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On the dissociation constants of BAPTA-type calcium buffers

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Abstract — We have determined or redetermined the calcium dissociation constants of seven BAPTA-type buffers with K_D's in the range from 0.4 μM to about 20 mM in 300 mM KCl. These include four newly synthesized ones: 5-nitro BAPTA; 5,5′-dinitro BAPTA; 5-methyl-5′-nitro BAPTA; and 5-methyl-5′-formyl BAPTA. Moreover, we tabulate dissociation constants or K_D's for BAPTA and eleven BAPTA-type buffers, compare most of them with an empirical curve based upon so-called Hammett values, and predict K_D's for several still unsynthesized but potentially valuable buffers.

The injection of calcium buffers into cells is an important method for controlling cytosolic calcium. Since Durham last surveyed the 'readily available chelators' for such purposes in this journal [1], Tsien has introduced the BAPTA-type chelators [2]. These have several important advantages. They are practically insensitive to intracellular pH changes, release Ca^{2+} far more quickly than do EDTA and EGTA [3], are far less metabolizable than citrate, and unlike NTA or citrate are not limited by the insolubility of their calcium complexes. Moreover, it should be quite practical to modify the BAPTA molecule so as to obtain a whole family of otherwise similar molecules with K_D's or calcium dissociation constants covering the biologically significant range from 10^{-7} to 10^{-2} molar.

More recently, injected BAPTA buffers have proven valuable for damping calcium transients [4–6]. Hence we tried to suppress calcium gradients (in fucoid eggs) by injecting both BAPTA and the then-available BAPTA-type buffers [7]. However, we found that injections of the BAPTA-type buffers with the highest available K_D's – 5,5′-dibromo and 4,4′-difluoro BAPTA with K_D's of 4 to 5 μM – were most effective biologically. So BAPTA-type buffers with yet higher K_D's were called for. Moreover, our quantitative analysis of the data called for more accurate K_D values particularly in the high ionic strength milieu within marine cells. These needs led us to the syntheses and measurements reported here and finally to the prescription for yet further syntheses which is likewise reported here. These results, in turn, led us to a far more satisfactory study of (putative) calcium gradient suppression in fucoid eggs [8]. As discussed below, these new buffers should prove widely useful in cell biology.

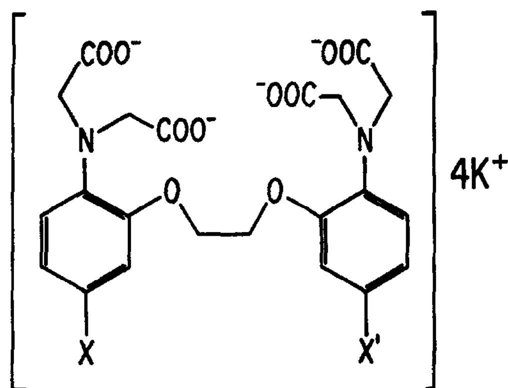

Fig. 1 Low affinity BAPTA buffers

Materials and Methods

Determining dissociation constants

For buffers with K_D's less than 100 μM, we used a method similar to that of Harrison and Bers [9] but used WPI calcium-selective electrodes calibrated via an Orion standard calcium chloride solution for pCa 1–4 and a solution of 1 mM dibromo BAPTA plus 0.5 mM $CaCl_2$ for pCa 5.8. K_D's of the very weak but strongly colored buffers mononitro and dinitro BAPTA were determined with the aid of a recording spectrophotometer: our Scatchard graphs plotted ΔAbs/[Ca] vs ΔAbs at 430 nm.

These measurements were done in 300 mM KCl in an effort to approximate the ionic strength inside the marine cells where most BAPTA-type buffer injections have been done. For the stronger buffers (where intracellular free Mg^{2+} can compete with intracellular free Ca^{2+} for buffer binding), the measurements were done with and without 1 mM Mg^{2+}. The 1 mM figure was used in an effort to approximate the free Mg^{2+} level inside marine cells. However, it should be noted that while 1 mM is a reasonable approximation for excitable marine cells [10], free Mg^{2+} may well be at least three-fold higher in marine eggs [11].

