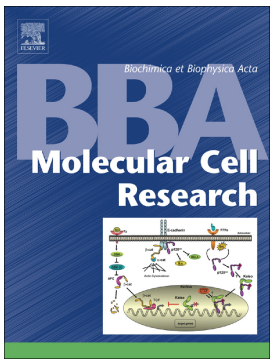
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# Cellular effects of BAPTA: are they only about $Ca^{2+}$ chelation?

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**Abstract**

Intracellular $Ca^{2+}$ signals play a vital role in a broad range of cell biological and physiological processes in all eukaryotic cell types. Dysregulation of $Ca^{2+}$ signaling has been implicated in numerous human diseases. Over the past four decades, the understanding of how cells use $Ca^{2+}$ as a messenger has flourished, largely because of the development of reporters that enable visualization of $Ca^{2+}$ signals in different cellular compartments, and tools that can modulate cellular $Ca^{2+}$ signaling. One such tool that is frequently used is BAPTA; a fast, high-affinity $Ca^{2+}$-chelating molecule. By making use of a cell-permeable acetoxymethyl ester (AM) variant, BAPTA can be readily loaded into the cytosol of cells (referred to as BAPTAi), where it is trapped and able to buffer changes in cytosolic $Ca^{2+}$. Due to the ease of loading of the AM version of BAPTA, this reagent has been used in hundreds of studies to probe the role of $Ca^{2+}$ signaling in specific processes. As such, for decades, researchers have almost universally attributed changes in biological processes caused by BAPTAi to the involvement of $Ca^{2+}$ signaling. However, BAPTA has often been used without any form of control, and in many cases has neither been shown to be retained in cells for the duration of experiments nor to buffer any $Ca^{2+}$ signals. Moreover, increasing evidence points to off-target cellular effects of BAPTA that are clearly not related to $Ca^{2+}$ chelation. Here, we briefly introduce $Ca^{2+}$ signaling and the history of $Ca^{2+}$ chelators and fluorescent $Ca^{2+}$ indicators. We highlight $Ca^{2+}$-independent effects of BAPTAi on a broad range of molecular targets and describe some of BAPTAi's impacts on cell functions that occur independently of its $Ca^{2+}$-chelating properties. Finally, we propose strategies for determining whether $Ca^{2+}$ chelation, the binding of other metal ions, or off-target interactions with cell components are responsible for BAPTAi's effect on a particular process and suggest some future research directions.

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## Introduction into intracellular $Ca^{2+}$ signaling

$Ca^{2+}$ is a multifunctional intracellular messenger that controls a wide spectrum of cell biological and physiological processes, including fertilization, cell division, muscle contraction, synaptic transmission, hormone release and immune responses.[1,2] Cells contain millimolar levels of phosphate, which is essential for numerous functions, including formation of molecules with high phosphoryl-transfer potential. Therefore, low cytosolic $Ca^{2+}$ concentrations ($[Ca^{2+}]_{cyt}$) are required because calcium phosphate has a low solubility constant and crystalline precipitates would cause cell damage and membrane ruptures.[3] In cells, basal $[Ca^{2+}]_{cyt}$ is approximately 100 nM[4], whereas $[Ca^{2+}]$ in the extracellular environment (1-2 mM) and in organellar stores such as the endoplasmic reticulum (ER)[5] and lysosomes[6] (approximately 500 μM) are much higher.

The major intracellular $Ca^{2+}$ storage organelle in non-excitable cells and neurons is the ER, which accumulates $Ca^{2+}$ through the actions of sarco-endoplasmic reticulum $Ca^{2+}$ ATPases (SERCA enzymes)[7] and ER luminal $Ca^{2+}$-binding proteins.[8] The ER does not function in isolation but operates within an inter-organellar signaling network, with organelle cross-talk established through membrane contact sites, for example with mitochondria, lysosomes, and the plasma membrane. Localized and specific exchanges of $Ca^{2+}$ occur at these membrane contact sites, thereby creating $Ca^{2+}$ signaling nano- and micro-domains that are key in sustaining specific cellular functions.[9]

The opening of various $Ca^{2+}$-permeable channels residing at organelles and the plasma membrane generates $[Ca^{2+}]_{cyt}$ increases with specific profiles in terms of amplitude, kinetics, frequency and subcellular location. Increases in $[Ca^{2+}]_{cyt}$ typically oscillate from 100 nM to ~1 μM in the bulk of the cytosol. Higher $[Ca^{2+}]_{cyt}$ concentrations occur in nano- and micro-domains within cells, such as the ER-mitochondrial interface (~100 nM to 10 μM range) and at the mouth of channel pores (~100 nM to >100 μM range).[2]

The unique spatiotemporal profile of intracellular $Ca^{2+}$ signals can lead to dichotomous outcomes. This becomes apparent at the ER-mitochondrial interface, where $Ca^{2+}$ released by ER-resident inositol 1,4,5-trisphosphate ($IP_3$) receptors ($IP_3Rs$) is efficiently transmitted to the mitochondria via the outer mitochondrial membrane-resident voltage-dependent anion channels 1 (VDAC1)[10,11] and the mitochondrial $Ca^{2+}$ uniporter[12]. On the one hand, ER-derived $[Ca^{2+}]_{cyt}$ oscillations leads to mitochondrial $Ca^{2+}$ uptake that promotes cell survival by driving mitochondrial bioenergetics and oxidative metabolism.[13] Abrogating ER-to-mitochondria $Ca^{2+}$ fluxes challenges cell survival and

requires cells to engage in other mechanisms to avoid cell death, such as autophagy.[14] On the other hand, ER-derived $Ca^{2+}$ signals can cause '$Ca^{2+}$ overload' in the mitochondrial matrix, triggering processes that induce cell death.[15,16]. It is not only the case that ER-derived $Ca^{2+}$ signals impact on mitochondrial function[17], but *vice versa* mitochondrial activity influences $Ca^{2+}$ release from the ER through localized redox signaling[18,19] and via $Ca^{2+}$ sequestration[20].

Furthermore, dysregulation of $Ca^{2+}$ signaling has been implicated in a variety of diseases and appears to be an important and proximal driver in the pathogenesis of neurodegeneration,[21,22] pancreatitis[23] and cancer.[16,24] Tools to intervene with $Ca^{2+}$ signaling are thus not only fundamental in elucidating the cellular implications of intracellular $Ca^{2+}$ signals, but they may also hold therapeutic potential to tackle pathogenesis and/or lessen disease burden.[25]

## A brief history of BAPTA

The study of intracellular $Ca^{2+}$ signaling takes an important place in cell biology, physiology, and disease conditions. Because $Ca^{2+}$ signaling is typically fast, transient, and localized, there is a need for chemical tools that chelate $Ca^{2+}$ with high affinity and rapid kinetics. Such $Ca^{2+}$-chelating tools would suppress $[Ca^{2+}]_{cyt}$ rises and alter the spatiotemporal properties of physiological $Ca^{2+}$ signals.[26]

A major challenge for the development of a specific $Ca^{2+}$ chelator is achieving selectivity of $Ca^{2+}$ over $Mg^{2+}$, which is 10,000-fold more abundant in the cytosol.[27] In this regard, it is important to know that the radius of the $Mg^{2+}$ ion (0.65 A) is considerably smaller than that of $Ca^{2+}$ (0.99 A). While ethylenediaminetetraacetic acid (EDTA) does not discriminate between $Mg^{2+}$ and $Ca^{2+}$, insertion of two ethylene glycol units between the chelating amino and acetic acid groups yields the larger ethylene glycol bis(2-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid (EGTA) molecule which selectively chelates $Ca^{2+}$. The tetracarboxylate moieties of EGTA are positioned in such a way that the four carboxyl groups together with two secondary amines and two ether oxygen atoms are appropriately orientated to bind the larger $Ca^{2+}$ ion with high affinity (~300 nM) and high selectivity over binding $Mg^{2+}$ (>$10^5$). While the larger ionic radius of $Ca^{2+}$ allows the four carboxyl groups to form a perfectly sized chelate, $Mg^{2+}$, with its smaller ionic radius, would force the four carboxyl groups closer together, leading to charge repulsion.[28] Yet, EGTA still has some disadvantages as a $Ca^{2+}$ chelator in a cellular context.

First, the molecule is not cell permeable due to its negative charges at neutral pH, limiting its application within living cells, unless microinjection or micro-pipetting is available. A simple solution for the intracellular loading of EGTA, and other $Ca^{2+}$ chelators and indicators, involves chemically

masking the tetracarboxylate $Ca^{2+}$-binding groups as acetoxymethyl (AM) esters, thereby enabling the lipophilic tetra-ester to cross the plasma membrane via diffusion.[26] Subsequently, non-selective, ubiquitously present, intracellular carboxylesterases remove the AM groups, and the resulting hemiacetal decomposes to yield methylene glycol (the hydrated form of formaldehyde) and the free carboxylates of EGTA, which now are available to bind and chelate $Ca^{2+}$. Esterification is the standard method for delivery of chemical $Ca^{2+}$ indicators and other anionic small molecules to living cells.[29] Once the AM compounds cross the plasma membrane and become de-esterified, they are trapped inside the cell due to their charged carboxylate groups. As a result, these $Ca^{2+}$ chelators and indicators accumulate within cells in a time- and concentration-dependent manner, with prolonged incubations causing intracellular concentrations to rise to high levels. For instance, an AM-ester of a compound added at an extracellular concentration of 10 μM results in the accumulation of the de-esterified, free acid form to intracellular concentrations of up several mM within 30-60 minutes.[30] It should be noted, however, that intracellular compartmentalization and export of intracellularly loaded compounds are common processes impacting $BAPTA_i$ loading. There are several transport mechanisms that perform this function, including members of the ATP-binding cassette (ABC) transporter family and anion transporter systems.[31–34] There are pharmacological and experimental approaches that can reduce the export of intracellularly loaded moieties, and it should not be taken for granted that compounds will be retained indefinitely within cells.[35]

A second disadvantage of EGTA is that the aliphatic amines necessary for binding $Ca^{2+}$ are alkaline (pKa values of 9.0 and 8.6)[28], causing the amines to be protonated at physiological pH. Due to the need for proton dissociation, EGTA binds $Ca^{2+}$ relatively slowly and its binding kinetics are sensitive to pH.

