Teaching the modes of Ca\(^{2+}\) transport between the plasma membrane and endoplasmic reticulum using a classic paper by Kwan et al.

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Submitted 7 April 2009; accepted in final form 6 May 2009

Liang W. Teaching the modes of Ca\(^{2+}\) transport between the plasma membrane and endoplasmic reticulum using a classic paper by Kwan et al. *Adv Physiol Educ* 33: 169–174, 2009; doi:10.1152/advan.00025.2009. —This teaching article uses the report by Kwan et al., “Effects of methacholine, thapsigargin, and La\(^{3+}\) on plasmalemmal and intracellular Ca\(^{2+}\) transport in lacrimal acinar cells,” where the effects of Ca\(^{2+}\)-mobilizing agents in regulating Ca\(^{2+}\) fluxes were examined under various conditions. Upper-level undergraduate and new graduate students in physiology are the target audience. Teaching and learning points are put forth in this article to illustrate 1) the characteristics of methacholine- and thapsigargin-induced Ca\(^{2+}\) responses, 2) the different endoplasmic reticulum Ca\(^{2+}\) stores accessible to methacholine and thapsigargin, 3) the inhibitory effects of La\(^{3+}\) on Ca\(^{2+}\) extrusion and Ca\(^{2+}\) influx, and 4) the facilitatory role of La\(^{3+}\) on endoplasmic reticulum Ca\(^{2+}\) recycling. Each of the above concepts is first explained with references to the figures adapted from the original article. A list of student learning questions then follows, where the answers are found in the teaching notes for the instructors. It is the objective of this article to make both teaching and learning Ca\(^{2+}\) regulation a rewarding experience for all.

Ca\(^{2+}\) recycling; capacitative Ca\(^{2+}\) entry; education

The important roles of Ca\(^{2+}\) in cell physiology cannot be emphasized more. In neuronal endings, Ca\(^{2+}\) entry triggers the vesicular release of neurotransmitters. In cardiac and smooth muscle, the coupling between Ca\(^{2+}\) influx and release governs contractility. In endothelial cells, Ca\(^{2+}\) mobilization precedes the synthesis and secretion of various vasoactive substances. A specific mode of Ca\(^{2+}\) influx, capacitative Ca\(^{2+}\) entry (CCE), is particularly important in nonexcitable cells, of which endothelial cells are an example. Other terms for CCE include store-operated and store-mediated Ca\(^{2+}\) entry (9). The prevailing significance of CCE in nonexcitable cells may be explained by the lack of functional voltage-gated Ca\(^{2+}\) entry in these cells. In such cells where electrical stimulation (via membrane depolarization) yields minimal or no Ca\(^{2+}\) movement, CCE provides the means to refill depleted intracellular Ca\(^{2+}\) stores.

It is currently accepted that depletion of the endoplasmic reticulum (ER) Ca\(^{2+}\) store brings about the redistribution of a Ca\(^{2+}\) sensor protein, stromal interacting molecule-1 (Stim1) from within the ER to areas closer to the plasma membrane (PM) (8). Without leaving the ER, Stim1 may interact with the PM protein Orai1, which is in close proximity with the ER Ca\(^{2+}\) sensor protein (5, 8). A diffusible Ca\(^{2+}\) influx factor, as proposed by Bolotina and Csutora (1), may also be involved in the activation of Orai1. Orai1 contains subunits that form pores on the PM that allow Ca\(^{2+}\) passage (6). The resulting Ca\(^{2+}\) current is commonly termed Ca\(^{2+}\) release-activated Ca\(^{2+}\) current (3) and forms the basis of CCE that was observed by Putney in 1986 (7).