Synthesis and characterization of new buffers

As was shown by Tsien in his original paper describing BAPTA [2], substituents on the aromatic rings influenced BAPTA's affinity for Ca^{2+}. It was found that electron withdrawing groups lower the affinity for Ca^{2+}, while substituents that donate electrons to the ring result in an increase in affinity. For example, replacement of the hydrogens *para* to the chelating nitrogens with methyl groups tripled the affinity for Ca^{2+}; while replacement of these hydrogens by bromine atoms lowered the affinity by about ten-fold.

The strongly electron withdrawing character of the nitro group makes it a good candidate for a substituent that would shift the dissociation constant into the high micromolar range. Nitration of the BAPTA structure was complicated by the susceptibility of the four ester groups to acid hydrolysis. A wide range of nitration conditions were examined before a sufficiently mild procedure was found. In the following procedures, nitration was always accompanied by partial hydrolysis which resulted in low overall yields. This hydrolysis seemed especially apparent when nitrating the 5-methyl BAPTA. In this case, the tetra *tert*-butyl ester of this compound was used for the reaction resulting in an improved yield when compared to the tetramethyl ester. The novel compounds synthesized are represented in Figure 1.

BAPTA tetramethyl ester was synthesized by the method of Tsien [2]. The 5-methyl BAPTA *t*-butyl ester was synthesized as an intermediate for the fluorescent Ca^{2+} probe, Indo-1 [12] with *t*-butyl bromoacetate replacing methyl bromoacetate in the alkylation reaction. The 5-methyl-5′-formyl BAPTA (ligand 1d) was the hydrolysis product of the formyl methyl BAPTA tetramethyl ester that was also described as an intermediate for Indo-1.

Solvents were used as commercially available. HPLC chromatograms were run on a Supelcosil LC-8-DB 150 mm x 4.6 mm ID column using methanol:water, 40:60, + 1% acetic acid. NMR spectra were run on a 360 MHz Nicolet NMR.

^{1}H NMR peaks are represented as follows on the ppm scale: spin multiplicity – s, singlet; d, doublet; b, broad. Relative numbers of protons are represented for each peak.

Ligand 1a (5-nitro BAPTA) synthesis

Ligand 1a (5-nitro BAPTA) was synthesized as follows: 1.0 g (1.87 mM) BAPTA tetramethyl ester was dissolved in 2.5 ml glacial acetic acid. 125 µl of 15.8 M HNO_3 was added by 5 µl aliquots to reach 1.98 mM HNO_3. After 10 min, TLC in ethyl acetate:hexanes (1:1) showed that some of the starting material remained. 25 µl (0.4 mM) additional HNO_3 was added. TLC after 5 min showed that almost all the starting ester had been converted to a mixture of mono- and di-nitrated products. The reaction was then quenched with 20 ml saturated $NaHCO_3$ and extracted with 70 ml ethyl acetate. The organic layer was washed with saturated $NaHCO_3$ and saturated NaCl, dried over Na_2SO_4, and evaporated to a yellow oil. This was chromatographically purified on a silica gel column of particle size 35–70 µm and 2.5 cm length and was packed in equal volumes of ethyl acetate, hexanes, and chloroform. This column gave 0.42 g that still contained a trace of the dinitro derivative. Another column was run in ethyl acetate, hexanes, and chloroform (1:2:1). This solvent resulted in a complete separation of the mono- and di-nitro compounds. The pure fractions of the mononitro were evaporated to a clear oil which solidifies on standing. The solid was washed with hexanes and filtered to give 0.32 g of light yellow solid, pure to TLC (30% yield), mp 148–150 °C NMR ($CDCl_3$, 360 MHz): 3.6–3.7 ppm (d,12H), 4.2 ppm (s,4H), 4.3 ppm (s,4H), 4.3–4.4 ppm (d,b,4H), 6.7 ppm (d,1H), 6.8–7.0 ppm (m,4H), 7.8 ppm (s,1H), 7.9 ppm (d,1H). An HPLC of the saponified ester showed that the purity was approximately 97%.

