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Calcium and Inositol 1,4,5-Trisphosphate–Induced Ca^{2+} Release

One of the pathways that regulates cytosolic free Ca^{2+} is that of inositol 1,4,5-trisphosphate ($InsP_3$) inducing intracellular stores to release Ca^{2+} (*1*). It has been suggested that the dependence of this release on the concentration of free Ca^{2+} in the cytoplasm comprises Ca^{2+}-induced activation of the $InsP_3$-gated channel in the presence of submicromolar concentrations of cytosolic Ca^{2+} (*2*, *3*). The immediate positive feedback control of $InsP_3$-induced Ca^{2+} release resulting from such activation would be important in the regulation of store discharge, and this control has been incorporated into different models describing the spatiotemporal aspects of Ca^{2+} signaling, for example, hormone-induced oscillation in cellular Ca^{2+} and propagation of Ca^{2+} waves (*1–3*).

Nevertheless, as the buffering of free Ca^{2+} at low concentrations generally involves the use of Ca^{2+} chelators, the validity of such experiments may be doubted if the chelators have any activity other than chelating. Recently, Richardson and Taylor showed that several chelators interfered with the $InsP_3$ receptor (*4*). In their experiments, the Ca^{2+}-free forms of the Ca^{2+} chelator BAPTA and the related fura-2 dye proved to be competitive antagonists of $InsP_3$ binding to its receptor. In contrast, EGTA [another Ca^{2+} chelator that has been widely used in most of the experiments supporting Ca^{2+} activation of the $InsP_3$-gated channel (*2*)] was found by Taylor and Richardson to have virtually no effect on $InsP_3$ binding to its receptor, as measured at low temperature under alkaline conditions (*4*).

We further investigated the effect of EGTA by directly measuring $InsP_3$-induced $^{45}Ca^{2+}$ efflux from microsomal stores derived from cerebellum, a tissue rich in $InsP_3$ receptors. We found that 1 mM EGTA greatly reduced the amount of $^{45}Ca^{2+}$ released during 2 s (Fig. 1). The dose dependence of $InsP_3$-induced $^{45}Ca^{2+}$ release was studied in two different media buffered at pCa 6.5 (20°C, pH 7.1), which contained either 30 μM Ca^{2+}-free EGTA and 30 μM CaEGTA or 1 mM Ca^{2+}-free EGTA and 1 mM CaEGTA. In the presence of the higher concentration of EGTA, the dose-response curve for $InsP_3$ was shifted upwards by one order of magnitude, which implies inhibition by EGTA. Artifactual effects of EGTA or CaEGTA on different systems are well documented (*5*).

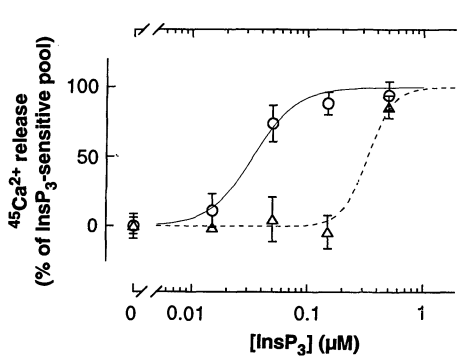

Fig. 1. Inhibition by EGTA per se of $InsP_3$-induced $^{45}Ca^{2+}$ release within 2 s from cerebellar microsomes. Microsomes, prepared from sheep brain (*7*), were actively loaded with $^{45}Ca^{2+}$, diluted in an $InsP_3$-free medium at pCa 9, and layered onto a nitrocellulose 0.45-μm filter. Most of the external $^{45}Ca^{2+}$ was washed out by a short rinse with the same $InsP_3$-free medium. The adsorbed microsomes were perfused with 2 ml of release medium containing different concentrations of $InsP_3$. Perfusion under controlled vacuum took about 2 s. The filter was counted with no additional rinsing, so that the perfusion-dependent drop in counted $^{45}Ca^{2+}$ reflected only the drop in trapped $^{45}Ca^{2+}$ during the 2-s perfusion. This drop is expressed as the fraction of the $InsP_3$-sensitive $^{45}Ca^{2+}$ pool released. The release medium contained 100 mM KCl, 20 mM NaCl, 5 mM $MgCl_2$, 25 mM Hepes-KOH (pH 7.1, 20°C), and either 30 μM EGTA plus 30 μM CaEGTA (○), or 1 mM EGTA plus 1 mM CaEGTA (△). In both cases, pCa was close to 6.5, as checked by fluorescence measurements after addition of fluo-3. The mean value, ± standard deviation, is indicated.

