Salud Mental 2014;37:103-110
ISSN: 0185-3325
DOI: 10.17711/SM.0185-3325.2014.013

Calcium release from intracellular pools promotes serotonin secretion at synaptic terminals

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Original article

Translation of the original version published in spanish in: Salud Mental 2014, Vol. 37 Issue No. 2.

SUMMARY

This work analyses the participation of calcium release from the endoplasmic reticulum in the serotonin release in synaptic terminals. Experiments were carried out in synapses formed in culture between serotonergic Retzius neurons and pressure mechanosensory neurons, isolated from the Central Nervous System of the leech. In this preparation, paired-pulse stimulation produced synaptic facilitation. Locking ryanodine receptors in a sub-conductance state by incubation with 100 µM ryanodine caused an elongation of the synaptic potential in response to presynaptic impulses, suggesting that calcium released through these channels may reach the vesicles and then promote secretion. By contrast, depletion of intracellular calcium pools by incubation with 500 nM thapsigargin gradually decreased paired-pulse synaptic facilitation and abolished extrasynaptic axonal serotonin release in response to trains of impulses. All this occurred without changes in the properties of the postsynaptic membrane, indicating that intracellular calcium release participates in a feedback mechanism that enhances presynaptic and perisynaptic release in serotonergic neurons.

Key words: Serotonin, synapse, calcium-induced calcium release, facilitation, endoplasmic reticulum.

RESUMEN

En este trabajo se estudió la participación que tiene la liberación de calcio del retículo endoplásmico en la liberación de serotonina en terminales sinápticas. Los experimentos se llevaron a cabo en sinapsis formadas en cultivo entre neuronas serotonérgicas de Retzius y neuronas mecanosensoriales sensibles a presión, aisladas del Sistema Nervioso Central de la sanguijuela. En esta preparación la estimulación con pares de impulsos produjo facilitación sináptica. La estabilización de los receptores de rianodina en un estado de sub-conductancia por la incubación con rianodina 100 µM produjo un alargamiento del potencial sináptico en respuesta a impulsos presinápticos, sugiriendo que el calcio liberado por estos canales puede alcanzar las vesículas y promover la secreción. En contraste, el vaciamiento de los depósitos intracelulares de calcio con tapsigargina 500 nM produjo una disminución gradual de la facilitación sináptica ante impulsos presinápticos pareados y abolió la liberación extrasináptica en el axón neuronal en respuesta a trenes de impulsos. Todo esto ocurrió sin cambios en las propiedades de la membrana postsináptica, lo cual sugiere que la liberación de calcio intracelular participa en un mecanismo de retroalimentación positiva que promueve la liberación presináptica y perisináptica en las neuronas serotonérgicas.

Palabras clave: Serotonina, sinapsis, liberación de calcio inducida por calcio, facilitación, retículo endoplásmico.

INTRODUCTION

Serotonin is a neurotransmitter and neuromodulator of great importance in the regulation of various physiological functions and behaviors in animals throughout the phylogenetic scale, including humans. For example, aggressive behavior and the establishment of social dominance are regulated by serotonin from crustaceans[1–3] to primates[4–7] where serotonin also regulates mood. Serotonin also regulates feeding,[8,9] sleep, attention,[10] anxiety,[11] circadian rhythms,[12] sexual behavior[13,14] and the generation of rhythmic motor patterns such as locomotion, mastication and breathing[14–17], among many others. In humans, changes in the serotonergic system are related to behavioral and neurological disorders including food, depression,[18] epilepsy,[19] schizophrenia,[20] and anxiety,[21] therefore, studying how its release is regulated in the nervous system could help developing treatments for this kind of pathology.

Serotonergic neurons secrete this monoamine from synaptic terminals,[22–24] where it acts as a neurotransmitter producing rapid and localized effects on postsynaptic terminals in fixed neural circuits, and also from extrasynaptic sites on soma,[25–30] axon and dendrites,[31] where it acts as a modulator causing slow and diffuse paracrine type effects.

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Received: October 8, 2013. Accepted: November 15, 2013.