The issues related to the basic nature of EGTA were tackled in seminal work of the late Roger Tsien by incorporating the aliphatic amine scaffold of EGTA into an aromatic framework, converting the alkyl amines into anilines. This resulted in 1,2-bis(*o*-aminophenoxy)ethane-*N,N,N′,N′*-tetraacetic acid, the BAPTA molecule.[26,28,36] BAPTA retains the distinctive size and shape of EGTA, which is responsible for its noteworthy selectivity for $Ca^{2+}$ over $Mg^{2+}$ (Fig. 1). However, the structural alteration imbues BAPTA with a few crucial advantages compared to EGTA. First, because the pKa of aniline nitrogens is lower than the pKa of aliphatic amines (the two highest pKa values being 6.4 and 5.5), the $Ca^{2+}$ binding to BAPTA is independent of pH within cells. Moreover, $Ca^{2+}$-binding kinetics of BAPTA are considerably increased compared to those of EGTA.[28] For this reason, $EGTA_i$ is considered unable to chelate fast, nanoscopic $Ca^{2+}$ transients while $BAPTA_i$ would be able to interfere with such $Ca^{2+}$

signals. In addition, the affinity of BAPTA for $Ca^{2+}$ can also be strengthened or weakened by electron-donating or -withdrawing substituents on the aromatic rings, respectively.

A third disadvantage of EGTA is the lack of a detectable optical change in the molecule upon binding of $Ca^{2+}$. In contrast, the aromatic rings of BAPTA provide a visual cue to monitor its $Ca^{2+}$ chelation. Upon complexation with $Ca^{2+}$, the lone pairs of electrons on BAPTA's nitrogens twist out of conjugation with the aromatic ring, shifting the absorbance band of BAPTA from a maximum of 254 nm in the absence of $Ca^{2+}$ to 203 nm when $Ca^{2+}$ is bound.[37] In theory, this absorbance peak shift could be used to assay $Ca^{2+}$ binding by BAPTA, were it not that the absorbance of BAPTA is concealed by the more abundant cellular proteins and nucleic acids that absorb in the same UV region of the spectrum.

To enable visualization of BAPTA in cellular systems, a benzofurane fluorophore was added to the molecule, resulting in the ratiometric indicator Fura-2.[36] Later, intensiometric fluorescent $Ca^{2+}$ indicators were developed, including Fluo-1, -2, -3, -4 (green) and Rhod-1 and -2 (red).[37] The latter one (Rhod-2) is positively charged, thereby accumulating in the mitochondrial matrix. New generations of $Ca^{2+}$ indicators include the Cal series of $Ca^{2+}$ indicators, which have a drastically higher quantum yield than e.g., Fluo-4 (0.75 vs 0.16). Such $Ca^{2+}$ indicators are generally loaded into the cell at low concentrations (1 μM extracellular AM concentrations for ~30 minutes), allowing monitoring of the spatial and temporal properties of intracellular $Ca^{2+}$ signals by measuring changes in fluorescence intensities or ratios. The fluorescence values from these $Ca^{2+}$ indicators can be readily calibrated into $Ca^{2+}$ concentrations by determining maximal and minimal fluorescence values from the $Ca^{2+}$ bound and $Ca^{2+}$-free indicator (typically denoted $F_{max}$ (or $R_{max}$) and $F_{min}$ (or $R_{min}$)).[38] An overview of the properties EDTA, EGTA, BAPTA and Fura-2 is provided in Fig. 2.

$Ca^{2+}$ indicators allow direct visualization of $Ca^{2+}$ signals at tissue, whole-cell, and microscopic levels. As such, these tools have provided a means to examine the involvement of $Ca^{2+}$ in cellular processes and caused a leap forward in the study of cellular $Ca^{2+}$ signaling. Whilst direct visualization of $Ca^{2+}$ signals using indicators is helpful in discerning the putative role of $Ca^{2+}$ in a process, a $Ca^{2+}$ signal by itself is not proof of a $Ca^{2+}$-mediated process. Therefore, *bona fide* $Ca^{2+}$ chelators are critical tools to abrogate $Ca^{2+}$ signals potently, efficiently, and rapidly, thereby elucidating the role of $Ca^{2+}$ signaling in physiological processes in the cell and/or mechanisms underlying pathogenesis.

It is experimentally challenging to directly visualize $Ca^{2+}$ signals within nano- and micro-domains using $Ca^{2+}$ indicators that are loaded into the bulk cytoplasm. However, there are several strategies that facilitate the measurement of $Ca^{2+}$ fluxes in these limited volumes. Primarily, this entails the use of

genetically-encoded $Ca^{2+}$ indicators (GECIs) that can be directed to specific locations. For instance, GECIs can be targeted to ER-mitochondrial contact sites using targeting sequences and chemical linkers [19,39] and to $Ca^{2+}$ signaling nanodomains established by single channel openings via their fusion to $Ca^{2+}$-permeable channels[40,41]. For a more elaborate discussion on the detection of nano- and microscopic $Ca^{2+}$ signals, we refer the reader to another review.[42] Despite these elegant solutions for monitoring $Ca^{2+}$ fluxes in nano- and micro-domains, it remains the case that the visualization of a $Ca^{2+}$ signal is not proof that $Ca^{2+}$ is involved in a specific cellular function. Rather, inhibition or buffering of $Ca^{2+}$ signals, or suppression of suspected downstream effectors is required; BAPTAi is commonly used to provide such experimental proof.

## BAPTA: is it only about $Ca^{2+}$ chelation?

As described above, intracellularly trapped BAPTA is instrumental to test the involvement of $Ca^{2+}$ in cellular processes. Standard conditions for loading BAPTA into cells involve applying 10 µM BAPTA-AM to the extracellular milieu, and incubating for 30 – 60 minutes to ensure sufficient chelation of $[Ca^{2+}]_{cyt}$. Researchers rely on BAPTA's selectivity (against $Mg^{2+}$) and specificity towards $Ca^{2+}$ to assign $Ca^{2+}$ signaling in a certain process that can be either blocked or stimulated by BAPTAi.

Although BAPTAi is a powerful tool for $Ca^{2+}$ research, there has been a growing body of literature pointing to off-target effects of BAPTA and its derivates.[43] Specifically, BAPTAi has been reported to directly influence a diverse set of proteins with divergent cellular function including metabolic enzymes, kinases, carriers, channels and pumps (Fig. 3).

*Effects of BAPTAi on metabolic enzymes, carriers, and cell viability*

A first study hinting towards $Ca^{2+}$-independent effects of BAPTAi was published in 1990 by Yoshito and colleagues, arguing that BAPTAi directly inhibited amylase release and reduced cellular ATP level in rat parotid cells.[44] This reduction of ATP levels by BAPTAi was later confirmed in multiple cell types by Saoudi et al.[45] In the same study, it was reported that BAPTAi, unrelated to its $Ca^{2+}$-chelating capacity, affected mitochondrial respiration, shape, and distribution, and dramatically depolymerized the cytoskeleton. The last observation was confirmed in 2009, when Furuta et al. found that BAPTAi, via a $Ca^{2+}$-independent mechanism, disrupted the cytoskeleton, thereby inhibiting insulin-mediated glucose transport by GLUT4 in adipocytes.[46] Later, Whitehead et al. also suggested that BAPTAi directly binds to, and inhibits, the glucose transporter GLUT4.[47] Lastly, BAPTAi, and some BAPTAi variants, have been shown to increase glutamate-induced excitotoxicity in hippocampal neurons[48] and to stimulate prostacyclin production by endothelial cells, with a cytotoxic effect being observed during prolonged loading[49].

More recently, we found that BAPTAi directly impacts and inhibits 6-phosphofructo-2-kinase/fructose-2,6-biphosphatase (PFKFB3).[50] Remarkably, the inhibition of *in vitro* PFKFB3 activity by BAPTA was lost by adding $Ca^{2+}$, thus indicating that the free acid form of BAPTA, but not the BAPTA-$Ca^{2+}$ complex, could inhibit PFKFB3. This enzyme operates in the proximal phase of the glycolytic pathway where it phosphorylates fructose-6-phosphate into fructose-2,6-bisphosphate. Fructose-2,6-bisphosphate is a potent allosteric activator of 6-phosphofructokinase-1 (PFK-1), which converts fructose-6-phosphate into fructose-1,6-bisphosphate, thereby stimulating glycolytic flux and sustaining cellular metabolism. Our work indicated an important role for adequate PFKFB3 activity in supporting the function of the energy sensing complex, the mammalian Target of Rapamycin Complex 1 (mTORC1), a pivotal energy sensing complex controlling protein translation. Inhibition of mTORC1 by BAPTAi (via binding to PFKFB3) particularly affected short-living proteins. Indeed, we found that BAPTAi evoked a rapid decline in MCL-1 protein levels, thereby resulting in cell death of MCL-1-dependent cancer cells. All these effects could be recapitulated by low $Ca^{2+}$-affinity BAPTA variants implying a $Ca^{2+}$-independent impact of BAPTAi on metabolism, translation, and cell death.