Compelling evidence of the modes of Ca\(^{2+}\) influx in CCE was presented by Kwan et al. in an APS classic paper in 1990 (4). The stated article has been cited 188 times as of March 2009 and is the subject of study in this teaching article, which is aimed at upper-level undergraduate and new graduate students in physiology. Although not a focus of the original article, the basic principles of cytosolic Ca\(^{2+}\) measurements warrant a brief introduction here. Kwan et al. (4) loaded lacrimal acinar cells with fura-2 and captured fluorescence signals with a spectrofluorimeter. Two wavelengths, 340 and 380 nm, are used alternately to excite fura-2-loaded cells typically. When fura-2 is unbound or Ca\(^{2+}\) free, fluorescence is emitted at 340-nm excitation. On the other hand, when fura-2 is bound by Ca\(^{2+}\), fluorescence is emitted at 380-nm excitation. The emitted fluorescence is captured at 500 nm. The fluorescence ratio between 340 and 380 nm is calculated and inserted into an equation described by Grynkiewicz et al. (2). Values of absolute Ca\(^{2+}\) concentration are determined using this equation. To make use of the equation, minimal and maximal fluorescence ratios are also needed, and these can be obtained by permeabilizing the cells and subjecting them to high Ca\(^{2+}\) with and without a chelator. The measurement of fluorescence ratios in determining relative Ca\(^{2+}\) concentration earns fura-2 the description of a ratiometric Ca\(^{2+}\) indicator. Another ratiometric dye commonly used is indo-1. Only one excitation wavelength (at 338 nm) is used with indo-1, but fluorescence signals are captured at two emission wavelengths, usually 405 and 485 nm. Ca\(^{2+}\)-free indo-1 emits fluorescence at 485 nm, whereas the Ca\(^{2+}\)-bound dye emits at 405 nm. Since leakage of dyes does not affect the calculated fluorescence ratios, ratiometric Ca\(^{2+}\) indicators remain very useful tools in cytosolic Ca\(^{2+}\) measurements.

Using Ca\(^{2+}\) mobilizing agents on lacrimal acinar cells, Kwan et al. demonstrated that ER Ca\(^{2+}\) refilling after store depletion occurs via a two-step process (4). First, extracellular Ca\(^{2+}\) enters the cytosol or, alternatively, Ca\(^{2+}\) released from the ER is retained in the cytosol. Cytosolic Ca\(^{2+}\) is then taken up by the ER to refill the Ca\(^{2+}\) store. Using the article by Kwan et al. (4) as a reference, I will propose some teaching and learning points on drug-induced Ca\(^{2+}\) responses, ER Ca\(^{2+}\) recycling, and CCE in the following areas: 1) methacholine (MeCh)-induced ER Ca\(^{2+}\) release and Ca\(^{2+}\) influx, 2) thapsigargin (TSG)-induced ER Ca\(^{2+}\) release and Ca\(^{2+}\) influx, 3) differences in ER Ca\(^{2+}\) stores activated by MeCh and TSG, 4) dual effects of La\(^{3+}\) in blocking Ca\(^{2+}\) extrusion and Ca\(^{2+}\) influx, and 5) facilitation of ER Ca\(^{2+}\) recycling by La\(^{3+}\).
Teaching points for student handout 1. Figure 1A (adapted from Fig. 1A of the original article) compares Ca\(^{2+}\) responses elicited by 3 \(\mu\)M MeCh in different extracellular Ca\(^{2+}\) environments. Trace 1 shows a biphasic MeCh-induced Ca\(^{2+}\) response with a sustained plateau after the initial upstroke. The plateau was maintained by continuous Ca\(^{2+}\) influx as 1 mM Ca\(^{2+}\) was present in the bathing solution. Upon repeated additions of the Ca\(^{2+}\) chelator EGTA (at points a and b; Fig. 1A), Ca\(^{2+}\) influx was diminished and eventually abolished. Trace 2 in Fig. 1A shows a MeCh-induced Ca\(^{2+}\) response obtained in nominal Ca\(^{2+}\)-free solution. No Ca\(^{2+}\) was added to the bathing solution in this case. In addition, 3 mM EGTA was added (at point c; Fig. 1A) to chelate any trace amounts of Ca\(^{2+}\) that may be present. The result was a transient MeCh-induced Ca\(^{2+}\) response with no plateau phase, i.e., no Ca\(^{2+}\) influx. A comparison between traces 1 and 2 reveals that the initial upstroke and decay phase of the Ca\(^{2+}\) response is contributed by ER Ca\(^{2+}\) release, independent of extracellular Ca\(^{2+}\). Trace 3 in Fig. 1A was obtained much like trace 2 except that EGTA was not added and 1 mM Ca\(^{2+}\) was added (at point d; Fig. 1A) to the bathing solution at the end of the transient Ca\(^{2+}\) response. This elicited a Ca\(^{2+}\) plateau to a level similar to that seen in trace 1 (before EGTA was added). A comparison between traces 1 and 3 indicates that the processes of ER Ca\(^{2+}\) release and Ca\(^{2+}\) influx can be functionally separated by manipulating the extracellular Ca\(^{2+}\) environment. Trace 3 in Fig. 1A also demonstrates an example of CCE when the MeCh-sensitive ER Ca\(^{2+}\) store is depleted.