Due to the anatomical complexity of the serotonergic neurons in vertebrates, the fine mechanisms of synaptic serotonin release have been studied mainly in synapses formed in culture between isolated identified neurons of the central nervous system of the leech *Hirudo medicinalis*.[32,33] The neurons in culture, being iso-potential, have considerable advantages for these studies. The basic mechanisms of release in these synaptic terminals are similar to those of conventionally studied synapses as the neuromuscular plate[34] and the squid giant synapses,[35,36] i.e., the release occurs in a quantum way, depending on calcium and on the presynaptic membrane potential.[37,38] In the active site of synaptic terminals the serotonin is released from clear synaptic vesicles, from which there is a pool ready to be released and a reserve pool.[39] Depending on the release probability and on the amount of available vesicles, there are short-term plasticity phenomena such as facilitation and depression.[40] As in many other synaptic terminals, clear vesicles in these terminals are surrounded by electron dense vesicles that also contain serotonin[39,41] and release it in presynaptic or extrasynaptic zones, also producing postsynaptic effects, although slower (Trueta and De-Miguel, in preparation).

Serotonergic terminals contain endoplasmic reticulum, which is an intracellular calcium pool.[42,43] The endoplasmic reticulum stores calcium in high concentrations due to the function of an ATPase that actively transports calcium from the cytoplasm to the lumen of the reticle against its concentration gradient.[42] The calcium of this intracellular reservoir may be released into the cytoplasm through channels called ryanodine receptors that open in response to moderate increases in cytoplasmic calcium concentration, producing the so-called calcium-induced calcium release (ICRC).[43] This mechanism plays a key role in secretion at the excitable endocrine cells[44,45] and in some neuronal types it has been shown that it contributes to the spontaneous release of neurotransmitter,[46–48] as well as to the synaptic facilitation[47] and to the release in response to trains of impulses.[49,50]

Furthermore, in some neurons that use electron dense vesicles, calcium release from intracellular pools is an important contribution to the increase in intracellular calcium concentration in response to the electrical activity[51] and it participate in the mobilization of these vesicles towards the membrane and its fusion with same in order to produce peptide secretion.[52–54] In the cell body of serotonergic Retzius neurons calcium the calcium-induced calcium release also has an important contribution to somatic secretion when producing mobilization of vesicles towards the membrane[55] (León-Pinzón et al. in preparation). The presence of endoplasmic reticulum cisternae in synaptic terminals of these neurons suggests that this calcium release mechanism could also participate in the regulation of synaptic release of serotonin, but this participation has not been studied. While synaptic secretion in response to a pulse is very fast and is unlikely to have a contribution from this calcium source, which is activated more slowly, calcium-induced calcium release could be involved in the mobilization of clear vesicles of the reserve pool and/or of the electron dense vesicles released in perisynaptic areas in response to repetitive electrical activity.

In the present work we study the participation of calcium-induced calcium release in the serotonin synaptic and perisynaptic release in response to pairs and trains of impulses. For this purpose we use synapses formed between serotonergic Retzius neurons and pressure mechanosensory neurons (P-cells), isolated from the Central Nervous System of the leech *Hirudo medicinalis* and making contact in culture. Presynaptic Retzius neuron was stimulated by the current injection with an intracellular electrode and the serotonin release was analyzed from synaptic potentials recorded in the postsynaptic P-cell. Calcium release from the endoplasmic reticulum was promoted using ryanodine, which stabilizes ryanodine receptors in a semi-open state, or was inhibited by using thapsigargin, which inhibits the calcium ATPase from the endoplasmic reticulum, resulting in the depletion of the intracellular pool through the passive calcium leak.

MATERIALS AND METHODS

Neuronal isolation and culture

The nerve ganglia chain forming the central nervous system of the leech was isolated and the ganglion capsules were opened using fine tweezers. After treatment with collagenase-dispase (2mg/ml), Retzius neurons (Rz) and pressure-sensitive neurons (P) were individually isolated by suction through a glass pipette. Neurons were seeded on culture dishes (Falcon, primary) covered with concanavalin-A, placing the axonal stump of the Retzius neuron in contact with the soma of P-neuron. Figure 1A shows an image of a pair of neurons Rz-P in culture. Neurons were recorded after 2-7 days in culture.