As described above, blocking ER-to-mitochondrial $Ca^{2+}$ transfer diminishes mitochondrial metabolism, leading to the activation of cell survival strategies such as autophagy. In fact, both decreased and increased cellular $Ca^{2+}$ signaling have been implicated in triggering autophagy in different cellular contexts.[51] However much of this work has been dependent on the use of BAPTAi to implicate $Ca^{2+}$ signals as a necessary factor for induction of autophagy. Indeed, BAPTAi seems to universally block autophagy no matter what pro-autophagic stimulus is used.[14,51] Therefore, further work will be needed to discern $Ca^{2+}$-dependent versus $Ca^{2+}$-independent effects of BAPTAi on autophagic flux.

*Effect of BAPTAi on other enzymes and kinases*

In 1993, it was shown that BAPTAi caused the translocation and inactivation of protein kinase C in macrophages.[52] This effect was independent of BAPTA's $Ca^{2+}$-chelating properties because the $Ca^{2+}$ ionophore A23187, which was used to increase $[Ca^{2+}]_{cyt}$, did not reverse the inhibitory effect of BAPTAi.[52]. Hardie reported that BAPTA inhibited phospholipase C activity in *Drosophila* photoreceptors unrelated to its role as a $Ca^{2+}$ chelator.[53]

*Effect of BAPTAi on ion channels*

Richardson and Taylor reported that BAPTA directly inhibited $IP_3$-mediated $Ca^{2+}$ release by acting as a competitive antagonist of $IP_3$ binding to its receptor.[54] $IP_3$Rs are tetrameric channels, and it is thought that all four subunits that form a functional $IP_3$R channel need to be occupied by $IP_3$ to induce

channel opening and $Ca^{2+}$ flux.[55] Thus, loss of binding of one $IP_3$ molecule from a tetrameric $IP_3R$ channel due to BAPTAi is sufficient to prevent that $IP_3R$ channel to open and participate in $Ca^{2+}$ release. In this way, BAPTA could have a profound inhibitory effect on $IP_3R$-mediated $Ca^{2+}$ release that could be mistaken for $Ca^{2+}$ chelation.

Apart from intracellular $IP_3Rs$, plasmalemmal ion channels are also affected by BAPTA in a $Ca^{2+}$-independent fashion. In 2007, Sobanov et al. and Tang et al. demonstrated that BAPTA directly blocked $Cl^-$ channels in adipocytes and hERG $K^+$ channels, respectively.[56,57]

*Effect of BAPTA on ion pumps*

In addition to ion channels, ion pumps can also be affected by BAPTA. These pumps are critical to maintain ionic gradients across membranes. P-type ATPases, for example, couple the hydrolysis of ATP to conformational changes that enable ion transport against electrochemical gradients.[7] A study performed by the Nedergaard lab in 2018 indicated that chemical $Ca^{2+}$ chelators, including BAPTA and fluorescent $Ca^{2+}$ indicators, directly inhibited the $Na^+/K^+$-ATPase independently of their $Ca^{2+}$-chelating properties in a broad spectrum of cell types including neurons, astrocytes, cardiomyocytes and kidney proximal tubule epithelial cells.[30] Consequently, the $Ca^{2+}$ indicators and BAPTAi altered the metabolic status of cells and impacted cellular viability in a concentration-dependent manner. These effects were not only found *in vitro* but also *in vivo* in mouse brain loaded with $Ca^{2+}$ indicators.[30]

*Chelation of ions other than $Ca^{2+}$ by BAPTA*

In addition to chelating $Ca^{2+}$, and its direct effects on proteins, BAPTA can bind transition metals such as $Zn^{2+}$, $Fe^{3+}/Fe^{2+}$ and $Cu^{2+}$ with high affinities.[58–60] For example, using $^1H$ NMR spectroscopy Sunayna and colleagues found that BAPTA also chelates $Zn^{2+}$, $Pb^{2+}$, $Ni^{2+}$, $Mn^{2+}$, and $La^{3+}$.[59] Employing spectrophotometry, Britigan et al., demonstrated that both ferrous ($Fe^{2+}$) and ferric ($Fe^{3+}$) iron can form complexes with BAPTA.[58] Consequently, BAPTAi may interfere with cellular processes through the chelation of ions other than $Ca^{2+}$.

BAPTA's chelation of ions other than $Ca^{2+}$ is a significant consideration given the importance of ions such as $Zn^{2+}$, $Fe^{3+}/Fe^{2+}$ and $Cu^{2+}$ in coordinating cell physiological processes.[61] This includes meiotic entry in the cell cycle[62], gastrin signaling by G cells in the gastric antrum[63], and sperm-induced $Ca^{2+}$ oscillations in eggs[64], as well as being co-factors for cell death and survival processes such as autophagy[65,66], ferroptosis[67,68], ferritinophagy[69,70] and cuproptosis[71].

Indeed, BAPTAi blocks meiotic entry in oocytes independently of $Ca^{2+}$ chelation; instead this effect was attributed to BAPTA's ability to chelate $Zn^{2+}$.[62] This was confirmed using TPEN (N,N,N',N'-tetrakis(2-pyridylmethyl)ethylenediamine), a transitional metal chelator with low affinity for $Ca^{2+}$ and high affinity for $Zn^{2+}$ (Kd of respectively 40 μM and 0.26 fM).[60] Since TPEN also inhibited meiosis, it was found that chelating $Zn^{2+}$ prevents the cell from entering meiosis by inhibiting the kinase cascade that leads up to meiotic entry. Of note, $Zn^{2+}$ homeostasis may intersect with $Ca^{2+}$ signaling as both chelating $Zn^{2+}$ with TPEN and $Zn^{2+}$ overload impede $IP_3R1$-driven $Ca^{2+}$ oscillations in mouse eggs exposed to sperm cells.[64] Thus, $Zn^{2+}$ chelators can also blunt $Ca^{2+}$ signaling through $Zn^{2+}$-dependent regulation of $Ca^{2+}$-transport systems such as $IP_3Rs$.

## What about BAPTA-based fluorescent $Ca^{2+}$ indicators?

The discussion above primarily focused on BAPTA, but since some commonly used fluorescent $Ca^{2+}$ indicators (such as Fluo-1, -2, -3, -4, Fura-2 and Rhod-1 and -2) are based on BAPTA, they too may exert cellular effects irrespective of their $Ca^{2+}$-binding properties. As described above, fluorescent $Ca^{2+}$ indicators can directly inhibit the $Na^+/K^+$-ATPase. Fura-2, in its $Ca^{2+}$-free form, can interact with myoplasmic proteins (aldolase, glyceraldehyde phosphate dehydrogenase and creatine kinase), BSA and poly-lysine.[72,73] Moreover, BAPTA-based $Ca^{2+}$ indicators reduced the contractility of cardiomyocytes in a concentration-dependent manner, partially due to direct inhibition of acto-myosin ATPase activity.[74]

It therefore appears that $Ca^{2+}$ indicators too have cellular actions that are independent of $Ca^{2+}$ chelation. However, such adverse effects have not been systematically investigated, and whether the cellular targets of $Ca^{2+}$ indicators overlap with those of BAPTA remains to be established. Since $Ca^{2+}$ indicators represent chemical modifications of BAPTA it is plausible that there will be differential targets. Moreover, in contrast to $Ca^{2+}$ chelators, $Ca^{2+}$ indicators are typically applied at lower concentrations and brief loading times. The milder conditions under which $Ca^{2+}$ indicators are used may avoid some off-target actions of these compounds. However, this is clearly not always the case since side-effects of $Ca^{2+}$ indicators under standard loading conditions have been observed for the inhibition of $Na^+/K^+$ ATPase[30] and acto-myosin ATPase.[74]