Figure 1B (adapted from Fig. 1B of the original article) shows that the Ca\(^{2+}\) response elicited by MeCh was due to the activation of muscarinic receptors. A prior incubation with atropine (a muscarinic antagonist) prevented MeCh-induced Ca\(^{2+}\) mobilization (trace 1; Fig. 1B). The addition of atropine during the plateau phase of the MeCh-induced Ca\(^{2+}\) response abolished the response completely (trace 2; Fig. 1B).

Student handout 1. Figure 1 is included in the handout.

1. Fill in the blanks (at points a–d) to indicate whether EGTA or Ca\(^{2+}\) was added in traces 1–3 in Fig. 1A.

2. Was extracellular Ca\(^{2+}\) present during the course of traces 1 and 2? How were the responses different and why?

3. In Fig. 1B, the addition of which drug (at points indicated by arrows) would have resulted in the responses seen in traces 1 and 2?

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**MeCh-Induced ER Ca\(^{2+}\) Release and Ca\(^{2+}\) Influx**

**TSG-Induced ER Ca\(^{2+}\) Release and Ca\(^{2+}\) Influx**

Teaching points for student handout 2. Figure 2A (adapted from Fig. 3A of the original article) shows the relationship between TSG concentration and the resultant Ca\(^{2+}\) responses in nominal Ca\(^{2+}\)-free solution followed by the addition of 3 mM Ca\(^{2+}\) to the bathing solution. By inhibiting ER Ca\(^{2+}\), ATPase so that cytosolic Ca\(^{2+}\) cannot be taken up into the ER, TSG causes a gradual depletion of the ER Ca\(^{2+}\) store. High TSG concentrations (0.7 and 1 \(\mu\)M) induced ER Ca\(^{2+}\) release to a similar extent (traces 1 and 2; Fig. 2A) with nearly equal time courses. The subsequent addition of 3 mM Ca\(^{2+}\) induced Ca\(^{2+}\) influx, as indicated by the sustained plateau phase. The submaximal TSG concentration of 0.3 \(\mu\)M elicited a slightly smaller maximal Ca\(^{2+}\) response with a longer time course (trace 3; Fig. 2A) and was followed by a plateau of similar amplitude as traces 1 and 2 in the presence of extracellular Ca\(^{2+}\). By 0.03 \(\mu\)M, TSG elicited a much slower Ca\(^{2+}\) response (trace 4; Fig. 2A). The maximum attained Ca\(^{2+}\) concentration [intracellular Ca\(^{2+}\)] was also lower than those in traces 1–3 but the subsequent plateau was higher. However, when taken from the net difference between the sustained plateau and end level of the preceding response from ER Ca\(^{2+}\) release (i.e., between points i and ii on traces 1–3 and points iii and iv on trace 4), the plateau was essentially of the same amplitude regardless of TSG concentration. Altogether, from traces 1–4, it can be concluded that TSG causes the ER Ca\(^{2+}\) store to deplete completely, which, in the presence of extracellular Ca\(^{2+}\), triggers CCE. Trace 5 in Fig. 2A shows marginal TSG-induced ER Ca\(^{2+}\) release and Ca\(^{2+}\) influx only due to the very low TSG concentration (0.01 \(\mu\)M) used.