Intracellular recording

The presynaptic neuron was stimulated by injecting depolarizing current pulses through an intracellular microelectrode, with a 20-30 MΩ resistance, filled with 3M potassium chloride, using an intracellular amplifier (Getting, model 5) in balance bridge mode. Pulses are used with duration of 100 ms to produce two action potentials; or of 200 ms to produce trains of 5-6 action potentials. The synaptic potentials were recorded in the P-cell with a microelectrode filled with 2M cesium chloride. The signals were acquired and digitized with a Digidata 1322 converter (Axon Instruments) at 10 KHz for their subsequent analysis.

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Pharmacology

After recording synaptic potentials in control conditions, 100 μM ryanodine or 500 nM thapsigargin were added. Ryanodine, at the concentration we use, stabilizes the channel of the ryanodine receptors in a sub-conductance state, producing a slow release of calcium into cytoplasm.[56–58] Thapsigargin blocks ATPases that pump calcium within endoplasmic reticulum; as the passive calcium leak is not compensated, the pool gradually empties.

Iontophoretic application of serotonin

Microelectrodes that are similar to those used for intracellular recording were filled with a solution of 5-hydroxytryptamine-HCl 150 mM. To retain the serotonin in the electrode a constant direct current of -1 nA was applied to the electrode. The tip of the electrode was placed near the soma of the postsynaptic neuron P. To produce serotonin output, 2 nA positive current pulses were applied with duration of 200 ms.

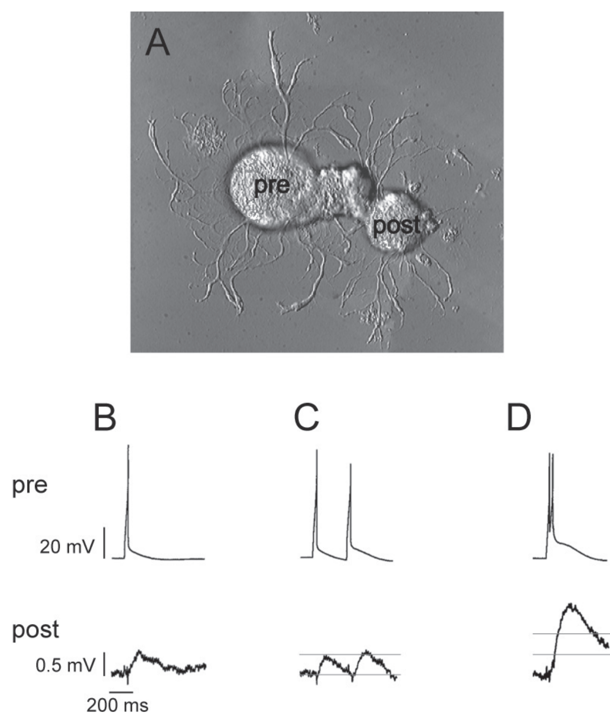

Figure 1. Facilitation in synapses between Retzius Neurons and P-Neurons in culture. **A**) Phase-contrast image of a pair of P-Retzius neurons being in contact in culture. **B-D**) Recording of the pre and post-synaptic membrane potential. The (pre) top traces show presynaptic action potential in the Retzius neuron and the (post) bottom traces show synaptic potentials recorded in P-neuron. In D, two action potentials with an interval of 30 ms caused facilitation. Note that the amplitude of the composite synaptic potential in D has an amplitude greater than the sum of the two individual synaptic potentials shown in C (marked by horizontal gray lines).

RESULTS

In pairs of neurons Rz-P (Figure 1A) that had formed synapses, the firing of an action potential by the presynaptic Retzius neuron evoked a potential synaptic in the postsynaptic neuron P (Figure 1B), produced by the serotonin presynaptic release. While a pair of action potentials produced at an interval of 300 ms or more in the presynaptic neuron produced two synaptic potentials of similar amplitude in the P-cell (Figure 1C), the reduction of this interval to 100 ms or less, produced synaptic facilitation, determined by the presence of a synaptic potential having greater amplitude than the sum of the two individual synaptic potentials (Figure 1D).