## Further considerations and future directions

Unfortunately, the above-mentioned studies have not received major attention in the $Ca^{2+}$ field. As a result, BAPTAi continues to be in widespread usage, generally without appropriate controls.[43] Hundreds of publications have used BAPTAi to delineate the involvement of $Ca^{2+}$ signals in specific

cellular processes.[75–78] This is concerning because the cellular actions of BAPTAi and related compounds are clearly not limited to $Ca^{2+}$ chelation.[45,46] Indeed, the dichotomy of BAPTA being both a $Ca^{2+}$ chelator and a protein-binding ligand can lead to different, yet not fully mutually exclusive, interpretations for the same experimental observations. For example, BAPTAi has been reported to decrease MCL-1-protein levels.[50,78] Bonnefond et al interpreted that $Ca^{2+}$ signaling regulates MCL-1-protein levels through an effect on mRNA translation via a pathway involving the $Ca^{2+}$-binding protein calmodulin.[78] Instead, our recent work revealed that both BAPTAi and low $Ca^{2+}$-affinity BAPTAi variants were equally potent in lowering MCL-1-protein levels and suppressing MCL-1 translation, pointing towards a $Ca^{2+}$-independent effect of BAPTAi on MCL-1-protein levels.[50] We postulated that this effect of BAPTA on MCL-1-protein levels is due to direct inhibition of PFKFB3 and subsequent mTORC1 inhibition. This mechanism may also account for our previous observations whereby BAPTAi boosted venetoclax-induced cell death in B-cell cancer cells, which we interpreted at the time of publication as an interplay between $Ca^{2+}$-signaling inhibition and BCL-2 inhibition in cell death.[79] We want to stress that our findings on BAPTAi inhibiting PFKFB3 do not exclude that $Ca^{2+}$ signaling can affect MCL-1 translation. Indeed, in the Bonnefond study, non-BAPTA-based tools interfering with $Ca^{2+}$ signaling (such as specific calmodulin kinase inhibitors or inhibitors of store-operated $Ca^{2+}$ entry ) also lowered MCL-1-protein levels.[78,80] Also in our study, while being much less effective than BAPTAi, EGTAi, which does not inhibit PFKFB3 enzyme activity, did evoke a moderate downregulation of MCL-1-protein levels, suggesting some link between $Ca^{2+}$ signaling and MCL-1 translation.[50] Moreover, the widely accepted role of $Ca^{2+}$ in regulation of cellular metabolism, which has been proposed by numerous studies based on BAPTA's actions, may be confounded by multiple direct BAPTA targets including PFKFB3[50],the $Na^+/K^+$-ATPase[30], and the other moieties mentioned above.

Whilst BAPTAi is a critical tool for dissecting $Ca^{2+}$-related cellular responses, relatively few studies have included controls for its potential off-target effects. Research that revealed such $Ca^{2+}$-independent events often did not characterize the exact molecular mechanism underlying the studied phenomena. More concerning is that the reported off-target effects of BAPTA are probably only the tip of the iceberg, with other $Ca^{2+}$-independent actions of BAPTA likely unreported. It is therefore difficult to assess to what extent the $Ca^{2+}$-independent effects of BAPTAi impinge on the results of published studies. In the following paragraphs we expand on different options to control for off-target effects when using BAPTA.

*The use of low $Ca^{2+}$ affinity BAPTA variants*

We propose that whenever BAPTAi is used to examine the role of $Ca^{2+}$ signaling in a certain cell physiological or pathophysiological process, it is critical to use BAPTA variants with varying affinities

for $Ca^{2+}$. Fortunately, several of such BAPTA variants are at our disposal, such as dibromo-BAPTA (DB-BAPTA, *K*d = 1.6 μM), difluoro-BAPTA (DF-BAPTA, *K*d = 635 nM) and tetrafluoro-BAPTA (TF-BAPTA, *K*d = 65 μM), of which the last is the most interesting one given its 400-fold lower affinity for $Ca^{2+}$ compared to BAPTA (*K*d = 160 nM) (Fig. 4). If the observed cellular response involves $Ca^{2+}$, TF-BAPTAi should not phenocopy the effect of BAPTAi.

Unfortunately, TF-BAPTA-AM is not commercially available and can only be obtained through custom chemical synthesis, which necessitates expensive bulk orders and long waiting times. Though TF-BAPTA has a *K*d for $Ca^{2+}$ that is approximately 400-fold lower than that of BAPTA, it is not possible to fully exclude that TF-BAPTA might impact the very high $Ca^{2+}$ concentrations in cellular nanodomains such near the mouth or in proximity of $Ca^{2+}$-permeable channels. Hence, further research on TF-BAPTAi's impact on $Ca^{2+}$-signaling nanodomains is certainly needed and high on the agenda. An additional BAPTA variant that could be used as a control is 'half-BAPTA' (*N*-(*o*-methoxyphenyl) iminodiacetic acid), which represents half of a full BAPTA molecule and has a low affinity for $Ca^{2+}$ (*K*d ~3 mM).[81] Half-BAPTA mimicked some of the deleterious effects of BAPTAi seen in hippocampal neurons, indicating that it may be a suitable control for $Ca^{2+}$-independent effects.[48]

*The use of EGTAi as $Ca^{2+}$-chelating control for BAPTAi*

EGTA may also be a potential control for assessing putative off-target effects of BAPTA. EGTA and BAPTA have different structures but similar affinities for $Ca^{2+}$. The forward $Ca^{2+}$ binding kinetic of BAPTA is ~40 times faster than that of EGTA[54], and as mentioned earlier, it is commonly thought that due to its more rapid binding of $Ca^{2+}$ only BAPTAi is suitable for chelating $Ca^{2+}$ in nano- and microdomains, whereas EGTAi is presumed to be ineffective. Indeed, modelling studies have shown that BAPTAi is more effective than EGTAi in constraining the amplitude and spatial spread of a $Ca^{2+}$ signal around a channel.[82] Moreover, there is clear evidence that BAPTAi can affect, physiological processes where EGTAi is ineffective. For example, $Ca^{2+}$-dependent inactivation of Orai1 store-operated $Ca^{2+}$ entry channels can be prevented by BAPTAi but not by EGTAi, owing to the much slower $Ca^{2+}$-chelating kinetics of the latter.[83] Observations such as these support the notion that differences in the effectiveness of BAPTAi versus EGTAi in altering $Ca^{2+}$ relates to $Ca^{2+}$ signals occurring in nanodomains. In essence, it is argued that if BAPTAi is inhibitory and EGTAi is not, there must be nanoscopic $Ca^{2+}$ signals coupling a $Ca^{2+}$ source and a downstream effector molecule that EGTAi is unable to influence.[55] However, EGTAi has been shown to buffer $Ca^{2+}$ signals within tens of nanometers of a $Ca^{2+}$ source.[84]

The effectiveness of EGTAi as a $Ca^{2+}$ chelator depends on multiple factors such as the proximity of a $Ca^{2+}$ source to its effector molecule, the affinity of the effector molecule for $Ca^{2+}$, the concentration of EGTAi, and the degree of saturation of EGTA with $Ca^{2+}$. EGTAi is more effective at restraining $Ca^{2+}$ signals if the $Ca^{2+}$ source has an intermittent ion flux that does not lead to saturation of binding by EGTA. For instance, using reaction diffusion simulations, it has been proposed that EGTAi can inhibit vesicular neurotransmitter release within the nanodomain of single voltage-gated $Ca^{2+}$ channels.[85]

It is therefore plausible that EGTAi could be a useful control for the $Ca^{2+}$ independent effects of BAPTAi since EGTAi may be as potent as BAPTAi in buffering $Ca^{2+}$ signals and inhibiting downstream responses in many situations. However, it is not always clear to what extent BAPTAi and EGTAi differentially impact microscopic and/or nanoscopic $Ca^{2+}$ signals. For this reason, a difference in the effectiveness of BAPTAi versus EGTAi in inhibiting a process could be difficult to interpret. Of note, in our work examining the effect of BAPTAi on glycolysis and mTORC1, EGTAi did not copy the inhibition of PFKFB3 activity. Moreover, EGTAi was much less effective in decreasing MCL-1-protein levels, while it was equally effective as BAPTAi in reducing agonist-evoked $Ca^{2+}$ signals.[42] Without the use of TF-BAPTAi, however, one would typically interpret such results as indicating that $Ca^{2+}$-signalling nanodomains were operative.

*The use of transition metal chelators as a control for BAPTA*

Non-specific effects due to chelation of transition metal ions by BAPTAi may be ruled out using chelators that have high affinity for such transition metals and low affinity for $Ca^{2+}$. As mentioned above, TPEN is an excellent tool to validate the contribution of $Zn^{2+}$ to a cellular process.[60] TPEN has a high selectivity for $Zn^{2+}$ over $Ca^{2+}$ ($1.5 \times 10^{12}$). Therefore, if a process is inhibited by TPEN, it implies that the process is regulated by $Zn^{2+}$.[62,63] To discriminate effects of BAPTA through $Cu^{2+}$ chelation versus $Ca^{2+}$ chelation, $Cu^{2+}$ chelators such as tetra thiomolybdate, trientine, and D-penicillamine can be considered.[71]

*Validation of effective $Ca^{2+}$ chelation by BAPTAi*

Another issue within the published literature involving BAPTAi is that the buffering of cytosolic $Ca^{2+}$ is generally assumed after BAPTA loading but is usually not demonstrated. Indeed, many studies that have employed intracellularly-loaded BAPTA have assumed that it was retained indefinitely over prolonged experimental time-courses, while in fact cells are known to extrude $Ca^{2+}$ chelators and $Ca^{2+}$ indicators rapidly. Indeed, we have found that, in HEK293 cells at 37°C, ~50% of the $Ca^{2+}$-chelating capacity of BAPTAi is lost within an hour after washing/incubating cells in medium without BAPTA-AM (Fig. 5). It is therefore recommended to test the extent of $Ca^{2+}$ chelation in experiments where

the extracellular BAPTA-AM is removed, or to maintain BAPTA-AM in the extracellular medium when performing long-term experimental analyses.