Ligand 1b (5,5′-dinitro BAPTA) synthesis

Ligand 1b (5,5′-dinitro BAPTA) was synthesized as follows: 1.0 g (1.87 mM) BAPTA tetramethyl ester was dissolved in 2.5 ml glacial acetic acid at room temperature. 250 µl (3.97 mM) HNO_3 was added slowly. After stirring for 15 min, a light yellow precipitate formed. TLC of the reaction mixture in 1:1 ethyl acetate:hexanes showed good conversion of the starting material to a single lower Rf yellow compound. The reaction was quenched with 25 ml saturated $NaHCO_3$ and extracted with 70 ml ethyl acetate. The organic layer was washed with saturated $NaHCO_3$ and saturated NaCl, then dried over Na_2SO_4, and evaporated to a brown oil. Residual acetic acid was removed under high vacuum. The resulting oil was dissolved in a minimum volume of ethyl acetate and precipitated with hexanes to give 0.81 g dinitro BAPTA, approximately 70% pure by TLC. This solid was further purified on silica gel packed in equal parts ethyl acetate, hexanes, and chloroform. The major yellow band was collected and evaporated to a clear yellow oil which solidified on standing. The solid was washed with hexanes and filtered to give 0.67 g of light yellow solid, pure to TLC (57% yield), mp 178–180 °C, NMR ($CDCl_3$, 360 MHz): 3.6–3.7 ppm (s,12H), 4.3 ppm (s,8H), 4.4 ppm (s,4H), 6.7 ppm (d,2H), 7.7 ppm (s,2H), 7.9 ppm (d,2H). An HPLC of the saponified material showed that the purity was approximately 98%.

Ligand 1c (5-methyl, 5′-nitro BAPTA) synthesis

Ligand 1c (5-methyl, 5′-nitro BAPTA) was synthesized as follows: 0.10 g (0.16 mM) 5-methyl BAPTA tetra *t*-butyl ester was dissolved in 1.0 ml glacial acetic acid. 50 µl (0.79 mM) HNO_3 was added by 5 µl aliquots over 5 min with rapid stirring. H_2SO_4 (50 µl) was added slowly. TLC in 1:1 ethyl acetate:hexanes showed good conversion of the starting material to a lower Rf yellow product. The reaction was immediately quenched with 10 ml saturated $NaHCO_3$. The aqueous layer was extracted with 40 ml ethyl acetate. The organic layer was washed one time with saturated $NaHCO_3$ and once with saturated NaCl, then dried over Na_2SO_4, and evaporated to an oil. Any residual acetic acid was removed under high vacuum and the resulting oil was purified on silica gel packed in an equal mixture of ethyl acetate, hexanes, and chloroform. The product eluted rapidly with good separation of the desired product from the more polar impurities. The pure fractions were evaporated to an orange oil which solidified on standing. This solid was washed with hexanes and filtered to give 45 mg of yellow solid, pure on TLC (42% yield), mp 120–122 °C, NMR ($CDCl_3$, 360