The synaptic knobs of the Retzius neurons contain endoplasmic reticulum cisternae at a distance greater than 500 nm of the active zones (see, for example, Figure 1 in reference [39]). Therefore, to study whether the release of calcium from such intracellular pools is involved in the neurotransmitter release, it was necessary to investigate if the calcium released through ryanodine receptors spreads to the sites where the vesicles are located. To this end, the postsynaptic responses were recorded after the presynaptic firing of simple action potentials, paired and in trains at a frequency of 20 Hz in control conditions and then the continuous output of calcium was induced from the endoplasmic reticulum into the cytoplasm, incubating pairs of neurons with 100 μM ryanodine, which stabilizes the channels of ryanodine receptors in a sub-conductance state.[56–58]

Under these conditions there was an elongation of 552 ± 174% in the decay phase of the synaptic potential, from 70.5 ± 28.9 ms in control conditions (n = 11; Figure 2A, left panel, black trace) to 389.7 ± 100.3 ms after five minutes (n=3, figure 2A, left panel, gray trace). To verify that this enlargement of the synaptic potential was not produced by a gradual decay of the state of the neurons over time, we recorded the postsynaptic responses of pairs of neurons that were not treated with ryanodine. In these cases, the average decay time of the synaptic potential increased only marginally during five minutes of recording (Figure 2B, black triangles) from 106.1 ± 21.6 ms to 131.2 ± 25.2 ms after five minutes of recording (n = 11).

The effect of ryanodine was most notable with pairs or trains of pulses in the presynaptic neuron (Figure 2). With paired pulses, the synaptic potential in the presence of ryanodine increased by 436 ± 168%, from 140.82 ± 19.3 ms to 689.2 ± 271.3 ms (n=5), while in control conditions it only changed from 168 ± 34.6 ms to 88.5 ± 28.1 ms (n=13). With trains of five or six pulses at 20 Hz, the ryanodine increased the decay time of the synaptic potential by 420 ± 105%, from 335.9 ± 116.7 ms to 1454.3 ± 325.8 ms (n=5), while in control conditions it changed from 392.7 ± 108 ms to 441.9 ± 100.1 ms (n=8).

To verify that the effects of ryanodine were not caused by changes in the electrical properties of the postsynaptic

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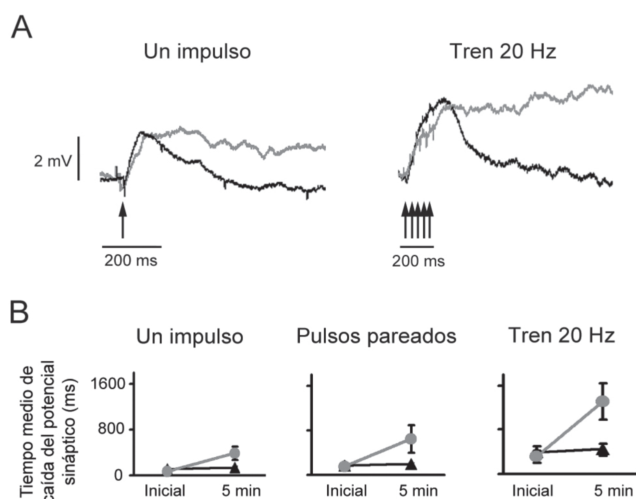

Figure 2. Calcium release through ryanodine receptors reaches the synaptic vesicles and promotes serotonin release. **A**) Synaptic potential recorded in the cell P, in response to an impulse (left) or a train of 6 impulses (right) in presynaptic Retzius cell under control conditions (black) and after 5 minutes of incubation with ryanodine 100 μM (gray). Stabilization of ryanodine receptors in a sub-conductance status prolonged ryanodine postsynaptic potential in the cell P. **B**) Average fall time (mean ± standard error, n=6 for each group) of the synaptic potential in response to an impulse, paired-pulse or trains of presynaptic impulses before (initial) and 5 minutes after addition of ryanodine 100 μM (gray circles). Control cells (black triangles) were recorded in the absence of ryanodine.