*Development of new BAPTA analogues*

Finally, we believe that a new generation of BAPTA analogues should be developed to avoid, or at least reduce, $Ca^{2+}$-independent effects on cellular targets. While it is likely that such new BAPTA analogues will chelate other metals ions in addition to $Ca^{2+}$, it may be possible to generate BAPTA variants that cannot bind certain protein targets. Such tools will better enable the characterization of the role of $Ca^{2+}$ in cellular processes.

## Concluding remarks

BAPTAi is a powerful, convenient, and universally used tool to study the involvement of $Ca^{2+}$ signaling in cellular processes. However, over the years evidence has accrued that BAPTAi displays $Ca^{2+}$-independent off-target interactions with a diverse set of proteins, as well as profound interactions with other metal ions, thereby potentially intervening with cell biological and physiological processes independently of its $Ca^{2+}$-chelating properties. A convenient way to avoid wrongly attributing cellular effects of BAPTAi to $Ca^{2+}$ signaling is to include low-affinity BAPTAi variants, EGTAi, and other metal ion chelators, as controls. Going forward, it may be necessary to reassess the role of $Ca^{2+}$ signaling in myriad cellular processes given that evidence for the involvement of $Ca^{2+}$ was based on BAPTAi.

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**Figure legends**

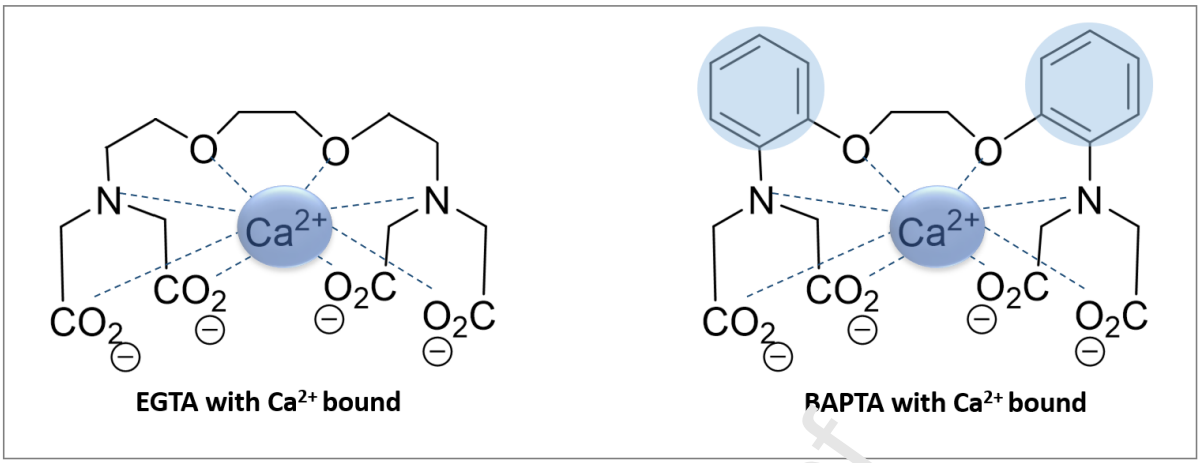



**Figure 1. Comparison between EGTA and BAPTA structures in their $Ca^{2+}$-bound state**. A key difference between EGTA and BAPTA is the presence of aromatic rings. Figure adapted from [43], created with Chemdraw.

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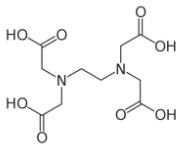
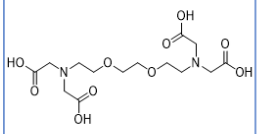
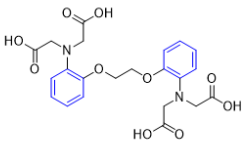
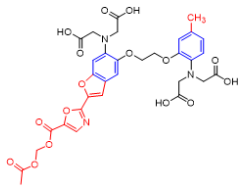
| | **EDTA** | **EGTA** | **BAPTA** | **Fura-2** |
|---|---|---|---|---|
| Structure |  |  |  |  |
| $Ca^{2+}$ affinity (*Kd*) | ~ 138 nM | ~ 60.5 nM | ~ 160 nM | ~ 145 nM |
| Selectivity | $Ca^{2+} = Mg^{2+}$ | $Ca^{2+} >>> Mg^{2+}$ | $Ca^{2+} >>> Mg^{2+}$ | $Ca^{2+} >>> Mg^{2+}$ |
| Kinetics | Slow | Slow | Fast | Fast |
| pH sensitivity | Yes | Yes | No | No |
| Fluorescent | No | No | No | Yes |

**Figure 2. Overview of the structural, chemical, and biophysical properties of EDTA, EGTA, BAPTA and Fura-2**.

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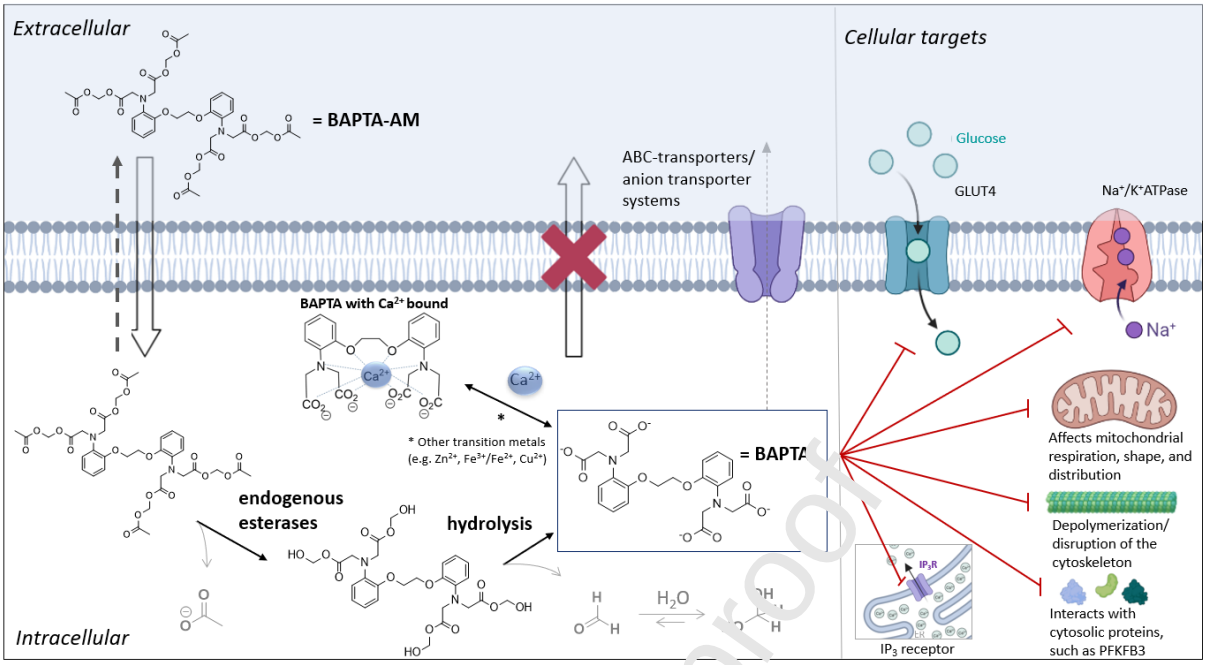



**Figure 3. Schematic representation of BAPTAi loading and its cellular targets**. BAPTA is loaded into cells as an acetoxymethyl ester (BAPTA-AM). This ensures membrane permeation of BAPTA. Once inside the cytosol, non-specific esterases cleave off the -AM moiety, yielding the intracellularly trapped, free acid form of BAPTA that is capable of chelating cytosolic $Ca^{2+}$. However, in addition to its $Ca^{2+}$-chelating properties, BAPTAi also directly impacts a broad spectrum of cellular targets and interferes with different processes. As such, BAPTA directly inhibits the activity of GLUT4, $Na^+/K^+$-ATPases and PFKFB3, affects mitochondrial functions and disrupts the cytoskeleton. Figure adapted from [37, 43]. Moreover, while the free acid form of BAPTA cannot leave the cell via diffusion, ABC transporters, among other mechanisms, may contribute to its extrusion over long-term periods.

