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CALCIUM BINDING TO FLUORESCENT CALCIUM INDICATORS: CALCIUM GREEN, CALCIUM ORANGE AND CALCIUM CRIMSON

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SUMMARY : The recently introduced fluorescent calcium sensitive indicators calcium green, calcium orange and calcium crimson suggest important improvements and advantages to detect small calcium transients at low indicator concentrations. Thermodynamic dissociation constants and dissociation rate constants of calcium green, calcium orange and calcium crimson were measured by use of fluorescence titration and stopped flow fluorescence, respectively. Calcium binding to the indicators conforms to a 1:1 calcium:indicator complex although at high concentrations of calcium the fluorescence properties deviate somewhat from the behaviour predicted by the simple model. Dissociation of the calcium-indicator complex was found to be monoexponential under all conditions examined. The affinity for calcium of the three indicators generally increases with raising temperatures (K_d at 11.5° C and 39.7° C (nM): 261, 180 for calcium green; 527, 323 for calcium orange; 281, 204 for calcium crimson) and pH (K_d at pH 6.42 and 7.40 (nM): 314, 226 for calcium green; 562, 457 for calcium orange; 571, 269 for calcium crimson). The changes of the thermodynamic dissociation constant are mainly caused by changes of the association rate constant. The temperature dependence of calcium binding to the indicators revealed that this process is entropically favoured at ambient temperature.

Fluorescent ion indicators are invaluable tools to measure intracellular ion concentrations. In particular, Ca^{2+} sensitive indicators allow the investigation of the spatial distribution of cytosolic free Ca^{2+} and the time course of calcium transients. Fluorescent Ca^{2+} sensitive indicators administered as acetoxymethyl esters and subsequently trapped by cells are widely used to monitor intracellular free Ca^{2+}. Temporal changes of intracellular free Ca^{2+} occur either as periodic processes (spiking) or can be observed as continuous rises and decreases of calcium concentration. The kinetics of Ca^{2+} binding to indicators determine the fastest process that can be observed in this way. The quantification of Ca^{2+} requires the knowledge of the thermodynamic dissociation constant of the indicator under the particular conditions of

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Abbreviations: EDTA, ethylenebis(nitrilo)N,N,N',N'-tetraacetic acid; EGTA, ethylene-bis(oxyethylenenitrilo)N,N,N',N'-tetraacetic acid; HEPES, 4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid; HPLC, high performance liquid chromatography; K_d, thermodynamic dissociation constant; k_{off}, dissociation rate constant; k_{on}, association rate constant; MES, 2-(N-morpholino) ethanesulfonic acid.

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the experiment, and, since intracellular pH may vary considerably (1), K_d values at different pH values have to be taken into consideration. Since some of the pK_a values of the Ca^{2+} binding groups are in the range of 6 to 8, Ca^{2+} binding to most Ca^{2+} indicators depends on pH.

In order to avoid interference with cellular fluorophores and to avoid the requirement of expensive UV-light equipment for fluorescence microscopy, indicators like fluo-3 (2) have been developed that combine the advantages of a high quantum yield, high specificity for Ca^{2+} and excitation with visible light which allows a high spatial and temporal resolution of calcium modulations in living cells. The more recently introduced fluorescent indicators calcium green, calcium orange and calcium crimson exhibit similar fluorescence properties as fluo-3, i.e. closely spaced excitation and emission wavelengths, no significant shift of the excitation and emission spectra but an increase of fluorescence intensity upon Ca^{2+} binding. Calcium green exhibits a five-fold enhanced fluorescence intensity when compared with fluo-3 at the same excitation and emission maxima. Thus, calcium green provides an excellent fluorescence signal even when used at low concentrations. According to the manufacturer, calcium orange and calcium crimson are more photostable than calcium green and fluo-3. Equilibrium Ca^{2+} binding and kinetics of Ca^{2+} binding to calcium green, calcium orange and calcium crimson were measured under a set of different conditions in order to judge whether these indicators are suitable for measurement of Ca^{2+} concentrations in vivo and in vitro.