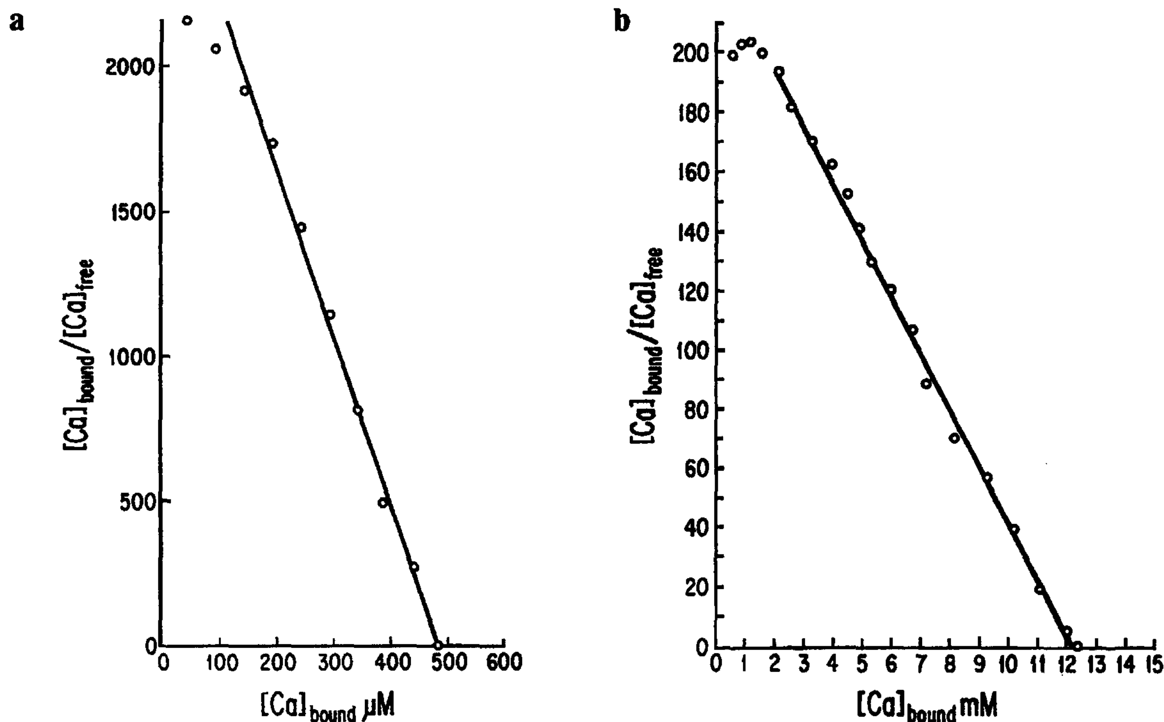

Fig. 2 Representative Scatchard plots of dissociation constants obtained via calcium selective electrodes
(a) 500 µM, 5,5′-dimethyl BAPTA in 10 ml 0.3 M KCl; pH of 7.0 via 10 mM HEPES; 24°C. This run gave a K_D value of 0.17 µM and a purity of 94%; a second run gave 0.17 µM and 104%
(b) 15 mM 5-methyl-5′-nitro BAPTA in 3 ml of 0.3 M KCl; pH of 7.0 via 10 mM HEPES; 22°C. K_D = 53 µM; purity = 81%

MHz): 1.8–1.9 ppm (d,36H), 2.4 ppm (s,3H), 3.8 ppm (s,4H), 4.4–4.6 ppm (d,b,4H), 6.7 ppm (d,1H), 6.9 ppm (s,1H), 7.2 ppm (s,1H), 7.8 ppm (s,1H), 7.9 ppm (d,1H). An HPLC of the saponified ester showed approximately 98% purity.

Hydrolysis of the tetramethyl ester of 5,5′-dinitro BAPTA

The tetramethyl ester of 5,5′-dinitro BAPTA was hydrolyzed as follows: 0.75 g (1.2 mM) was suspended in 5 ml dioxane containing 3 ml methanol. 0.4 g (6 mM) KOH in 1 ml of water was added and the solid slowly dissolved into a dark red solution while stirring. The mixture reaction was stirred overnight at room temperature, the organic solvents were removed, and the resulting red liquid was purified on Sephadex LH-20 in deionized water. The red band was collected, acidified to pH 3, and filtered to give 550 mg (80% yield) bright yellow solid after drying. NMR (DMSO, 360 MHz): 4.2 ppm (s,8H), 4.3 ppm (s,4H), 6.6 ppm (d,2H), 7.6 ppm (s,2H), 7.8 ppm (d,2H), 12.6 ppm (s,b,4H).