neuron, responses of P-cells were recorded before and after the application of the drug (Figure 3). Action potentials produced by depolarizing pulses in the postsynaptic neuron in control conditions (Figure 3A, black trace) and in the presence of ryanodine (Figure 3A, gray trace) were identical, discarding possible effects on the postsynaptic electrical activity induced by ryanodine. In addition, the input resistance and the time constant of the postsynaptic membrane to a pulse of hyperpolarizing current also remained unchanged (Figure 3B, black trace) in the presence of ryanodine (Figure 3B, gray trace). Moreover, the postsynaptic neuron's response to the application of serotonin via iontophoresis was neither altered by the ryanodine (Figure 3C), discarding changes in the postsynaptic sensitivity to serotonin after treatment with ryanodine. All this suggests that the calcium released from the endoplasmic reticulum through ryanodine receptors, reaches the release sites in the synaptic terminals and that it is capable of promoting fusion of the vesicles with the membrane.

To verify that the presynaptic electrical activity stimulates the calcium-induced calcium release and that this mechanism is involved in the release of serotonin, intracellular calcium pools were emptied using thapsigargin. Since such depletion occurs slowly, responses were recorded every five minutes for at least 30 minutes.

After 15 minutes of incubation with thapsigargin the generation of synaptic potentials was kept in response to a pulse, but the composite synaptic potential, in response to paired pulses decreased by 33%, showing a decrease in synaptic facilitation after 15 minutes of incubation with thapsigargin (Figure 4A). This was not caused by a declining status of neurons, since in registered pairs in the absence of thapsigargin the amplitude of the composite synaptic potential decreased only marginally even after 45 minutes of recording (Figure 4B).

This result suggests that the release of calcium from intracellular pools plays an important role in the facilitation of synaptic release of serotonin before the repetitive electrical activity.

Some pairs of Retzius-P neurons do not form synapses, given that no synaptic potentials are produced in response to a presynaptic impulse. However, subsequent stimulation

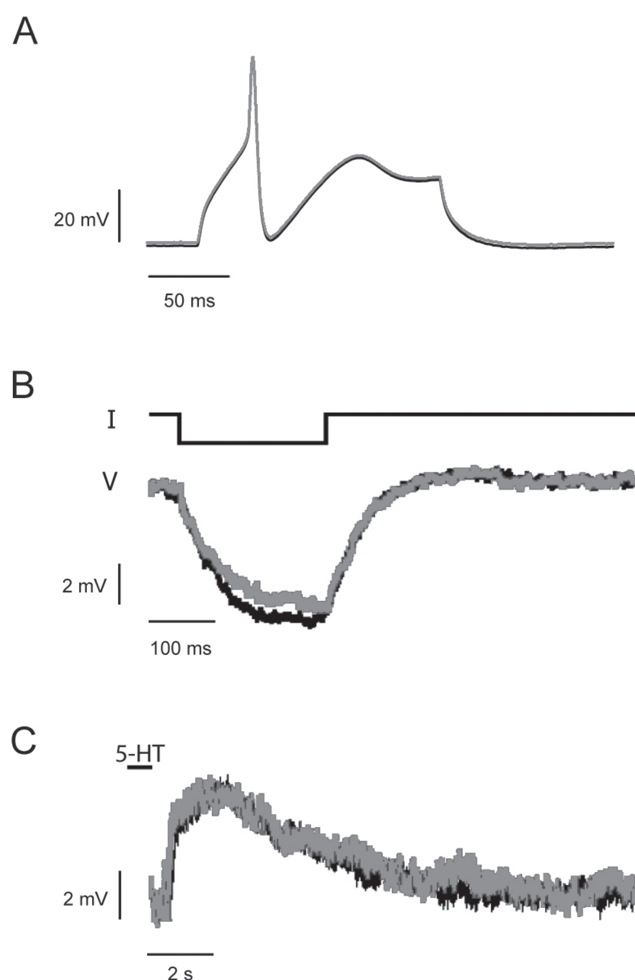

Figure 3. Ryanodine does not affect the properties of the membrane of the postsynaptic cell. Voltage response of P-cell to a depolarizing current pulse (**A**), to a hyperpolarizing current pulse (**B**) and to an iontophoretic serotonin pulse (5-HT, black bar), (**C**) before (black traces) and 10 minutes after addition of ryanodine 100 μM (gray traces). Ryanodine did not change the shape of the action potential, neither the input resistance nor the time constant of the membrane, nor the amplitude or time course of the response to 5-HT.

