorescence of the calcium-free indicator as measured at wavelength 1 and 2 and R_{max}, the ratio of the fluorescence of the calcium-bound indicator as measured at wavelength 1 and 2, or F_{min}, the fluorescence of the calcium-free indicator and F_{max}, the fluorescence of the calcium-bound indicator (2) were measured in the same cuvette by the addition of EGTA or EDTA and then calcium. R_{min} and R_{max} were also determined by independent measurements in the absence of added calcium. In these cases, R_{min} measurements at pH 6.5, 6.0 and 5.5 were made with KEGTA and 0.1 g K-Chelex-100 since the ability of EGTA alone to complex calcium is compromised at low pH. Mn (indo-1 and fura-2) or Co (fluo-3) was added to determine the presence of fluorescent indicator forms that would not bind calcium; this was <3% of the total signal. This fluorescence was subtracted from measurements used to determine the K_d.

To determine the K_d, the specific activity of calcium was determined from the measurement of radioactivity of first aliquot after the subtraction of the radioactivity of the buffer: the total calcium present was the sum of added $^{40}Ca/^{45}Ca$ admixture, the residual calcium in the Chelex-100 and calcium present in the buffer and cuvette. The radioactivity of second aliquot was used to determine the calcium concentration in the solution. Rearranging the equation from (2), where S_{f2}/S_{b2} is the ratio of fluorescence of calcium free indicator and calcium-bound indicator, both measured at wavelength 2:

the ratio of bound/free indicator = b/f =

$$[(R-R_{min})/(R_{max}-R)] \times (S_{f2}/S_{b2}) \text{ for fura-2 or indo-1 or}$$

$$(F-F_{min})/F_{max}-F \text{ for fluo-3.}$$

Therefore the fraction of total indicator bound = B = (b/f)/(b/f)+1.
To correct for the calcium bound by the indicator and determine the K_d, the following equation was solved:

$$B = 1/(1+(K_d/(C-(B \times I)))),$$

where B = fraction of indicator bound, C = total calcium in the solution, I = indicator concentration.

To compare the results obtained with Chelex-100 to that of calcium-EDTA buffers, calcium-EDTA buffers were prepared using equilibrium constants obtained from (7) and corrected for ionic strength and temperature as described in (4). EDTA purity was determined via the ammonium oxalate method (8). Free calcium was calculated using a computer program based on (9).

Perfused heart experiments- The hearts of male 200-250 gm Sprague Dawley rats were excised, placed in oxygenated HEPES-Tyrode's and then cannulated via the aorta and perfused at 60 mm Hg in a system modified from (10). The hearts were paced at 300 bpm and force measured via an apical strain gauge linked to a recorder. The hearts were enclosed in a Radnoti water-jacketed chamber that had a port in the side through which a bifurcated quartz randomized fiber optic cable (Dolan Jenner, Woburn, MA) was placed on the left ventricular epicardial surface. One branch of the cable was connected to the excitation monochromator of the Aminco-Bowman fluorometer, the light was carried to the cardiac surface and the emitted light was transferred through the second branch of the cable to the emission monochromator of the fluorometer. Cardiac fluorescence was continually monitored throughout the experiment. After 15 minutes, the hearts were infused with 2 μM indo-1 A/M and fluorescent pH indicator BCECF A/M for 30 minutes, followed by a 15 minute washout period. BCECF fluorescence was measured at 500 nm excitation and 550 nm emission. Global ischemia was then initiated on experimental preparations by clamping the aortic cannula. Indo-1 fluorescence was calibrated using K_ds of 212nM (pH7.1) and 482 nM (pH 6.0), determined in the presence of 1 mM Mg, 0.1 M KCl at 37° as described above. Since Chelex-100 does bind magnesium and other di- and tri-valent cations, to determine a K_d in a magnesium-containing solution required a measurement of the K_d with Chelex-100 in the selected buffer in the absence of magnesium. EGTA and calcium were added to a sample of the buffer and the calcium activity determined using the K_d previously acquired. Magnesium was then added, a new fluorescence ratio measured and the previously known free calcium level used to determine the K_d in the presence of magnesium, correcting for the slight changes in calcium and magnesium activity due to the presence of EGTA. BCECF was calibrated through the use of a 10 μM ionomycin buffer system recirculated for 10 minutes at the end of the experiment that equilibrated calcium and pH.