The free acids of these compounds were converted to their potassium salts by the addition of stoichiometric amounts of KOH in water followed by lyophilization to a red solid.

Results

Figure 2 shows two representative Scatchard plots which were obtained via electrode-monitored titrations. These results proved to be highly reproducible. Figure 3 shows representative spectra obtained to determine the K_D of dinitro BAPTA.

Our main results are shown in Table 1. These were determined, or in one case corrected for, an ionic strength of 0.3 M and a pH of 7.0. For buffers with a calcium K_D less than 2 µM, they were

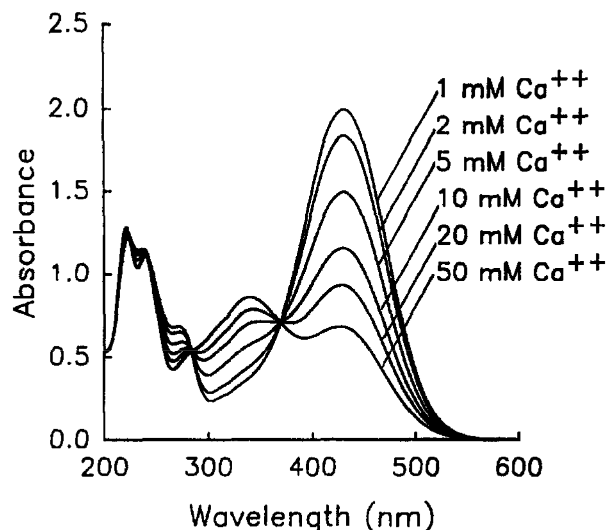

Fig. 3 Representative effect of changing [Ca] on the absorption spectrum of 100 μM 5,5′-dinitro BAPTA. Spectra were measured at an ionic strength of 0.1 (obtained by adding KCl), pH 7.0 (via 10 mM HEPES), and 20°C. A Scatchard plot of these data yielded a K_D of about 6 mM

determined or corrected for media containing 1 mM Mg^{2+} as well as for Mg^{2+}-free media; for K_D's above 2 μM, the effect of 1 mM Mg^{2+} should be negligible and was not studied.

In the case of dibromo BAPTA, our results can be directly compared with those of Harrison and Bers [9]. At an ionic strength of 0.3 M, we find a K_D of 3.57 ± 0.05 μM while they report 3.47 μM; at 0.1 M ionic strength, we find 1.47 ± 0.05 μM while they report 1.45 ± 0.06 μM. This excellent agreement provides considerable confidence in our results. Moreover, they report a purity of 97 ± 1% (n = 4) while we found 97% and 102% in two determinations. Together, this reproducibility, the apparent stability, and the relatively high purity of commercially available dibromo BAPTA leads us to propose its use as a basic standard in physiological work with calcium.

In Table 2, we combine our K_D data with those for three other commercially available buffers, Nitr-5 and its photolysis product, as well as Fura-2. Moreover, we present these K_D's for ionic

Table 1 Dissociation constants, purity, and molecular weights of nine BAPTA-type calcium buffers obtained from Molecular Probes. Measured in 300 mM KCl, 10 mM HEPES pH buffer set at pH 7.0, 22 ± 1°C, and with or without 1 mM $MgCl_2$. All measurements are ours except for two marked by an asterisk which were taken from Harrison and Bers [9]. The Mg^{2+} effect should be negligible on the weaker buffers