Also shown in Fig. 2A is that the effective CCE was stimulated by 0.03–1 \(\mu\)M TSG. Figure 2B (adapted from Fig. 3B of the original article) compares 0.3 \(\mu\)M TSG-elicited Ca\(^{2+}\) responses in varying extracellular Ca\(^{2+}\) concentrations. The increase in extracellular Ca\(^{2+}\) concentration from 0 to 3 mM was reflected by the higher amplitudes of the TSG-induced sustained Ca\(^{2+}\) plateau in the various traces [with extracellular Ca\(^{2+}\) concentrations: trace 1 (0 mM + EGTA), trace 2 (0 mM), trace 3 (0.3 mM), trace 4 (1 mM), and trace 5 (3 mM); Fig. 2B]. The amount of Ca\(^{2+}\) influx is thus dependent on extracellular Ca\(^{2+}\) concentration. For trace 2, where extracellular Ca\(^{2+}\) was absent until the addition of 3 mM Ca\(^{2+}\), the resultant Ca\(^{2+}\) plateau reached a similar level to that of trace 5, the extracellular Ca\(^{2+}\) concentration of which was 3 mM throughout. This serves as further evidence of the importance of extracellular Ca\(^{2+}\) concentration in regulating the amount of Ca\(^{2+}\) influx after ER Ca\(^{2+}\) store depletion. Thus, if 3 mM Ca\(^{2+}\) is added in traces 3 and 4 (at points a; Fig. 2B), the subsequent plateau would also reach a similar level to that of trace 5. For trace 1, no plateau would be expected even if 3 mM Ca\(^{2+}\) is added since the bathing solution contains EGTA also. The addition of EGTA in trace 5 abolished the Ca\(^{2+}\) plateau, again
and the individual TSG concentrations used in each trace.

...complete ER Ca^{2+} sensitive to MeCh was depleted. Finally, the addition of 3 mM Tris elicited Ca^{2+} release directly. Cells were permeabilized with saponin to facilitate the delivery of IP$_3$ and heparin (an IP$_3$ receptor antagonist), both of which are membrane impermeable. Figure 3C shows the partial ER Ca^{2+} release upon the addition of IP$_3$ to mimic MeCh stimulation at the muscarinic receptor. The subsequent addition of TSG further induced ER Ca^{2+} release. However, no IP$_3$-induced ER Ca^{2+} release was visible when TSG was added first to elicit a response (Fig. 3D).

Student handout 3. Figure 3 is included in the handout. 1. Given 0.015, 0.03, and 1 µM of TSG were added in Fig. 3, A and B, match the TSG concentration with the correct trace. 2. Why is the last Ca^{2+} response in trace 4 smaller than those in traces 1–3 in Fig. 3A?

...why is the last Ca^{2+} response in trace 4 smaller than those in traces 1–3 in Fig. 3A?

3. Why do the amplitudes of the MeCh-induced Ca^{2+} responses differ in Fig. 3B despite the same MeCh concentration being added?

4. Both IP$_3$ (used in Fig. 3, C and D) and MeCh (used in Fig. 3, A and B) elicited the same kind of responses with respect to TSG addition before or after treatment. In terms of Ca^{2+}-mobilizing effects, how is IP$_3$ related to MeCh?

5. What can be concluded about the nature of the MeCh-sensitive ER Ca^{2+} store in relation to the TSG-sensitive one?

Dual Effects of La^{3+} in Blocking Ca^{2+} Extrusion and Ca^{2+} Influx

Teaching points for student handout 4. We have seen in the above sections that the extent of CCE, or Ca^{2+} influx after the MeCh-sensitive ER Ca^{2+} store was depleted, and this was not enough to trigger maximal CCE, as seen in traces 1–3.