Results

By adjusting the amount of Chelex-100 and calcium added, it was possible to adjust calcium levels from <100 to >10000 nM. The binding affinity of Chelex-100 decreased ~ 4-fold between pH 7.4 and 5.5, as compared to an ~ 5000 fold or ~ 400 fold decline in EGTA or EDTA affinity, respectively. The fluorescent indicators did not alter counting efficiency and absorbance and fluorescence measurements did not reveal indicator binding to Chelex-100.

Table 1. Effects of pH on the K_ds (in nM) of fluo-3, fura-2 and indo-1 at 22° and 37°C as determined by Chelex and EDTA assays

	22°C				
Indicator & assay	pH 7.40	7.00	6.50	6.00	5.50
Fluo-3, Chelex	608±14 (39)	641±21 (22)	1114±25 (15)	2046±70 (15)	5793±129 (33)
EDTA	462±15 (6)	516±17 (3)	771±1 (3)	1639±35 (3)	5415±252 (6)
Fura-2, Chelex	173±5 (38)	194±4 (10)	299±17 (9)	471±25 (11)	1317±70 (22)
EDTA	144±5 (6)	142±3 (3)	202±8 (3)	362±30 (3)	1022±16 (6)
Indo-1, Chelex	236±7 (24)	280±13 (18)	411±19 (12)	619±40 (12)	2030±92 (21)
EDTA	191±5 (6)	190±1 (3)	297±6 (3)	532±7 (3)	1628±101 (6)
	37°C				
Indicator & assay	pH 7.22	6.80	6.34	5.84	5.36
Fluo-3, Chelex	552±23 (9)	677±31 (9)	1175±16 (9)	2353±93 (9)	7246±116 (15)
EDTA	407±24 (3)	500±3 (3)	903±6 (3)	2293±55 (3)	6042±51 (3)
Fura-2, Chelex	138±5 (6)	167±11 (6)	244±5 (6)	527±34 (6)	1243±49 (12)
EDTA	130±7 (3)	134±3 (3)	221±2 (3)	431±4 (3)	1188±29 (3)
Indo-1, Chelex	178±4 (6)	247±19 (6)	328±7 (6)	528±29 (6)	1773±42 (15)
EDTA	179±2 (3)	197±5 (3)	349±7 (3)	639±10 (3)	1680±46 (3)

K_d values are expressed in nM as the mean±s.e.m. with n=number of individual measurements (Chelex) or number of individual titrations (EDTA). Each EDTA titration was composed of 4-10 K_d determinations that were used to determine a mean value.

As illustrated in Table 1, the effect of pH on increasing the K_ds for fura-2, indo-1 and fluo-3 is apparent once the pH drops below 7.0. This occurs at both 22° and 37° and in measurements made with Chelex or with EDTA buffers. The magnitude of these changes increases rapidly as the solution is made more acidic. There is also a reduction of absolute fluorescence in the calcium bound forms that occurs at pH 6.0 and below for all indicators, as well as increases in the values of R_{min} and F_{min}. The binding affinity of all indicators increased slightly as the temperature was increased from 22 to

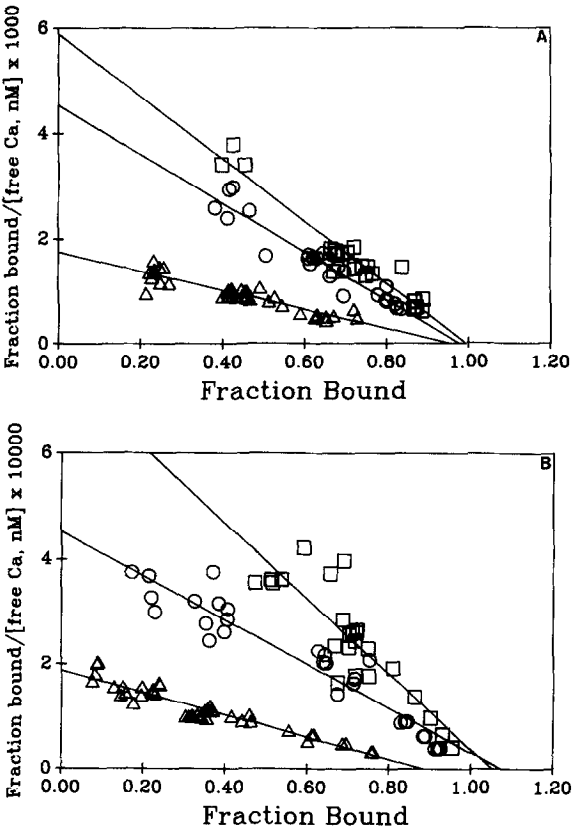

Figure 1. Effect of pH on the binding of indicator to calcium expressed as a Scatchard plot. Using the Chelex-100 assay, fluo-3 (△), fura-2 (○) and indo-1 (□) were measured at 22° at either pH 7.4 (A) or pH 5.50 (B) over a wide range of free calcium. Individual determinations were plotted and fitted via least squares regression.