BAPTA substitute	*Mol. Wt.*	*Method*	*Purity*[1]	*Dissociation Constant K_D (μM)*	
				No Mg^{2+}	*1 mM Mg^{2+}*
5,5′-dimethyl[2]	654	Ca electrode	≥95%	0.16	0.44
BAPTA itself[2]	626	Ca electrode	≥90%	0.59*	0.70
5-methyl-5′-formyl	668	Ca electrode	78%	0.60	(0.71)[3]
5,5′-difluoro[2]	662	Ca electrode	72%	0.61	(0.72)[3]
5,5′-dibromo[2]	784	Ca electrode	97 ± 1%*	3.6	
4,4′-difluoro[2]	662	Ca electrode	88%	4.6	
5-methyl-5′-nitro[2]	685	Ca electrode	81%	53	
5-mononitro[2]	670	Spectral	88%	94	
5,5′-dinitro	716	Both	≈80%	20,000?[4]	

[1]The impurities of the potassium salts as determined by our Scatchard plots should consist largely or entirely of water of hydration associated with the acid groups. Such water can raise the mass of the molecule by up to approximately 20%, resulting in an apparent impurity when the anhydrous molecular weights are used in these determinations

[2]Minimal toxicity established. See Discussion

[3]Inferred from the Mg^{2+} effect on BAPTA

[4]Inferred from our measurement of 7 ± 1 mM in 100 mM KCl questionably multiplied by a factor of 3 taken from [9], Figure 6

Table 2 A compilation of dissociation constants, K_D of commercially available BAPTA-type buffers with corrections for intracellular ionic conditions, i.e., 1 mM Mg^{2+}, pH 7.0, and 100 mM or 300 mM ionic strength. Also corrected for 22°C. Figures in parentheses are relatively uncertain. Primary references were used to obtain the data tabulated

	K_D (μM) in		References	
Buffer	*100 mM KCl*	*300 mM KCl*	*Primary*	*Other*
5,5′-dimethyl BAPTA	(0.15)	0.44	*, [9]	[2]
Fura-2	0.17	0.60	[12, 9]	
BAPTA	(0.21)	0.70	*, [9]	[2]
5-methyl-5′-formyl BAPTA	(0.24)	0.71	*, [9]	
5,5′-difluoro BAPTA	(0.25)	0.72	*, [9]	[12]
Nitr-5	(0.40)	(1.6)	[13], *, [15]	
5,5′-dibromo BAPTA	1.5	3.6	*	[9]
4,4′-difluoro BAPTA	(1.7)	4.6	*	[14]
Nitr-5 photolysis product	6.3	16	[13], [15]	
5-methyl-5′-nitro BAPTA	22	53	*	
5-mononitro BAPTA	(40)	(94)	*	
5,5′-dinitro BAPTA	7,000	(20,000)	*, [3]	

*This paper

conditions that should generally apply to marine cells (0.3 M ionic strength) as well as terrestrial ones (0.1 M ionic strength). We would like to point out that the photolysis product of Nitr-5 fills a substantial gap in the list of available K_D's. More important, it could imaginably be introduced when and where desired by first injecting Nitr-5 and then later illuminating all or part of a dividing system. This would reduce local calcium gradients [8] as well as transiently raising calcium.

Discussion

Applications

Recently we completed a study involving the injection of seven different BAPTA-type calcium buffers into fucoid eggs [8]. Critical final intracellular concentrations of each buffer prove to block development as well as cell division for up to two weeks. This critical inhibitory concentration is lowest for two buffers with K_D's of 4 to 5 μM and rises steadily as the K_D's shift below or above this optimal value to ones as low as 0.4 μM or as high as 94 μM. An analysis of these results indicated that the buffers act by facilitating free calcium diffusion within the eggs and thus by suppressing the formation of steady calcium gradients. This analysis then suggested that injections of BAPTA-type buffers will provide "an effective, general test for the actions of relatively steady, cytosolic calcium gradients. Much as cytochalasin immersal serves to test for actions of an actin cytoskeleton, so 'baptism' may test for the actions of such calcium gradients". Some limited support for this proposition has already been provided by studies of nuclear envelope breakdown in dividing sea urchin eggs [16].