Materials and Methods

All buffers were made up with deionized water purified to a resistance of 16 MOhm/cm or higher by use of a Barnstead NANOpure cartridge system from SKAN AG, Switzerland. Buffers were prepared by adding calculated amounts of NaOH to a solution of 50 mM HEPES or MES, assuming a pK_a of 7.55 for HEPES and 6.15 for MES at ambient temperature (3). The ionic strength was adjusted to 0.175 M by addition of NaCl. The pH values were determined after the experiments and confirmed to be constant over at least 7 days. For the experiments at different temperatures the pH was adjusted at the desired temperature in order to avoid artifacts caused by the temperature dependence of the pK_a values of HEPES and MES. A chelating chromatography gel was prepared according to (4) using 25 mM potassium phosphate at pH 6 instead of dimethyl formamide (4). All buffers were passed through a column of chelating gel before use.

Calcium green, calcium orange and calcium crimson were purchased as potassium salt from Molecular Probes Inc., Eugene, Oregon. Calcium green was dissolved in water-free dimethyl sulfoxide, calcium orange and calcium crimson in pure water, at a final concentration of 1 mM, and stored at -75° C. The quality of the indicators was controlled by reversed phase HPLC (Merck-Hitachi L-6200 HPLC pump module equipped with a Merck-Hitachi L-4200 detector) using a LiChrospher (Merck) 100 RP-18 column (5 μm particle size, 1.5 ml column volume). A solution of 10% 10 mM Na-citrate buffer at pH 3.0 and 90% acetonitril was used as solvent. 20 μl of a solution containing 20 μM indicator was applied and eluted isocratically using a flow rate of 1 ml/min. The elution volumes were 1.87 ml, 3.14 ml, and 1.98 ml for calcium green, calcium orange and calcium crimson, respectively.

Equilibrium measurements were performed by addition of $CaCl_2$ in 200 nM steps (12 steps in total) to a solution containing 500 nM indicator. Fluorescence of calcium green, calcium orange and calcium crimson was excited at 507 nm, 551 nm and 589 nm, and observed at 530, 574 and 607 nm, respectively. Fluorescence measurements were performed with a Perkin-Elmer 650-10S spectrofluorometer interfaced via an analog-to-digital converter (PC-28 from Instrumatic AG, Switzerland) to a microcomputer (Twix 88-XT personal computer). 2 nm slits were used on both the excitation and emission sides.

Fluorescence data were corrected for dilution which was in no case greater than 6.5%. Titrations were evaluated by non-linear least-squares fit (5) to the following equation: $F = F_{min}(1-x) + F_{max}x$ with $x = (b - SQRT(b^2 - 4c))/2$, $b = 1 + ([Ca^{2+}]_t + K_d)/[I]_t$ and $c = [Ca^{2+}]_t/[I]_t$. $[I]_t$ is the total concentration of the indicator, $[Ca^{2+}]_t$ the total calcium concentration, K_d the dissociation constant and $x = [I\text{-}Ca^{2+}]/[I]_t$, i.e. the degree of saturation of the indicator. $[Ca^{2+}]_t$ is the sum of the amount of Ca^{2+} present in the buffer (denoted $[Ca^{2+}]_{res}$) and the purposely added $CaCl_2$. $[Ca^{2+}]_{res}$ was treated as a fit parameter and was found to be between 100 and 250 nM. F denotes the measured fluorescence and F_{min} the fluorescence of unliganded indicator, which was determined after addition of excess EDTA (final concentration 1 mM). F_{max}, the fluorescence of indicator saturated with Ca^{2+}, was determined by the fit procedure.