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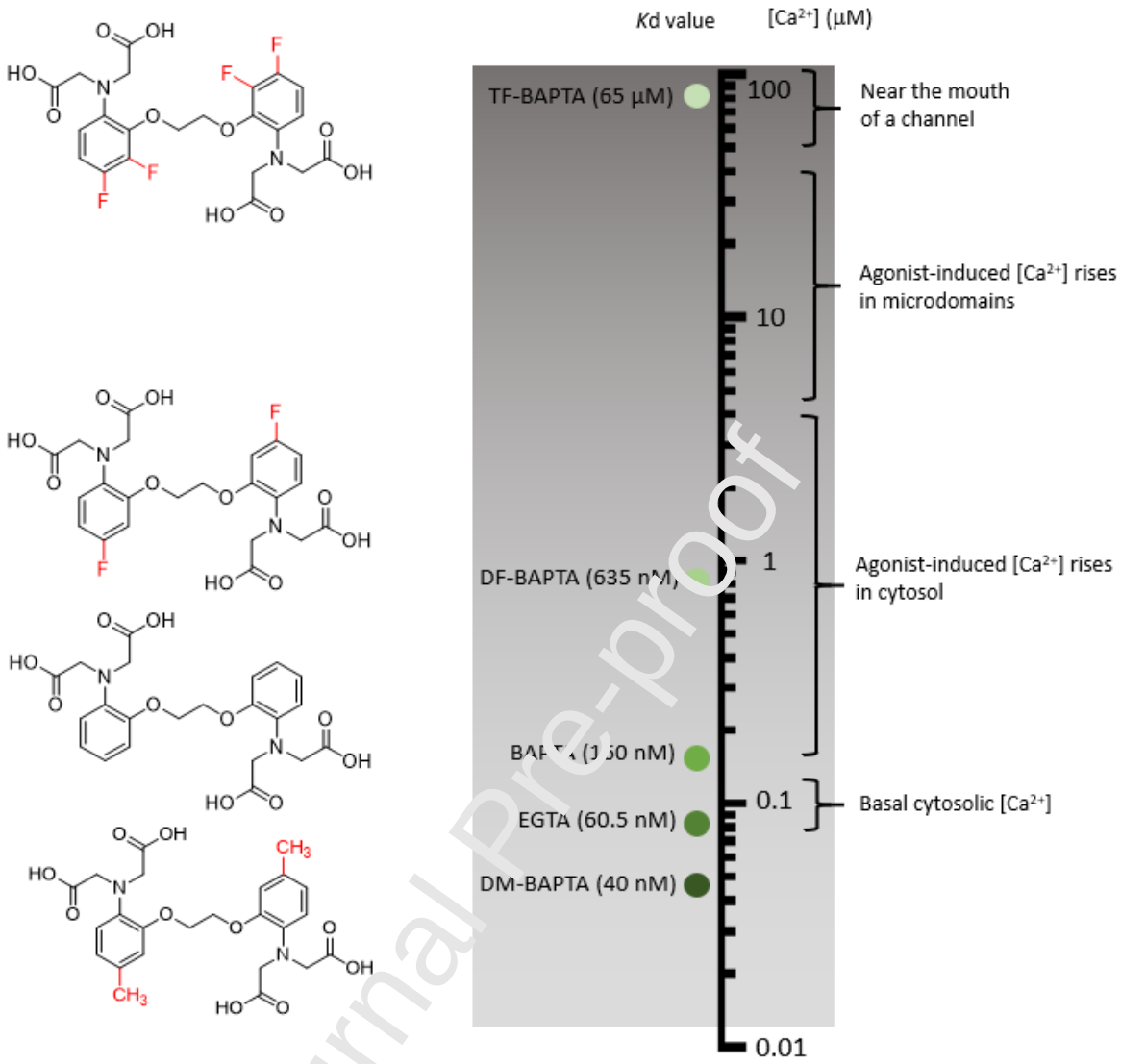



**Figure 4. $Ca^{2+}$-chelating compounds and their corresponding *K*d values in relation to intracellular $Ca^{2+}$ concentrations**. Schematic overview of the chemical structures and *K*d values for $Ca^{2+}$ binding for BAPTA, its derivatives and EGTA in relation to cytosolic $Ca^{2+}$ concentrations in cells and their nano- and micro- domains. The affinity of TF-BAPTA is 500 to 600-fold lower than the basal cytosolic $Ca^{2+}$ concentrations.

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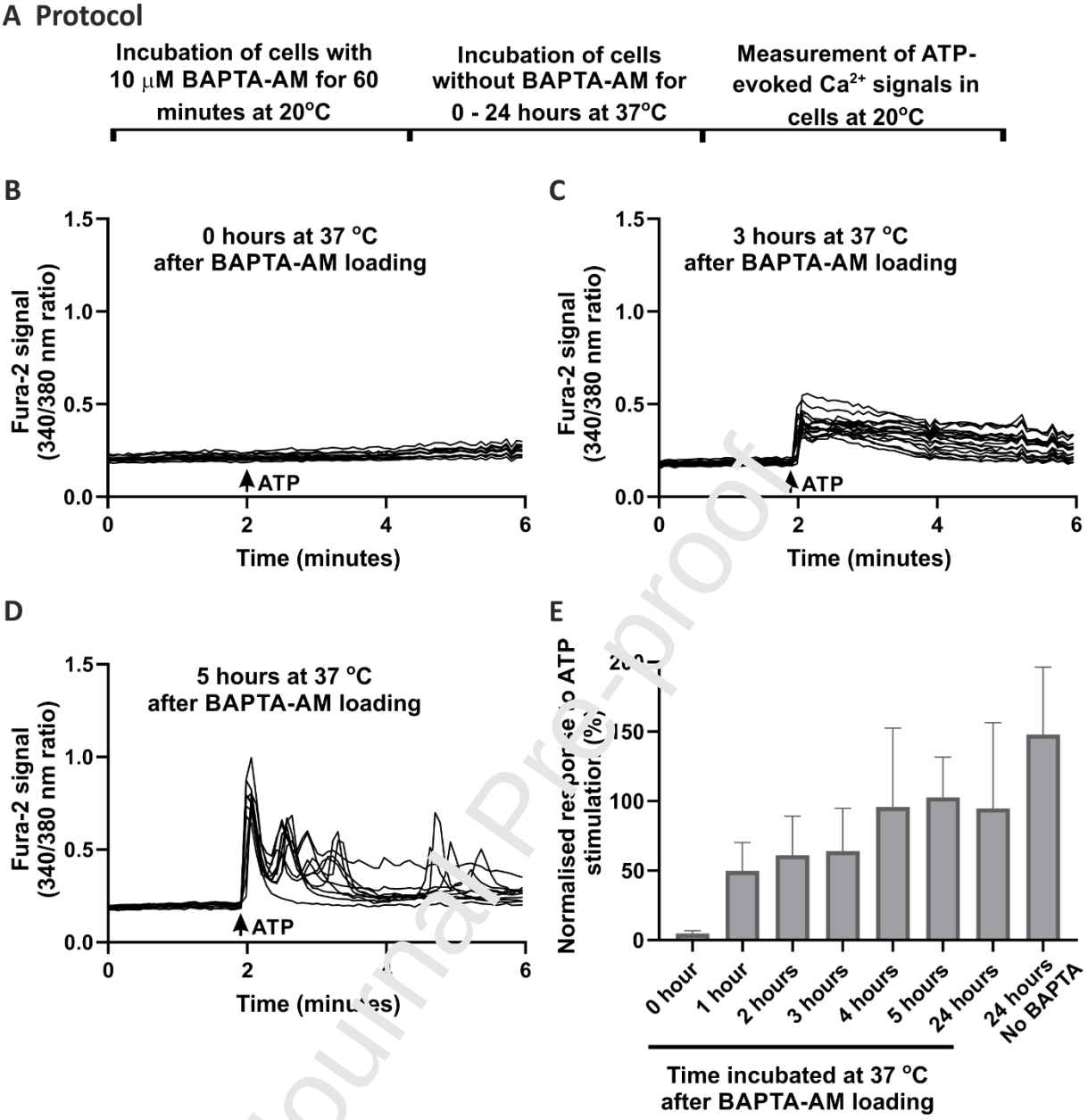



**Figure 5. Buffering of ATP-evoked $Ca^{2+}$ signals by intracellularly-loaded BAPTA in HEK293 cells, and progressive loss of buffering post loading**. Panel A shows the protocol used to examine the time-dependent buffering of $Ca^{2+}$ signals by BAPTAi. Panels B – D illustrate $Ca^{2+}$ responses recorded during the experiment. Panel E shows aggregated data (mean ± SD) for the ATP-evoked $Ca^{2+}$ responses at the times indicated. The $Ca^{2+}$ responses in Panel E reflect the integrated $Ca^{2+}$ signal that was recorded for 10 minutes post ATP addition. BAPTA was loaded into cells as an acetoxymethyl ester (BAPTA-AM; 60 minutes with 10 μM at 20°C). We have empirically found that neither BAPTA, nor fluorescent $Ca^{2+}$ indicators such as Fura-2, are extruded at 20°C. Following BAPTA loading, the extracellular BAPTA-AM was washed away, and cells were placed in an incubator at 37°C for the times shown in Panel E. Following the 37°C incubation, cells were loaded with Fura-2-AM (30 minutes with 1 μM at 20°C), and challenged with 100 μM ATP, as indicated in Panels B – D.