Preliminary experiments suggested that when the inhibitory effect of EGTA was taken into account, free Ca^{2+} was a poor activator of $InsP_3$-induced Ca^{2+} release (not shown), which is consistent with previous results by Meyer and co-workers (*6*). They found no influence of free Ca^{2+} in the range of 150 to 800 nM on the kinetics of Ca^{2+} release from permeabilized basophilic leukemia cells into Ca^{2+}-depleted media in which the only buffer for Ca^{2+} was fluo-3, present at a small concentration, around 1 μM. Do these results negate the activating effects of Ca^{2+} on $InsP_3$-induced Ca^{2+} release described previously? At least, they call for reexamination of the possible artifactual effects of Ca^{2+} chelators or Ca^{2+} probes in those experiments, as such effects likely account for part of the previously observed activation by Ca^{2+}. The Ca^{2+} sensitivity of the $InsP_3$ receptor might well have been overestimated.

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Response: After examination of Combettes and Champeil's legitimate concerns, we conclude that artifactual effects of Ca che-

lators were not responsible for our observations of Ca-dependent activation and inactivation of IP_3-mediated ^{45}Ca release from brain-derived microsomal vesicles, for two reasons: the conditions under which our experiments were performed did not favor an alteration of IP_3 binding by EGTA; and, more fundamentally, our own observations of the effects of EGTA do not coincide with those observed by Combettes and Champeil. The majority of the observations in our report (*1*) of Ca^{2+}-dependent activation of IP_3-induced $^{45}Ca^{2+}$ release were done with the use of 1 μM IP_3 and 1 mM total EGTA. The decrease in IP_3 sensitivity that Combettes and Champeil observed (their figure 1) at a higher total EGTA concentration (2 mM), and with 300 nM free Ca^{2+}, still resulted in maximal activation of $^{45}Ca^{2+}$ release with a lower [IP_3] (0.5 μM) than was used in our experiments. Data in our report (*1*) and additional unpublished results confirm that at high and low free [Ca^{2+}], 1 μM IP_3 produced near-maximal activation of IP_3-mediated $^{45}Ca^{2+}$ release with 1 mM total EGTA. Thus, the IP_3 and EGTA concentrations we used favored a saturating interaction of IP_3 with its receptor, and our measurements should not have been affected by a decrease in apparent affinity for IP_3 caused by EGTA. Under these conditions we found the Ca^{2+} dependence of both the maximum observed rate of $^{45}Ca^{2+}$ release and the cumulative $^{45}Ca^{2+}$ release over 2 s in the presence of 1 μM IP_3 to be highly dependent on extravesicular [Ca^{2+}] [figure 3C in (*1*)].