Stopped flow experiments were preformed as described in (6). Dissociation rate constants were measured by mixing a solution containing $CaCl_2$ (0.1 mM) and indicator (1 μM) with a solution containing 10 mM EDTA using a mixing ratio of 1:1. Fluorescence of calcium green, calcium orange and calcium crimson was excited at 507 nm, 546 nm (mercury line) and 581 nm (mercury line), respectively and collected above 530 nm, 570 nm and 605 nm, respectively, using cut-off filters. Stopped flow data were least-squares fitted to single or double exponentials using the program described in (5).

Results and Discussion

The excitation maxima of calcium green, calcium orange and calcium crimson were found at 507 nm, 551 nm, and 589 nm, respectively, and emission maxima were found at 530, 574 and 607 nm, respectively. Equilibrium binding was measured by fluorescence titration, without using Ca^{2+} buffers. For these experiments the concentration of Ca^{2+} was lowered by means of chromatography with a chelating gel (cf Materials and Methods). Thus, artifacts caused by calculating dissociation constants of buffering chelators were eliminated. The K_d value of fluo-3 determined in 50 mM HEPES, 150 mM NaCl, at pH 7.40 and 25°C (457±21 nM) agrees with K_d values determined under comparable conditions by other methods (6,7). During titrations, the total Ca^{2+} concentration was between 100 nM and 2.8 μM. Within this range the titrations conformed to a simple 1:1 binding of Ca^{2+} to the indicator. However, if the concentration of Ca^{2+} was increased above 3 μM, corresponding to about 95% saturation at a K_d of 100 nM and a concentration of indicator of 500 nM, fluorescence of each of the indicators was found to be higher than predicted. At very high Ca^{2+} concentrations (1 mM or higher, concentration of indicator 500 nM) the observed fluorescence exceeds F_{max} by about 7%, for all three indicators, even when they are purified by means of reversed phase HPLC. Since the concentration of Ca^{2+} does not significantly influence the absorbance spectrum of the indicators (data not shown), effects originating from absorbance changes are unlikely. The K_d values listed in Table 1 and 2 were determined by titration at low saturation, i.e. between 0 and 90%. K_d and k_{off} values determined with purified and unpurified indicator were the same within the experimental error (data not shown). Care was taken to assure a complete equilibration after each addition of $CaCl_2$ during the titrations. Each value of Table 1 and 2 represents the average of four titrations.

The fluorescence of the unliganded indicator relative to that of the Ca^{2+}-indicator complex (termed F_{rel} in Table 1 and 2) was found to decrease with increasing pH and increasing temperature. However, F_{rel} of calcium crimson did not change uniformly at different pH values but decreased substantially at elevated temperature (Table 1 and 2).

TABLE 1 . pH dependence of Ca^{2+} binding to calcium green, calcium orange and calcium crimson[a]

pH	Buffer[b]	Calcium Green		Calcium Orange		Calcium Crimson	
		K_d nM	F_{rel}[c] %	K_d nM	F_{rel} %	K_d nM	F_{rel} %
5.78	M	660±56	17.1	1124±116	38.1	2010±198	27.4
6.00	M	481±30	15.0	876±58	36.7	1182±42	28.7
6.21	M	359±26	12.7	688±33	35.6	876±94	28.7
6.42	M	314±11	11.6	562±21	35.2	571±60	29.4
6.61	M	285±4	10.7	487±8	34.9	456±44	30.3
6.80	M	258±12	10.1	464±27	34.4	457±29	30.3
6.81	H	258±25	10.2	513±41	34.6	469±14	29.6
7.00	H	268±18	9.4	461±36	33.7	398±23	29.8
7.20	H	269±10	9.1	434±21	33.8	300±12	31.4
7.40	H	226±9	9.1	457±13	33.2	269±18	30.2
7.61	H	218±13	9.2	420±36	33.4	247±40	28.5
7.82	H	208±2	9.1	424±7	33.3	147±3	28.3
8.03	H	235±17	9.0	419±2	33.3	101±8	26.1

[a]Each value represents the average of four titrations.