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# References


1. Berridge, M. J., Lipp, P. & Bootman, M. D. The versatility and universality of calcium signalling. *Nature Reviews Molecular Cell Biology* vol. 1 11–21 (2000).
2. Bootman, M. D. & Bultynck, G. Fundamentals of Cellular Calcium Signaling: A Primer. *Cold Spring Harb. Perspect. Biol.* **12**, (2020).
3. Carafoli, E. & Krebs, J. Why calcium? How calcium became the best communicator. *J. Biol. Chem.* **291**, 20849–20857 (2016).
4. Bonora, M. *et al.* Subcellular calcium measurements in mammalian cells using jellyfish photoprotein aequorin-based probes. *Nat. Protoc.* **8**, 2105–2118 (2013).
5. Krebs, J., Agellon, L. B. & Michalak, M. Ca(2+) homeostasis and endoplasmic reticulum (ER) stress: An integrated view of calcium signaling. *Biochem. Biophys. Res. Commun.* **460**, 114–121 (2015).
6. Lloyd-Evans, E. & Waller-Evans, H. Lysosomal Ca2+ Homeostasis and Signaling in Health and Disease. *Cold Spring Harb. Perspect. Biol.* **12**, 1–20 (2020).
7. Chen, J., Sitsel, A., Benoy, V., Sepúlveda, M. R. & Vangheluwe, P. Primary Active Ca2+ Transport Systems in Health and Disease. *Cold Spring Harb. Perspect. Biol.* **12**, (2020).
8. Wang, W. A. & Agellon, L. B. Organellar Calcium Handling in the Cellular Reticular Network. *Cold Spring Harb. Perspect. Biol.* **11**, (2019).
9. Csordás, G., Weaver, D. & Hajnóczky, G. Endoplasmic Reticulum-Mitochondrial Contactology: Structure and Signaling Functions. *Trends Cell Biol.* **28**, 523–540 (2018).
10. Rizzuto, R., Brini, M., Murgia, M. & Pozzan, T. Microdomains with High Ca2+ Close to IP3-Sensitive Channels that Are Sensed by Neighboring Mitochondria. *Science (80-. ).* **262**, 744–747 (1993).
11. Csordás, G., Thomas, A. P. & Hajnóczky, G. Quasi-synaptic calcium signal transmission between endoplasmic reticulum and mitochondria. *EMBO J.* **18**, 96–108 (1999).
12. Williams, G. S. B., Boyman, L., Chikando, A. C., Khairallah, R. J. & Lederer, W. J. Mitochondrial calcium uptake. *Proc. Natl. Acad. Sci. U. S. A.* **110**, 10479–10486 (2013).
13. Gherardi, G., Monticelli, H., Rizzuto, R. & Mammucari, C. The Mitochondrial Ca2+ Uptake and the Fine-Tuning of Aerobic Metabolism. *Front. Physiol.* **11**, (2020).
14. Cárdenas, C. *et al.* Essential regulation of cell bioenergetics by constitutive InsP3 receptor Ca2+ transfer to mitochondria. *Cell* **142**, 270–283 (2010).
15. Giorgio, V., Guo, L., Bassot, C., Petronilli, V. & Bernardi, P. Calcium and regulation of the mitochondrial permeability transition. *Cell Calcium* **70**, 56–63 (2018).
16. Danese, A. *et al.* Cell death as a result of calcium signaling modulation: A cancer-centric

prospective. *Biochim. Biophys. acta. Mol. cell Res.* **1868**, (2021).

17. Loncke, J. *et al.* Balancing ER-Mitochondrial Ca 2+ Fluxes in Health and Disease. *Trends Cell Biol.* **31**, 598–612 (2021).
18. Booth, D. M., Enyedi, B., Geiszt, M., Várnai, P. & Hajnóczky, G. Redox Nanodomains Are Induced by and Control Calcium Signaling at the ER-Mitochondrial Interface. *Mol. Cell* **63**, 240–248 (2016).
19. Booth, D. M., Várnai, P., Joseph, S. K. & Hajnóczky, G. Oxidative bursts of single mitochondria mediate retrograde signaling toward the ER. *Mol. Cell* **81**, 3866-3876.e2 (2021).
20. Duchen, M. R. Mitochondria and calcium: from cell signalling to cell death. *J. Physiol.* **529**, 57 (2000).
21. Popugaeva, E., Pchitskaya, E. & Bezprozvanny, I. Dysregulation of Intracellular Calcium Signaling in Alzheimer's Disease. *Antioxid. Redox Signal.* **29**, 1176–1188 (2018).
22. Calvo-Rodriguez, M., Kharitonova, E. K. & Bacskai, B. J. In vivo brain imaging of mitochondrial Ca2+ in neurodegenerative diseases with multiphoton microscopy. *Biochim. Biophys. acta. Mol. cell Res.* **1868**, (2021).
23. Petersen, O. H., Gerasimenko, J. V., Gerasimenko, O. V., Gryshchenko, O. & Peng, S. The roles of calcium and ATP in the physiology and pathology of the exocrine pancreas. *Physiol. Rev.* **101**, 1691–1744 (2021).
24. Marchi, S., Giorgi, C., Galluzzi, L. & Pinton, P. Ca2+ Fluxes and Cancer. *Mol. Cell* **78**, 1055–1069 (2020).
25. Silvestri, R., Nicolì, V., Gangadharannambiar, P., Crea, F. & Bootman, M. D. Calcium signalling pathways in prostate cancer initiation and progression. *Nat. Rev. Urol. 2023* 1–20 (2023) doi:10.1038/s41585-023-00738-x.
26. Tsien, R. Y. A non-disruptive technique for loading calcium buffers and indicators into cells. *Nat. 1981 2905806* **290**, 527–528 (1981).
27. Romani, A. M. P. Cellular magnesium homeostasis. *Arch. Biochem. Biophys.* **512**, 1–23 (2011).
28. Tsien, R. Y. New calcium indicators and buffers with high selectivity against magnesium and protons: design, synthesis, and properties of prototype structures. *Biochemistry* **19**, 2396–2404 (1980).
29. Tian, L. *et al.* Selective esterase-ester pair for targeting small molecules with cellular specificity. *Proc. Natl. Acad. Sci. U. S. A.* **109**, 4756–4761 (2012).
30. Smith, N. A. *et al.* Fluorescent Ca2+ indicators directly inhibit the Na,K-ATPase and disrupt cellular functions. *Sci. Signal.* **11**, (2018).
31. Di Virgilio, F., Fasolato, C. & Steinberg, T. H. Inhibitors of membrane transport system for organic anions block fura-2 excretion from PC12 and N2A cells. *Biochem. J.* **256**, 959 (1988).

32. Lüders, A. K., Saborowski, R. & Bickmeyer, U. Inhibition of multidrug/xenobiotic resistance transporter by MK571 improves dye (Fura 2) accumulation in crustacean tissues from lobster, shrimp, and isopod. *Comp. Biochem. Physiol. Part C Toxicol. Pharmacol.* **150**, 368–371 (2009).
33. Scherer, C., Wiltshire, K. & Bickmeyer, U. Inhibition of multidrug resistance transporters in the diatom Thalassiosira rotula facilitates dye staining. *Plant Physiol. Biochem.* **46**, 100–103 (2008).
34. Ouanounou, A. *et al.* ACCUMULATION AND EXTRUSION OF PERMEANT Ca 2+ CHELATORS IN ATTENUATION OF SYNAPTIC TRANSMISSION AT HIPPOCAMPAL CA1 NEURONS. (1996).
35. Bootman, M. D., Rietdorf, K., Collins, T., Walker, S. & Sanderson, M. Loading Fluorescent Ca2+ Indicators into Living Cells. *Cold Spring Harb. Protoc.* **2013**, pdb.prot072801 (2013).
36. Grynkiewicz$, G., Poenie, M. & Tsienb, R. Y. THE JOURNAL OF BIOLOGICAL CHEMISTRY A New Generation of Ca2+ Indicators with Greatly Improved Fluorescence Properties*. **260**, 1385 (1985).
37. Zhou, X., Belavek, K. J. & Miller, E. W. Origins of Ca2+Imaging with Fluorescent Indicators. *Biochemistry* **60**, 3547–3554 (2021).
38. Bootman, M. D., Rietdorf, K., Collins, T., Walker, S. & Sanderson, M. Converting fluorescence data into Ca2+ concentration. *Cold Spring Harb. Protoc.* **2013**, 126–129 (2013).
39. Csordás, G. *et al.* Imaging interorganelle contacts and local calcium dynamics at the ER-mitochondrial interface. *Mol. Cell* **39**, 121–132 (2010).
40. Dynes, J. L., Amcheslavsky, A. & Cahalan, M. D. Genetically targeted single-channel optical recording reveals multiple Orai1 gating states and oscillations in calcium influx. *Proc. Natl. Acad. Sci. U. S. A.* **113**, 440–445 (2016).
41. Tay, L. H. *et al.* Nanodomain $Ca^{2+}$ of $Ca^{2+}$ channels detected by a tethered genetically encoded $Ca^{2+}$ sensor. *Nat. Commun.* **3**, (2012).
42. Mulier, M., Vriens, J. & Voets, T. TRP channel pores and local calcium signals. *Cell Calcium* **66**, 19–24 (2017).
43. Bootman, M. D., Allman, S., Rietdorf, K. & Bultynck, G. Deleterious effects of calcium indicators within cells; an inconvenient truth. *Cell Calcium* vol. 73 82–87 (2018).
44. Yosuke, T. & Yoshito, M. Inhibitory effects of loading with the calciumchelator 1,2-bis(o-aminophenoxy)ethane-N, N, N, N',N'-tetraacetic acid (bapta) on amylase release and cellular atp level in rat parotid. *Biochem. Pharmacol.* **39**, 1775–1779 (1990).
45. Saoudi, Y. *et al.* Calcium-independent cytoskeleton disassembly induced by BAPTA. *Eur. J. Biochem.* **271**, 3255–3264 (2004).
46. Furuta, A. *et al.* Microtubule disruption with BAPTA and dimethyl BAPTA by a calcium chelation-independent mechanism in 3T3-L1 adipocytes. *Endocr. J.* **56**, 235–243 (2009).

47. Whitehead, J. P. *et al.* The role of Ca2+ in insulin-stimulated glucose transport in 3T3-L1 cells. *J. Biol. Chem.* **276**, 27816–27824 (2001).