Figure 3A shows that a portion of the Ca^{2+} store (from within the ER or other intracellular Ca^{2+} compartments) was not accessible to MeCh and was released only upon TSG application. That the TSG-sensitive ER Ca^{2+} store also includes the MeCh-sensitive one is demonstrated in Fig. 3B. The application of 0.1 µM TSG elicited a transient Ca^{2+} response that was not followed by a MeCh-induced response (trace 1; Fig. 3B), suggesting that most if not all of the ER Ca^{2+} store was already depleted by TSG stimulation. A lower TSG concentration (0.03 µM) also depleted most of the ER Ca^{2+} store (trace 2; Fig. 3B). The subsequent addition of MeCh only generated a very small response as a result (trace 2). At 0.015 µM, TSG induced partial depletion of the ER Ca^{2+} store only, leaving plenty of Ca^{2+} to be released by MeCh stimulation (trace 3; Fig. 3B). It is interesting to note that after the additions of both TSG and MeCh, the ER Ca^{2+} store was depleted to a similar extent, allowing a comparable amount of Ca^{2+} influx afterward with 3 mM extracellular Ca^{2+}. The amplitudes of CCE, after ER Ca^{2+} store depletion stimulated by TSG and MeCh, were also comparable among traces 1–3 in Fig. 3A and traces 1–3 in Fig. 3B. In the traces mentioned, MeCh induced partial depletion of the ER Ca^{2+} store while the remaining ER Ca^{2+} was released by the addition of TSG. In the absence of TSG (trace 4; Fig. 3A), the ER still contained a significant amount of Ca^{2+} after MeCh stimulation, thus reducing the CCE response.

The distinction between MeCh-sensitive and MeCh-insensitive (i.e., portion of the TSG sensitive) ER Ca^{2+} stores is also demonstrated in Fig. 3, C and D. Subsequent to the activation of the muscarinic receptor by MeCh, inositol 1,4,5-trisphosphate (IP$_3$) is synthesized and was used here to trigger ER Ca^{2+} release directly. Cells were permeabilized with saponin to facilitate the delivery of IP$_3$ and heparin (an IP$_3$ receptor antagonist), both of which are membrane impermeable. Figure 3C shows the partial Ca^{2+} release upon the addition of IP$_3$ to mimic MeCh stimulation at the muscarinic receptor. The subsequent addition of TSG further induced ER Ca^{2+} release. However, no IP$_3$-induced ER Ca^{2+} release was visible when TSG was added first to elicit a response (Fig. 3D).

Student handout 4. Figure 2 is included in the handout.

1. Fill in the blanks (at points a–c) to indicate whether EGTA or Ca^{2+} was added in Fig. 2, A and B.

2. Rank the order of TSG concentration (0, 0.015, 0.03, 0.3, 0.7, and 1 µM) added in traces 1–6 in Fig. 2A.

3. In Fig. 2A, trace 4 shows a TSG-elicited Ca^{2+} response that is smaller and slower than those in traces 1–3. Did complete ER Ca^{2+} depletion occur? Justify your answer.

4. In Fig. 2B, predict the level of Ca^{2+} influx for traces 1, 3, and 4 if the extracellular Ca^{2+} concentration is increased to 3 mM at point c.

Differences in ER Ca^{2+} Stores Activated by MeCh and TSG

Teaching points for student handout 3. Figure 3 is adapted from Figs. 4 and 5 of the original article. The range of TSG concentrations used was from 0.01 to 1 µM, which corresponds with traces 2–6. No TSG was present in trace 1. B: effects of varying extracellular Ca^{2+} concentrations on TSG responses. The Ca^{2+} concentrations used were as follows: 0.3 mM in trace 3, 1 mM in trace 4, and 3 mM in trace 5. No Ca^{2+} was present in traces 1 and 2. EGTA was present in trace 1 only. See the teaching notes for the identity of substances added at points a–c, points i–iv, and the individual TSG concentrations used in each trace.

Demonstrating the source of Ca^{2+} being extracellular during the plateau phase.

Student handout 2. Figure 2 is included in the handout.

1. Match the TSG concentration with the correct trace.

2. Why is the last Ca^{2+} response in trace 4 smaller than those in traces 1–3 in Fig. 3A?

3. Why do the amplitudes of the MeCh-induced Ca^{2+} responses differ in Fig. 3B despite the same MeCh concentration being added?

4. Both IP$_3$ (used in Fig. 3, C and D) and MeCh (used in Fig. 3, A and B) elicited the same kind of responses with respect to TSG addition before or after treatment. In terms of Ca^{2+}-mobilizing effects, how is IP$_3$ related to MeCh?