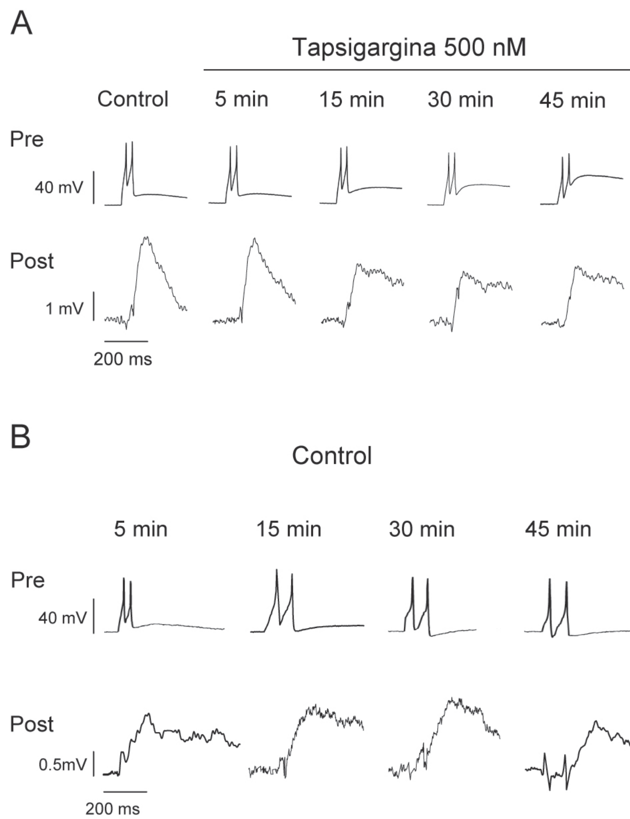

Figure 4. Calcium-induced calcium release promotes synaptic facilitation in response to paired-pulses. **A**) Recordings of pre-and postsynaptic membrane potential in response to application of presynaptic depolarizing pulses of 100 ms before (control) and during incubation with thapsigargin 500 nM. Note that after 15 minutes of incubation, the amplitude of the composite synaptic potential decreased. **B**) Control registers in the absence of thapsigargin, during the same time that the pairs of neurons with the drug were recorded. The amplitude of the synaptic potential was maintained along the recording.

produces a slow depolarization attributable to the release of serotonin from electron dense presynaptic vesicles (Figure 5A, Control). In such connections thapsigargin gradually decreased postsynaptic responses until abolishing them completely after 20 minutes of incubation (Figure 5A). However, in neurons pairs not treated with thapsigargin, responses were kept during 30 minutes (Figure 5B). As with ryanodine application, electrical responses from the postsynaptic neuron were not affected by incubation with thapsigargin (not shown). These results suggest that the release of calcium from intracellular pools is critical to produce the movement and fusion of electron dense vesicles in extrasynaptic release sites on the neuronal axon.

DISCUSSION

The results shown in this paper indicate that the calcium-induced calcium release modulates synaptic and extrasynaptic axonal release of serotonin. Elongation of synaptic potentials in pairs of neurons treated with ryanodine suggests that the calcium release from the endoplasmic reticulum through ryanodine receptors reaches synaptic vesicles and can participate in its delayed fusion with the membrane. Also decreased serotonin release after depletion of intracellular calcium pools suggests that the calcium-induced calcium release participates in serotonin release at the terminals in response to repetitive electrical activity.