Limitations

We feel that Tables 1 and 2 provide reasonably reliable values for the K_D's of all of the readily available BAPTA-type buffers when measured in vitro under intracellular ionic conditions. It is true that in vivo there are serious indications that Fura-2, at least, can show substantial binding to cytoskeletal elements. Moreover, when this occurs, there is reason to suspect that this binding substantially weakens it, i.e., raises its K_D [18, 19]. On the other hand, measurements of the free calcium level in *Xenopus* oocytes using Fura-2 give essentially the

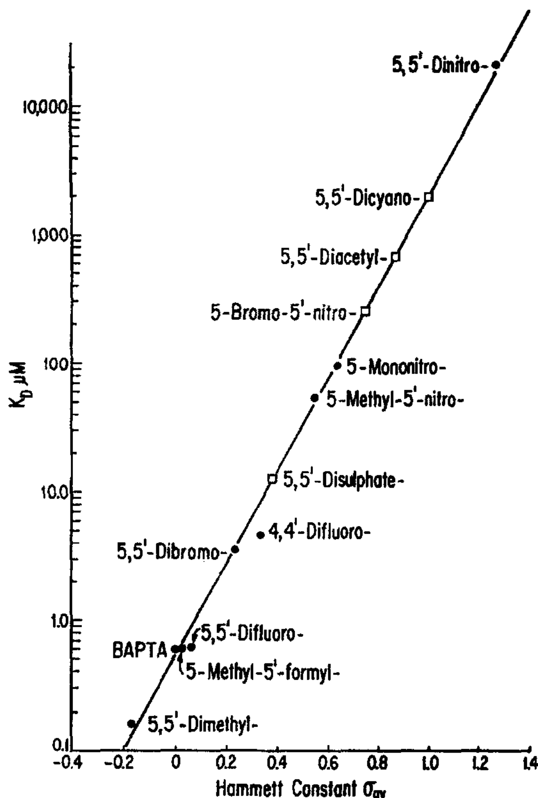

Fig. 4 Dissociation constants of BAPTA-type buffers vs Hammett constants of 5,5′ (*para*) or 4,4′ (*meta*) substituents. Filled circles, measured K_D; open squares, predicted K_D. All at 0.3 M ionic strength, 22°C, pH 7.0, no added Mg^{2+}. Hammett constant, σ^{*}_{para} in Table 7 of [14], is used where two values are listed for the same substituent. Where a buffer bears two different substituents, their average is used

same value as those obtained with either calcium microelectrodes or aequorin [19]. This indicates that in this egg, at least, in vitro and in vivo K_D's of Fura-2 are the same. On the other hand, free Mg^{2+} may well be as high as 3–4 mM in marine eggs [11], so the K_D's in such cells for the stronger buffers – with in vitro K_D's less than 1 µM – may prove substantially higher than those shown in the last column of Table 1.

Another consideration is toxicity. Because such buffers must be used at cytosolic concentrations of 1 to 10 mM, it is particularly important to have evidence that they are not toxic to cells, either through some unsuspected reaction unrelated to calcium chelation or through some contaminant. In the cases of all of the buffers marked with an asterisk in Table 1, injection into developing fucoid eggs has provided such evidence [8]; however, this same test, with our presently available sample of 5,5′-dinitro BAPTA, indicates that it is toxic at cytosolic concentrations above about 5 mM.

Future possibilities – the synthesis of useful new BAPTA-type buffers

Figure 4 shows a semilog plot of K_D's vs the available Hammett values [20] for the *para* (i.e., 5,5′), as well as one *meta* (i.e., 4,4′) substituents on various BAPTA-type buffers. It shows nine known K_D's in the range from about 10^{-7} to 10^{-2} molar, falling rather close to a straight line. This shows that this empirical relationship should provide a useful guide toward synthesizing new BAPTA-type buffers with desired K_D's. Four such buffers that should be relatively stable inside cells are also graphed.

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