5. What can be concluded about the nature of the MeCh-sensitive ER Ca^{2+} store in relation to the TSG-sensitive one?
store depletion, depends on extracellular Ca\(^{2+}\) concentration. Another factor, cytosolic Ca\(^{2+}\) removal, also determines the amplitude and rate of CCE. Figure 4 (adapted from Figs. 6 and 7 of the original article) made use of La\(^{3+}\) to show the importance of both Ca\(^{2+}\) influx and cytosolic Ca\(^{2+}\) retention in generating Ca\(^{2+}\) responses.

Figure 4A shows that La\(^{3+}\) (from 0.03 to 0.5 mM) blocked Ca\(^{2+}\) influx in the presence of 3 mM extracellular Ca\(^{2+}\) after MeCh-induced ER Ca\(^{2+}\) release in nominal Ca\(^{2+}\)-free solution (traces 2–4). Trace 1 in Fig. 4A shows the presence of CCE, which increased in amplitude as the extracellular Ca\(^{2+}\) concentration was raised, when La\(^{3+}\) was not added. In addition to blocking Ca\(^{2+}\) influx, La\(^{3+}\) was shown to retard the Ca\(^{2+}\) decay phase after MeCh stimulation. Cytosolic Ca\(^{2+}\) extrusion was prevented by La\(^{3+}\). More cytosolic Ca\(^{2+}\) was retained in the cytosol as the La\(^{3+}\) concentration was increased from 0.03 to 0.5 mM in traces 2–4 (Fig. 4A). More Ca\(^{2+}\) is taken up by the ER and is available for release, contributing to the elevated Ca\(^{2+}\) responses elicited by MeCh in traces 3 and 4 (compared with trace 2).

A typical biphasic Ca\(^{2+}\) response elicited by MeCh in 1 mM extracellular Ca\(^{2+}\) is shown in Fig. 4B in trace 1. The sustained plateau phase (immediately after the initial upstroke) indicates that Ca\(^{2+}\) influx after the ER Ca\(^{2+}\) store is partially depleted by MeCh. Increasing the extracellular Ca\(^{2+}\) concentration to 2 mM results in greater Ca\(^{2+}\) influx (trace 1), as shown earlier. In trace 2 in Fig. 4B, 0.5 mM La\(^{3+}\) was added before the MeCh response. As cytosolic Ca\(^{2+}\) removal was inhibited, a larger Ca\(^{2+}\) response is seen (trace 2). The higher peak Ca\(^{2+}\) and slower Ca\(^{2+}\) decay indicate greater Ca\(^{2+}\) availability in the cytosol that is taken up by the ER for release. The addition of 2 mM extracellular Ca\(^{2+}\) afterward failed to elicit a response (trace 2; Fig. 4B), in agreement with the responses shown in Fig. 4A.

Knowing the potential facilitatory effect of La\(^{3+}\) on ER Ca\(^{2+}\) uptake and release, it would be interesting to examine whether cytosolic Ca\(^{2+}\) retention is affected by the extent of ER Ca\(^{2+}\) depletion. The rapid initial upstroke of the MeCh-induced Ca\(^{2+}\) response prevents the application of La\(^{3+}\) at different points of the ER Ca\(^{2+}\)-depleting process. The time course of the TSG-induced Ca\(^{2+}\) response is much slower, and so the ER Ca\(^{2+}\)-ATPase inhibitor was used here. Figure 4C shows traces of TSG-elicited Ca\(^{2+}\) responses in nominal Ca\(^{2+}\)-free solution when 0.5 mM La\(^{3+}\) was added at different points of ER Ca\(^{2+}\) depletion. Trace 1 in Fig. 4C shows that when La\(^{3+}\) was added before TSG, the resultant Ca\(^{2+}\) response was much greater and had a very slow decay phase. If La\(^{3+}\) was added after the TSG-induced Ca\(^{2+}\) maximum had been reached, a secondary Ca\(^{2+}\) response was seen followed by a slow decay phase (trace 2; Fig. 4C). The addition of La\(^{3+}\) after the completion of the TSG response failed to elicit any response (trace 3; Fig. 4C). Altogether, blockade of Ca\(^{2+}\) extrusion is most effective before ER Ca\(^{2+}\) release, i.e., when [Ca\(^{2+}\)]\(_i\) is at the resting level. After exiting the ER,
Ca^{2+} would be lost to the extracellular space, and the addition of La^{3+} would not retain the Ca^{2+} in the cytosol (trace 3; Fig. 4C). If Ca^{2+} extrusion is stopped prematurely by La^{3+}, some Ca^{2+} retention would occur, and subsequent ER Ca^{2+} release would be enhanced (trace 2). By preventing any Ca^{2+} extrusion before stimulating ER Ca^{2+} release, the amount of Ca^{2+} mobilized to the cytosol would be maximal (trace 1).