[b]M = MES buffer, H = HEPES buffer. The indicated pH and an ionic strength of 0.175 M was adjusted by addition of NaOH and NaCl, respectively.

[c]Fluorescence intensity of the unliganded indicator relative to that of the Ca^{2+}-indicator complex. The relative standard deviation of this value was in no case greater than 3%.

Kinetics of Ca^{2+} dissociation from calcium green, calcium orange and calcium crimson were found to be monoexponential whereas fluo-3 exhibits biexponential kinetics of dissociation (6,8). The k_{off} values listed in Table 3 and 4 were obtained from 8 to 10 transients which were averaged before evaluation. The relative standard deviation of the rate constants determined from individual replicates were about 3 to 5%.

TABLE 2 . Temperature dependence of Ca^{2+} binding to calcium green, calcium orange and calcium crimson[a]

T °C	Calcium Green		Calcium Orange		Calcium Crimson	
	K_d nM	F_{rel}[b] %	K_d nM	F_{rel} %	K_d nM	F_{rel} %
11.5	261±20	10.0	527±40	35.9	261±14	36.2
16.0	285±19	9.5	544±37	34.4	278±33	33.5
20.8	242±11	9.4	495±16	33.6	263±19	31.3
25.7	235±19	9.1	403±28	33.5	253±7	29.4
30.3	208±10	9.0	384±11	33.6	257±13	26.9
35.0	221±13	8.6	380±25	32.4	221±24	25.7
39.7	180±12	8.6	323±5	32.4	204±10	24.5

[a]Each value represents the average of four titrations. All titrations were performed in 50 mM HEPES buffer at pH 7.40 and an ionic strength of 0.175 M.

[b]Fluorescence intensity of the unliganded indicator relative to that of the Ca^{2+}-indicator complex. The relative standard deviation of this value was in no case greater than 3%.

TABLE 3 . pH dependence of the dissociation and association rate constants of Ca^{2+} binding to fluorescent indicators

pH	Buffer[a]	Calcium Green		Calcium Orange		Calcium Crimson	
		k_{off} s^{-1}	k_{on} $10^9 M^{-1}s^{-1}$	k_{off} s^{-1}	k_{on} $10^9 M^{-1}s^{-1}$	k_{off} s^{-1}	k_{on} $10^9 M^{-1}s^{-1}$
5.78	M	239	0.36	314	0.28	444	0.22
6.00	M	209	0.43	288	0.33	442	0.37
6.21	M	202	0.56	267	0.39	387	0.44
6.42	M	198	0.63	270	0.48	358	0.63
6.61	M	186	0.72	266	0.55	332	0.73
6.80	M	182	0.71	256	0.55	319	0.70
6.81	H	179	0.69	258	0.50	307	0.65
7.00	H	181	0.68	255	0.55	284	0.71
7.20	H	176	0.65	236	0.54	260	0.87
7.40	H	178	0.79	233	0.51	232	0.86
7.61	M	177	0.82	237	0.56	198	0.80
7.82	H	178	0.85	242	0.57	171	1.16
8.03	H	178	0.76	248	0.59	148	1.47

[a]cf Table 1.

Dissociation rate constants were found to be independent of the concentration of chelator (final concentrations between 0.5 and 50 mM) and on the type of chelator used (EDTA or EGTA, not shown). EDTA was used in the present study because EGTA has a low affinity for Ca^{2+} at pH below 6. Association rate constants were calculated by dividing dissociation rate constants by the respective thermodynamic dissociation constants.