Calcium release from the endoplasmic reticulum plays a key role in excitable endocrine cells[44,45] and also in the extrasynaptic release of neurotransmitters in the soma of several neuronal types, including Retzius neurons,[55,59–61] as well as in glial cells.[62] Particularly, the calcium-induced calcium release provides a positive feedback mechanism

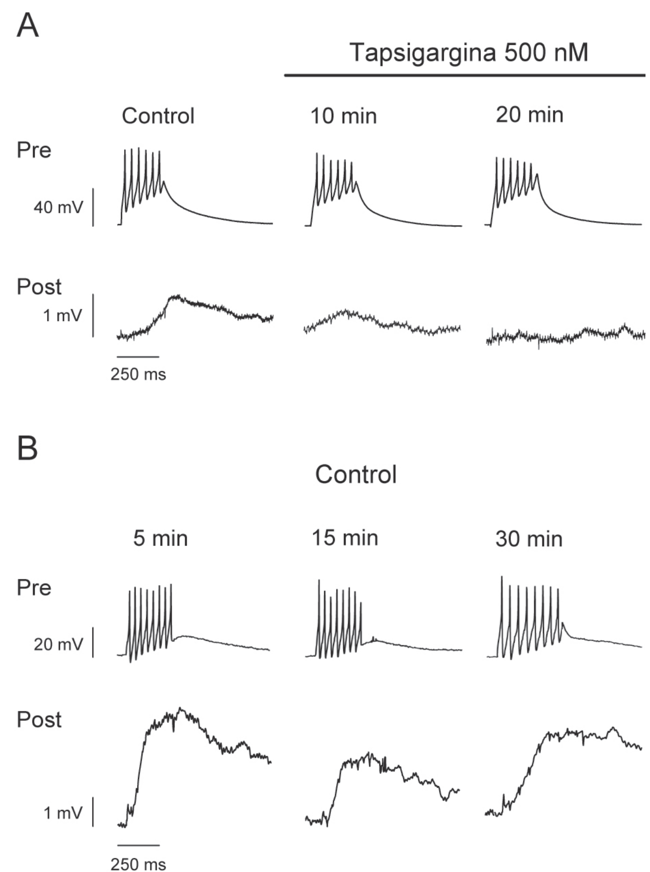

Figure 5. Calcium-induced calcium release is necessary for extrasynaptic release from electron dense vesicles. Recordings of pre-and postsynaptic membrane potential in response to application of presynaptic depolarizing pulses of 200 ms in neuron pairs where only extrasynaptic release occurs (note the slow postsynaptic response) considering trains of impulses. **A**) Responses in a pair of neurons before (Control) and during incubation with thapsigargin 500 nM. Note that the postsynaptic response was completely abolished after 20 minutes of incubation with thapsigargin. **B**) Responses in a pair of control neurons, in the absence of the drug. Note that the postsynaptic response was maintained throughout the entire recording.

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that amplifies the intracellular calcium signals caused by the transmembrane entry of this ion through voltage-dependent channels. Although in many neurons the presence of intracellular calcium pools has been described and even their participation in the generation of postsynaptic signals in dendrites,[63–66] their role in neurotransmitter release at the presynaptic terminals has been little studied. The presence of endoplasmic reticulum by the presynaptic terminals of Retzius serotonergic neurons suggests that functionally the calcium release of these compartments could have effects on the mobilization of vesicles and the release of serotonin, as happens in parasympathetic, cerebellar and hippocampal neurons, where calcium release from intracellular pools promotes transmitter release.[46,47,50] However, since endoplasmic reticulum cisternae are not very close to release areas and since calcium diffusion in cytoplasm is limited, it was necessary to determine first whether the calcium released from this pool reaches the release sites to be able to participate in exocytosis. Synaptic potential elongation in the presence of ryanodine suggests that when a calcium release occurs through ryanodine receptors, calcium may reach vesicles in the release sites and maintain the release of serotonin after electrical stimulation. Ryanodine stabilizes the channels of ryanodine receptors in a sub-conductance status where calcium may flow continuously, but at a slower rate than through the fully open channel. In various mammalian cell types, ryanodine, at the concentration used in this work, blocks ryanodine receptors, preventing the calcium-induced calcium release. However, leech neurons are little sensitive to many of the drugs used to block ion channels, therefore, it is not surprising that this high concentration of ryanodine does not block the channels, but stabilizes them in the semi-open status, as with ryanodine at lower concentrations in mammalian cells. Nevertheless, to verify this effect it is necessary to perform measurements of intracellular calcium signals in response to electrical stimulation in the presence of ryanodine in Retzius neurons.