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**Fig. 4.** Dual effects of La^{3+} on MeCh- and TSG-elicited Ca^{2+} responses. A: MeCh-elicited Ca^{2+} responses in nominal Ca^{2+}-free solution with varying La^{3+} concentrations added. Ca^{2+} (1 mM) was added sequentially to the bathing solution in trace 1 after the MeCh response. In traces 2–4, 3 mM Ca^{2+} was added extracellularly. B: comparison of MeCh-elicited Ca^{2+} responses in 1 mM Ca^{2+} with and without prior application of La^{3+} followed by the addition of 2 mM Ca^{2+} to the extracellular space. C: effects of La^{3+} on TSG-elicited Ca^{2+} responses in nominal Ca^{2+}-free solution. In traces 1–3, La^{3+} was added at different points as indicated. See the teaching notes for the individual La^{3+} concentrations used and the corresponding traces.

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**Fig. 5.** Effect of La^{3+} on ER Ca^{2+} recycling. A: Ca^{2+} responses elicited by MeCh (traces 2 and 3) and TSG (traces 1–3) with the application of La^{3+} (points a–c). In traces 2 and 3, atropine (ATR) was added to terminate the MeCh response. B and C: successive MeCh-elicited Ca^{2+} responses (with washouts in between) in nominal Ca^{2+}-free solution. La^{3+} was present in one of the graphs (B or C; see the teaching notes). See the teaching notes for the points of La^{3+} application and the corresponding traces.
Based on the above, in reference to Fig. 4C, the Ca\(^{2+}\) responses resulting from the addition of La\(^{3+}\) at other points can be predicted. Two sample scenarios are given. First, La\(^{3+}\) is added at point a (Fig. 4C), i.e., slightly after the initiation of the TSG response. The Ca\(^{2+}\) response would follow that of trace 2 up to point a, where it would deflect upward due to La\(^{3+}\)-induced Ca\(^{2+}\) retention, followed by a slow decay phase. The maximal Ca\(^{2+}\) attained will be higher than that in trace 2 since less Ca\(^{2+}\) is lost to the extracellular space before La\(^{3+}\) is added. On the other hand, maximal Ca\(^{2+}\) will be lower than that of trace 1, where no Ca\(^{2+}\) extrusion ever took place. In the second scenario, La\(^{3+}\) is added at point b (Fig. 4C), i.e., at the peak of the TSG response. A secondary Ca\(^{2+}\) response may or may not be visible because significant ER Ca\(^{2+}\) release would have already occurred and was thus lost to the extracellular space. The addition of La\(^{3+}\) may only retain the remaining Ca\(^{2+}\) and retard the decay phase of the response.

Student handout 4. Figure 4 is included in the handout.
1. What effects does La\(^{3+}\) have in regulating Ca\(^{2+}\) flux in a cell?
2. In Fig. 4A, which traces were under the influence of La\(^{3+}\)? Of these traces, which one was obtained with the highest and lowest La\(^{3+}\) concentration, respectively?
3. Explain why traces 3 and 4 in Fig. 4A show a slower Ca\(^{2+}\) decay phase.
4. Explain why trace 2 in Fig. 4B fails to show a response to the addition of 2 mM Ca\(^{2+}\).
5. In Fig. 4C, why do the responses in traces 1–3 differ when La\(^{3+}\) was added at different points?
6. With reference to Fig. 4C, predict the Ca\(^{2+}\) response if La\(^{3+}\) is added at points a and b.