48. Abdel-Hamid, K. M. & Baimbridge, K. G. The effects of artificial calcium buffers on calcium responses and glutamate-mediated excitotoxicity in cultured hippocampal neurons. *Neuroscience* **81**, 673–687 (1997).

49. Boeynaems, J. M., Heilporn, S., Broeders, F. & Braekman, J. C. Enhancement of the endothelial production of prostacyclin by substituted derivatives of BAPTA-AM. *Eur. J. Pharmacol.* **233**, 13–20 (1993).

50. Sneyers, F. *et al.* Intracellular BAPTA directly inhibits PFKFB3, thereby impeding mTORC1-driven Mcl-1 translation and killing MCL-1-addicted cancer cells. *Cell Death Dis.* (2023) doi:10.1038/s41419-023-06120-4.

51. Bootman, M. D., Chehab, T., Bultynck, G., Parys, J. B. & Rietdorf, K. The regulation of autophagy by calcium signals: Do we have a consensus? *Cell Calcium* **70**, 32–46 (2018).

52. Dieter, P., Fitzke, E. & Duyster, J. BAPTA induces a decrease of intracellular free calcium and a translocation and inactivation of protein kinase C in macrophages. *Biol. Chem. Hoppe. Seyler.* **374**, 171–174 (1993).

53. Hardie, R. C. Inhibition of phospholipase C activity in Drosophila photoreceptors by 1,2-bis(2-aminophenoxy)ethane N,N,N',N'-tetraacetic acid (BAPTA) and di-bromo BAPTA. *Cell Calcium* **38**, 547–556 (2005).

54. Richardson, A. & Taylor, C. W. Effects of Ca2+ chelators on purified inositol 1,4,5-trisphosphate (InsP3) receptors and InsP3-stimulated Ca2+ mobilization. *J. Biol. Chem.* **268**, 11528–11533 (1993).

55. Alzayady, K. J. *et al.* Defining the stoichiometry of inositol 1,4,5-trisphosphate binding required to initiate Ca2+ release. *Sci. Signal.* **9**, (2016).

56. Sabanov, V. & Nedergaard, J. Ca(2+) -independent effects of BAPTA and EGTA on single-channel Cl(-) currents in brown adipocytes. *Biochim. Biophys. Acta* **1768**, 2714–2725 (2007).

57. Tang, Q. *et al.* The membrane permeable calcium chelator BAPTA-AM directly blocks human ether a-go-go-related gene potassium channels stably expressed in HEK 293 cells. *Biochem. Pharmacol.* **74**, 1596–1607 (2007).

58. Britigan, B. E., Rasmussen, G. T. & Cox, C. D. Binding of iron and inhibition of iron-dependent oxidative cell injury by the 'calcium chelator' 1,2-bis(2-aminophenoxy)ethane N,N,N',N'-tetraacetic acid (BAPTA). *Biochem. Pharmacol.* **55**, 287–295 (1998).

59. Adoni, S., Mologu, T. & Igumenova, T. Metal Ion Sensing by BAPTA: Investigation Using 1H NMR Spectroscopy. *J. Student Res.* **11**, (2022).

60. Arslan, P., Virgilio, F. Di, Beltrame, M., Tsien, R. Y. & Pozzans, T. Cytosolic Ca2+ Homeostasis in

Ehrlich and Yoshida Carcinomas. *J. Biol. Chem.* **260**, 2719–2727 (1985).

61. Sekler, I., Sensi, S. L., Hershfinkel, M. & Silverman, W. F. Mechanism and regulation of cellular zinc transport. *Mol. Med.* **13**, 337–343 (2007).
62. Sun, L., Chai, Y., Hannigan, R., Bhogaraju, V. K. & Machaca, K. Zinc regulates the ability of Cdc25C to activate MPF/cdk1. *J. Cell. Physiol.* **213**, 98–104 (2007).
63. Chang, M., Xiao, L., Shulkes, A., Baldwin, G. S. & Patel, O. Zinc Ions Mediate Gastrin Expression, Proliferation, and Migration Downstream of the Cholecystokinin-2 Receptor. *Endocrinology* **157**, 4706–4719 (2016).
64. Hiroki, A., Emily, L. & A., F. R. Zn2+ is Essential for Ca2+ Oscillations in Mouse Eggs. *Elife* **12**, (2023).
65. Liuzzi, J. P., Guo, L., Yoo, C. & Stewart, T. S. Zinc and autophagy. *Biometals* **27**, 1087–1096 (2014).
66. Qi, J. *et al.* MCOLN1/TRPML1 finely controls oncogenic autophagy in cancer by mediating zinc influx. *Autophagy* **17**, 4401–4422 (2021).
67. Jiang, X., Stockwell, B. R. & Conrad, M. Ferroptosis: mechanisms, biology and role in disease. *Nat. Rev. Mol. Cell Biol.* **22**, 266–282 (2021).
68. Du, Y. & Guo, Z. Recent progress in ferroptosis: inducers and inhibitors. *Cell death Discov.* **8**, (2022).
69. Tang, M., Chen, Z., Wu, D. & Chen, L. Ferritinophagy/ferroptosis: Iron-related newcomers in human diseases. *J. Cell. Physiol.* **233**, 9179–9190 (2018).
70. Liu, J. *et al.* Autophagy-Dependent Ferroptosis: Machinery and Regulation. *Cell Chem. Biol.* **27**, 420–435 (2020).
71. Chen, L., Min, J. & Wang, F. Copper homeostasis and cuproptosis in health and disease. *Signal Transduct. Target. Ther.* **7**, (2022).
72. Bancel, F., Salmon, J. M., Vigo, J., Vo-Dinh, T. & Viallet, P. Investigation of noncalcium interactions of fura-2 by classical and synchronous fluorescence spectroscopy. *Anal. Biochem.* **204**, 231–238 (1992).
73. Konishi, M., Olson, A., Hollingworth, S. & Baylor, S. M. Myoplasmic binding of Fura-2 investigated by steady-state fluorescence and absorbance measurements. *Biophys. J.* **54**, 1089–1104 (1988).
74. Robinson, P. *et al.* Comparing the effects of chemical Ca 2+ dyes and R-GECO on contractility and Ca 2+ transients in adult and human iPSC cardiomyocytes. *J. Mol. Cell. Cardiol.* **180**, 44–57 (2023).
75. Turner, C., Connell, J., Blackstone, K. & Ringler, S. Loss of calcium and increased apoptosis within the same neuron. *Brain Res.* **1128**, 50–60 (2007).

76. Williams, J., Hou, Y., Ni, H. & Ding, W. Role of intracellular calcium in proteasome inhibitor-induced endoplasmic reticulum stress, autophagy, and cell death. *Pharm. Res.* **30**, 2279–2289 (2013).

77. Zhang, L., Cheng, X., Xu, S., Bao, J. & Yu, H. Curcumin induces endoplasmic reticulum stress-associated apoptosis in human papillary thyroid carcinoma BCPAP cells via disruption of intracellular calcium homeostasis. *Medicine (Baltimore).* **97**, (2018).

78. Bonnefond, M.-L. *et al.* Calcium signals inhibition sensitizes ovarian carcinoma cells to anti-Bcl-xL strategies through Mcl-1 down-regulation. *Apoptosis 2015 204* **20**, 535–550 (2015).

79. Vervloessem, T., Ivanova, H., Luyten, T., Parys, J. B. & Bultynck, G. The selective Bcl-2 inhibitor venetoclax, a BH3 mimetic, does not dysregulate intracellular Ca2+ signaling. *Biochim. Biophys. Acta - Mol. Cell Res.* **1864**, 968–976 (2017).

80. Bonnefond, M. L. *et al.* Inhibition of store-operated channels by carboxyamidotriazole sensitizes ovarian carcinoma cells to anti-BclxL strategies through Mcl-1 down-regulation. *Oncotarget* **9**, 33896 (2018).

81. Kao, J. P. Y., Li, G. & Auston, D. A. Practical Aspects of Measuring Intracellular Calcium Signals with Fluorescent Indicators. in *Methods in Cell Biology* 113–152 (2010).

82. Mulier, M., Vriens, J. & Voets, T. TRP channel pores and local calcium signals. *Cell Calcium* **66**, 19–24 (2017).

83. Zhang, X. *et al.* A calcium/cAMP signaling loop at the ORAI1 mouth drives channel inactivation to shape NFAT induction. *Nat. Commun. 2019 101* **10**, 1–13 (2019).

84. Nakamura, Y., Reva, M., David, X. & Digregorio, A. Cellular/Molecular Variations in Ca 2 Influx Can Alter Chelator-Based Estimates of Ca 2 Channel-Synaptic Vesicle Coupling Distance. (2018) doi:10.1523/JNEUROSCI.2061-17.2018.

85. Nakamura, Y. EGTA Can Inhibit Vesicular Release in the Nanodomain of Single Ca2+ Channels. *Front. Synaptic Neurosci.* **11**, (2019).


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The authors declare that they have no conflict of interest to report.

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**Highlights**

- Intracellular $Ca^{2+}$ signals control a plethora of cell physiological processes.
- Intracellular BAPTA loading is a widely used approach to implicate the involvement of $Ca^{2+}$ signaling in a particular process.
- Accumulating evidence indicates that intracellular BAPTA exerts $Ca^{2+}$-independent effects by directly impacting molecular targets.
- Future perspective on the use of intracellular BAPTA is discussed.

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