Since ryanodine binds to its receptors in the inner part of the reticular membrane, it is necessary to stimulate the neurons electrically in order that the compound is introduced and it opens the channels. Thus it is expected that only calcium release occurs after the electrical stimulation that produces transmembrane calcium entry and then an increase in the cytoplasmic calcium concentration sufficient to activate ryanodine receptors. Furthermore, the effect of ryanodine is observed tens of milliseconds after the action potential and it is most evident in response to repetitive stimulation, presumably because the calcium entering via the membrane needs to be diffused up to the endoplasmic reticulum to be able to activate the ryanodine receptors. Once this occurs, the calcium released also takes time to diffuse to the active region. In the case in which the calcium evokes the release of electron dense vesicles, the scheme is more complex and delayed as these vesicles are not in contact with the plasma membrane and their fusion requires prior transport to the membrane, thereby adding a delay to the process.

In various preparations which have been used to study the mechanisms of synaptic neurotransmitter release, facilitation is produced by the residual calcium remaining in the terminal after a nerve impulse, when a second impulse occurs before calcium concentration has returned to its basal levels.[34] This is the case of Retzius neurons hereby studied.[40] The amount of adding residual calcium to calcium entering the membrane in response to a second pulse increases the probability of neurotransmitter release,[67] resulting in the fusion of a greater number of vesicles with the membrane in response to the second impulse. The gradual decrease of the facilitation herein noted after draining the pool through the incubation with thapsigargin suggests that the calcium-induced calcium release contributes to synaptic facilitation. This could occur if the calcium released from the endoplasmic reticulum is added to residual calcium, helping to increase the probability of release. It is also possible that the calcium released from intracellular pools promotes the mobilization of vesicles from the reserve pool to the releasable pool, increasing the number of vesicles available for release.

The inhibition produced by the thapsigargin of postsynaptic responses in those cases where no synapses were formed and only perisynaptic release occurs, suggests that calcium release from intracellular pools is needed to produce the movement of electron dense vesicles towards extrasynaptic release sites and their fusion with the membrane. The involvement of calcium-induced calcium release in the mobilization of electron dense vesicles also occurs in the soma of Retzius neurons themselves during somatic secretion,[55] as well as in other preparations where the release occurs from this type of vesicles.[53,68] It is possible that the calcium release from the endoplasmic reticulum also participates in the fusion process of the electron dense vesicles with the plasma membrane, as the transmembrane influx of calcium in response to electrical stimulation possibly ends before these vesicles reach the membrane, whereas the intracellular calcium release is slower, but suitable to the time course of mobilization and fusion of this type of vesicles.

The depletion of intracellular calcium pools using thapsigargin is one of the most commonly used manipulations to eliminate the contribution of these pools and to study the role they perform in several physiological phenomena.[42] Since depletion occurs slowly, it was necessary to record the postsynaptic responses during tens of minutes, which allows the possibility that the status of neurons diminishes over time. However, control neurons that were recorded in the absence of the drug showed no significant decay in the amplitude of synaptic potentials in 30 minutes of recording, suggesting that the effects observed in neurons treated with thapsigargin were specifically produced by the drug when emptying calcium pools.

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Since the decrease in the amplitude of the postsynaptic responses in the presence of thapsigargin occurred in about 15 minutes and no change was observed thereafter, it seems that the depletion of calcium intracellular pools was completed during this time.

The effects of calcium release from the endoplasmic reticulum apparently are purely presynaptic effects upon the release of the neurotransmitter and not upon the sensitivity of the postsynaptic membrane to serotonin or upon their electrical properties, since the electrical characteristics of the postsynaptic P-neurons and their sensitivity to serotonin were not affected by ryanodine or thapsigargin.

In summary, the results shown in this paper suggest that the calcium-induced calcium release participates in a positive feedback mechanism that increases neurotransmitter release and offer a complementary explanation to the hypothesis of residual calcium.

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Translation of the original version published in spanish in:
Salud Mental 2014, Vol. 37 Issue No. 2.

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Declaration of conflict interest: None

Translation of the original version published in spanish in:
Salud Mental 2014, Vol. 37 Issue No. 2.