More recently we examined the effects of Ca^{2+} chelators on $^{45}Ca^{2+}$ release elicited by IP_3. We varied EGTA and BAPTA concentrations from 0 to 10 mM in superfusion buffers with a specified free Ca^{2+} concentration. Our purpose was to characterize the effects of graded changes in Ca^{2+} concentration (which result from the noninstantaneous mixing of these buffers in the superfusion chamber) on the kinetics of IP_3-mediated $^{45}Ca^{2+}$ release (*2*). Nonetheless, these experiments support the contention that neither EGTA nor BAPTA (*2*) is inhibitory. At time = 0 the superfusion valve was switched from a buffer containing 1 mM EGTA with no added Ca^{2+} to a buffer containing 5 μM IP_3 at 300 nM free Ca^{2+} and the indicated concentration of EGTA or BAPTA (Fig. 1). In general, the magnitude and kinetics of $^{45}Ca^{2+}$ release were similar under each of these conditions: increasing the EGTA concentration up to 10 mM did not inhibit IP_3-mediated $^{45}Ca^{2+}$ release (Fig. 1A). The slower rate of rise and slightly diminished peak release rate observed on switching to 300 nM free Ca^{2+} buffered with EGTA concentrations below 1 mM (Fig. 1B) [which resulted from a slower rate of change in free [Ca^{2+}] under these conditions (*2*)] is the opposite of what would be expected if high concentrations of EGTA were inhibitory. Furthermore, with 5 μM IP_3 at 300 nM Ca^{2+}, BAPTA (Fig. 1A) did not inhibit IP_3-induced $^{45}Ca^{2+}$ release as might be predicted based on the results of Richardson and Taylor (*3*). No inhibitory effects of EGTA or BAPTA were observed with 5 μM IP_3 at either ~10 nM Ca^{2+} (Fig. 1C) or 10 μM Ca^{2+} (not shown). Thus, as illustrated by the potentiating effect of 300 nM Ca^{2+} (compare Figs. 1A and 1B with 1C), over a range of EGTA and BAPTA concentrations, an increase in extravesicular [Ca^{2+}] rapidly potentiated and modulated the time course of IP_3-mediated $^{45}Ca^{2+}$ release, as we originally reported (*1*).

These results support the idea that a rapid increase in cellular free [Ca^{2+}] would trigger Ca^{2+} release by IP_3, such that the IP_3 receptor would mediate an IP_3-dependent Ca^{2+}-induced Ca^{2+} release (*1*). As we hypothesized, this type of regulation may be important for the formation of oscillations and waves in cytosolic Ca^{2+} (*4*) and has been incorporated into a mathematical model that accurately reproduces Ca oscillations and waves in *Xenopus* oocytes (*5*).

Although our results are not necessarily incompatible with those of Combettes and Champeil, we and others (*1*, *6*) have observed a lower affinity for IP_3 activation of Ca^{2+} release than that cited in their comment. The decrease in IP_3 sensitivity produced by free EGTA might be expected to be more apparent at lower Ca^{2+} concentrations because, for a given total [EGTA], free [EGTA] would be higher. At 10 μM Ca^{2+} and 1 mM total EGTA (~30 μM free EGTA), we observed half maximal activation of the maximum rate and cumulative amount of $^{45}Ca^{2+}$ release (measured over 2 s) at between 200 and 300 nM IP_3 [figure 2 of (*1*)]. At 10 nM and 300 nM Ca^{2+}, the apparent affinity of IP_3 for cumulative measurements of $^{45}Ca^{2+}$ release over a 2-s period was higher (EC_{50} = 100 to 200 nM) than for activation of the maximum rate of $^{45}Ca^{2+}$ release (EC_{50} = 600 to 700 nM). The difference in the apparent affinity of IP_3 for initial