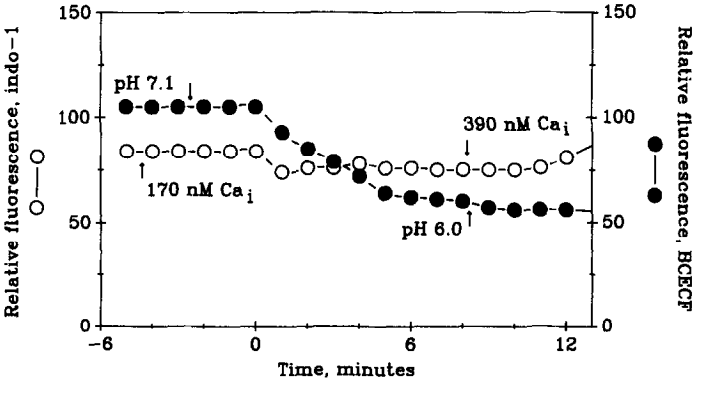

Figure 2. Effect of pH on the interpretation of fluorescence measurements in the isolated rat heart. The figure depicts the results of a representative experiment in which indo-1 and BCECF were simultaneously introduced into the cells of a perfused rat heart and indicator fluorescence was monitored at the epicardial surface. Control intracellular calcium activity Ca_i was 170 nM, calculated with an indo-1 K_d of 212 nM at an intracellular pH of 7.1. At time 0, the heart was made globally ischemic by clamping the aortic cannula. Intracellular pH declined to 6.0, increasing the K_d of indo-1 to 482 nM. The calculated intracellular calcium activity was then determined to be 390 nM.

37°C, resulting in a decrease in the K_d. A similar temperature dependency was noted with BAPTA and dibromo-BAPTA (11).

In Figure 1, Scatchard plots were used to determine whether the binding stoichiometry of one calcium ion to one ligand was altered at acidic pH. There was no apparent deviation from a single line regression at pH 5.5 or 7.4 for fura-2, fluo-3 or indo-1 at 22° or any other combination of pH or temperature tested (data not shown). As depicted in Figure 2, intracellular acidification such as that occurring in the ischemic heart can significantly alter the K_d of the indicator, resulting in little change in indicator fluorescence in the presence of a large increase in intracellular calcium activity.

Discussion

The method presented here to measure the K_ds for fluorescent calcium indicators provides a reasonable alternative to the use of EDTA or EDTA buffer systems in a number of cases. It is not necessary to determine the binding capacity of the resin, in comparison to the requisite determination of EDTA or EGTA purity (8) or use of a calcium electrode (12). This method also does not depend on the assumption of accurate knowledge of the stability constants of the chelator or methods to extrapolate differences between standard and experimental conditions of ionic strength or pH (4). Chelex-100 can still bind calcium at pH 5.0 and below, so extending the range of the Chelex method beyond that of EDTA or EGTA. The only assumptions made are readily verifiable: that the indicator concentration must be in a range that yields linear increases in fluorescence, that the total amount of calcium added is known and that the chelator is in equilibrium with the calcium. The use of relatively large amounts of added calcium insures that the effects of adventitious calcium are minimized and the use of radioisotope permits accurate determinations that are in accord with previous K_d measurements (2,13). This method may also be used to determine the K_d of any optical probe that binds a divalent cation and by comparing K_d results may be used to examine extrapolations made for EDTA and EGTA buffer calculations. This method could also be used to calibrate calcium electrodes.

It is not unexpected that these indicators are sensitive to changes in pH, given the sensitivity of the prototype, BAPTA (11). Those experiments, and those of (3,14), and the present results are in contrast to (15). In that case an attempt to determine the pH sensitivity of indo-1 was made using a single intermediate ratio measurement between R_{max} and R_{min}; that ratio was so close to the R_{min} that the differences in K_d were apparently not discernable. This assumption that the K_d for indo-1 did not change then affected their interpretation of alterations in the calcium transients that occurred during ischemic episodes in the perfused rabbit heart. Without the appropriate K_d corrections, interpretation of both qualitative and quantitative data from experiments with cells or organelles with a normally or transiently acidic intracellular pH is likely to be suspect.

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