Table 1 shows that the affinity for Ca^{2+} of the indicators decreases at acidic pH. Stopped flow measurements reveal that the decrease in affinity is mainly due to a decrease of k_{on} since k_{off} values increase only slightly at acidic pH (Table 3). Similar observations have been made earlier with fluo-3, indo-1 and fura-2 (8). The K_d values of the indicators were found to decrease with increasing temperature (Table 2). The influence of temperature on k_{on} is more pronounced than that on k_{off} (Table 4). A similar behaviour has been reported for fluo-3, indo-1, fura-2 (8) and azo-1 (9). Ca^{2+} binding to the indicators is an endothermic process, i.e. ΔH is positive in all cases (Table 5). Since the contribution of entropy to the reaction (about -45 kJ/mol at

TABLE 4 . Temperature dependence of the dissociation and association rate constants of Ca^{2+} binding to fluorescent indicators

T °C	Calcium Green		Calcium Orange		Calcium Crimson	
	k_{off} s^{-1}	k_{on} $10^9 M^{-1}s^{-1}$	k_{off} s^{-1}	k_{on} $10^9 M^{-1}s^{-1}$	k_{off} s^{-1}	k_{on} $10^9 M^{-1}s^{-1}$
11.8	105	0.51	147	0.28	145	0.56
16.0	124	0.44	164	0.30	159	0.57
20.5	139	0.57	198	0.40	187	0.71
25.2	173	0.74	228	0.57	204	0.81
29.9	192	0.92	275	0.72	236	0.92
34.8	244	1.10	298	0.78	256	1.16
39.7	282	1.57	356	1.10	305	1.50

TABLE 5 . Activation energies and thermodynamic parameters of Ca^{2+} binding to fluorescent indicators[a]

Indicator	Mode[b]	E_a kJ mol^{-1}	ΔS^+ J $mol^{-1}K^{-1}$	ΔH kJ mol^{-1}	ΔS J $mol^{-1}K^{-1}$
Calcium green	a	32.2±3.9	24.8±1.0	5.9±4.0	147.1±1.1
	d	26.3±0.9	-122.3±0.4		
Calcium orange	a	37.4±2.4	39.2±0.7	13.7±2.5	167.8±0.8
	d	23.7±0.7	-128.6±0.4		
Calcium crimson	a	26.2±2.1	5.5±0.6	6.9±2.2	149.7±0.7
	d	19.3±0.7	-144.2±0.3		

[a]Activation energies (E_a) were calculated by linear regression of a plot of ln(k) versus 1/T, where k is the indicated rate constant (k_{on} or k_{off}) and T the absolute temperature, using the expression $\delta \ln(k)/\delta(1/T) = -E_a/R$, where R is the gas constant. The entropy of activation (ΔS^+), the enthalpy (ΔH) and entropy (ΔS) of binding were calculated according to (9).

[b]a=association, d=dissociation.

ambient temperature) is larger than that of enthalpy (about +10 kJ /mol) the process of Ca^{2+} binding is driven entropically. Ca^{2+} binding to fluo-3, fura-2, indo-1 and azo-1 is also endothermic and entropically favoured (8,9).

The measurements presented above demonstrate that calcium green, calcium orange and calcium crimson are well suited to monitor fast changes in Ca^{2+} concentration. Since the fluorescence of calcium green is about five-fold higher than that of fluo-3 this indicator is particularly suited for monitoring Ca^{2+} concentrations. However, all three indicators exhibit elevated fluorescence when saturated with Ca^{2+}. It is conceivable that the commercial preparations of the indicators contain degradation products or impurities which have a lower affinity for Ca^{2+}. HPLC purified indicators still exhibit enhanced fluorescence at high saturation, although to a smaller extent when compared to untreated indicator. Thus, impurities or decay products may be responsible in part for the elevated fluorescence at saturating Ca^{2+} concentrations. Since no difference in K_d and k_{off} values was found when analyzing purified and unpurified indicator these contaminations are neglectable under the conditions used for the titrations and stopped flow experiments. However, if the hydrolysis products of acetoxymethyl esters of the indicators also exhibit elevated fluorescence at high saturation, then corrections for high Ca^{2+} concentrations may be required for measurements of cytosolic Ca^{2+}.

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