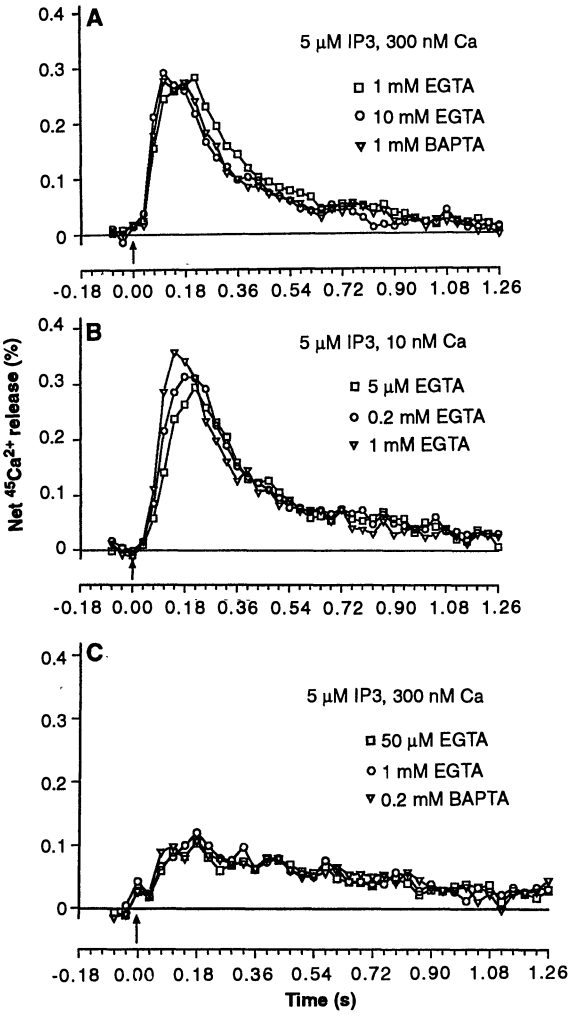

Fig. 1. The effects of EGTA and BAPTA concentration, at a specified free [Ca^{2+}], on IP_3-mediated ^{45}Ca release. In these experiments, vesicles were prepared as in our report (*1*). The introduction of stimulation buffers is indicated by upward arrows. The free [Ca^{2+}] in each of the buffers was monitored using a Ca^{2+}-selective electrode (*1*). Data are presented as single superfusion experiments, with comparable buffer flow rates for each experiment. Experiments depicted in (A) and (B) were done on different days so the amplitudes shown of $^{45}Ca^{2+}$ release are only semiquantitatively comparable. (**A**) At t = 0, the superfusate was switched to a buffer containing 5 μM IP_3, 300 nM free Ca^{2+}, and 1 mM EGTA (□), 10 mM EGTA (○), or 1 mM BAPTA (▽). (**B**) At t = 0, the superfusate was switched to a buffer containing 5 μM IP_3, 300 nM free Ca^{2+}, and 5 μM EGTA (□), 0.2 mM EGTA (○), or 1 mM EGTA (▽). (**C**) At t = 0, the superfusate was switched to a buffer containing 5 μM IP_3, 10 nM free Ca^{2+}, and 50 μM EGTA (□), 1 mM EGTA (○), or 0.2 mM BAPTA (▽).

rates and cumulative $^{45}Ca^{2+}$ release at low but not high $[Ca^{2+}]$ appears to be a result of the regulation by extravesicular $[Ca^{2+}]$ of the kinetics of $^{45}Ca^{2+}$ release: at 10 μM Ca^{2+}, IP_3-mediated $^{45}Ca^{2+}$ release is transient, due to the rapid onset of Ca^{2+}-dependent inactivation (*1*), whereas at lower free $[Ca^{2+}]$, $^{45}Ca^{2+}$ release persists throughout the exposure to IP_3. In any case, in all of these measurements we observed a lower apparent affinity of IP_3 for receptor activation than that obtained by Combettes and Champeil. However, because our data and those of others (6–8) suggest that factors such as the time interval over which Ca^{2+} release is measured and the extravesicular Ca^{2+} concentration, as well as receptor subtype, influence the apparent affinity of IP_3, it is not clear whether inhibition by EGTA contributed to the lower apparent affinity of IP_3 we observed.

The basis for the difference in the effects of EGTA in our experiments and those described by Combettes and Champeil is unclear. A clearer picture of the kinetics of the interaction of EGTA with the IP_3 receptor would be beneficial in this regard. Differences between the experimental systems, such as time resolution, buffer composition (for example, their use of Mg^{+2} in the release medium), or vesicle preparation, might well be involved.

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