Facilitation of ER Ca\(^{2+}\) recycling by La\(^{3+}\)

Teaching points for student handout 5. The concept of blocking Ca\(^{2+}\) extrusion to retain Ca\(^{2+}\) for ER Ca\(^{2+}\) recycling was confirmed in the experiments shown in Fig. 5A (adapted from Figs. 8A, 9, and 10 of the original article). Figure 5A compares the effect of La\(^{3+}\) before and after MeCh application. Complete Ca\(^{2+}\) retention by La\(^{3+}\) added at point c before any ER Ca\(^{2+}\) release, was obtained in trace 3 in Fig. 5A. Maximal Ca\(^{2+}\) mobilization after TSG application was seen as a result. The addition of La\(^{3+}\) before MeCh (at point a) yielded a Ca\(^{2+}\) response that decayed slowly (trace 2) compared with trace 1, where La\(^{3+}\) was added only after the MeCh response (at point b; Fig. 5A). The subsequent TSG-induced Ca\(^{2+}\) response in trace 2 was also larger than in trace 1. The larger TSG response in trace 2 indicates Ca\(^{2+}\) was retained after MeCh-induced release. Trace 2 shows direct evidence of the retained Ca\(^{2+}\) being taken up by the ER for further release or, in other words, ER Ca\(^{2+}\) recycling. In trace 1, after MeCh stimulation, Ca\(^{2+}\) loss to the extracellular space was not prevented, resulting in a less filled ER Ca\(^{2+}\) store and thus a smaller TSG response.

Figure 5, B and C, can be discussed in parallel to highlight the facilitatory role of La\(^{3+}\) in ER Ca\(^{2+}\) recycling in the absence of extracellular Ca\(^{2+}\). After a transient Ca\(^{2+}\) response was elicited and after a washout period in nominal Ca\(^{2+}\)-free solution, MeCh was unable to cause further Ca\(^{2+}\) release (Fig. 5B). However, in the presence of La\(^{3+}\), MeCh not only elicited successive Ca\(^{2+}\) responses, but the responses were sustained also (Fig. 5C). The responses in Fig. 5C again demonstrate that La\(^{3+}\) inhibits cytosolic Ca\(^{2+}\) extrusion, allowing ER Ca\(^{2+}\) recycling to occur for subsequent MeCh-induced release. If extracellular Ca\(^{2+}\) is added (arrows in Fig. 5), CCE is expected under the conditions shown in Fig. 5B but not Fig. 5C, because La\(^{3+}\) blocks Ca\(^{2+}\) influx in the latter.

Student handout 5. Figure 5 is included in the handout.
1. In each of the three traces shown in Fig. 5A, La\(^{3+}\) was added at different points (points a–c). Match the point of La\(^{3+}\) addition with the correct trace. Explain how La\(^{3+}\) affects MeCh- and TSG-induced Ca\(^{2+}\) responses.
2. In Fig. 5, B and C, which graph has La\(^{3+}\) added? Describe how La\(^{3+}\) affects the first and second, if any, MeCh-induced Ca\(^{2+}\) response.
3. What can be concluded about the effect of La\(^{3+}\) on ER Ca\(^{2+}\) recycling?
4. If extracellular Ca\(^{2+}\) is added at the end of the experimental trace (indicated by arrows) in both Fig. 5, B and C, what can be expected of the Ca\(^{2+}\) level?

SUMMARY

This teaching article outlines how the concept of intracellular Ca\(^{2+}\) movements, including ER Ca\(^{2+}\) release, ER Ca\(^{2+}\) recycling, and CCE, can be taught using figures adapted from Kwan et al. (8). This article is divided into five sections, each consisting of an explanation of the figures that serve as teaching notes followed by a student handout with learning questions. For those interested in the field of Ca\(^{2+}\) regulation pertaining to CCE, this article (as well as the original article) is particularly helpful in understanding the pathway of Ca\(^{2+}\) transport in refilling the ER after store depletion.

REFERENCES