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Cholesterol as a key player in the balance of evoked and spontaneous glutamate release in rat brain cortical synaptosomes

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ABSTRACT

Membrane rafts are domains enriched in sphingolipids, glycolipids and cholesterol that are able to compartmentalize cellular processes. Noteworthy, many proteins have been assigned to membrane rafts including those related to the control of the synaptic vesicle release machinery, which is a important step for neurotransmission between synapses. In this work, we have investigated the role of cholesterol in key steps of glutamate release in isolated nerve terminals (synaptosomes) from rat brain cortices. Incubation of synaptosomes with methyl-β-cyclodextrin (MβCD) induced glutamate release in a dose-dependent fashion. HγCD, a cyclodextrin with low affinity for cholesterol, had no significant effect on spontaneous glutamate release. When we evaluated the effects of MβCD on glutamate release induced by depolarizing stimuli, we observed that MβCD treatment inhibited the KCl-evoked glutamate release. The glutamate release induced by MβCD was not altered by treatment with EGTA nor with EGTA-AM. The KCl-evoked glutamate release was no further inhibited when synaptosomes were incubated with MβCD in the absence of calcium. We therefore investigated whether the cholesterol removal by MβCD changes intrasynaptosomal sodium and calcium levels. Our results suggested that the cholesterol removal effect on spontaneous and evoked glutamate release might be upstream to sodium and calcium entry through voltage-activated channels. We therefore tested if MβCD would have a direct effect on synaptic vesicle exocytosis and we showed that cholesterol removal by MβCD induced spontaneous exocytosis and inhibited synaptic vesicle exocytosis evoked by depolarizing stimuli. Lastly, we investigated the effect of protein kinase inhibitors on the spontaneous exocytosis evoked by MβCD and we observed a statistically significant reduction of synaptic vesicles exocytosis. In conclusion, our work shows that cholesterol removal facilitates protein kinase activation that favors spontaneous synaptic vesicles and consequently glutamate release in isolated nerve terminals.

1. Introduction

Lipid rafts are cholesterol and sphingolipid enriched membrane microdomains that segregate proteins on plasma membrane (Simons and Ikonen, 1997). These microdomains were renamed to membrane rafts since they are associated to signaling proteins such as ion channels, membrane receptors and transporters (Pike, 2006). During the last decade, many studies performed in nerve cells revealed that membrane raft plays an important role in neurotransmission by regulating synapse function in the central and peripheral nervous system. Indeed, in a pioneering work, Zamir and Charlton (2006) revealed that cholesterol depletion by methyl-β-cyclodextrin (MβCD) blocked the action potential-evoked transmission at crayfish neuromuscular junction. These authors also observed that MβCD treatment increased the spontaneous transmitter release, which involves some, but not all the steps in Ca^{+2} triggered exocytosis. Using another preparation, Wasser et al. (2007) described that cholesterol depletion of synaptic vesicle with MβCD impaired evoked neurotransmission and augmented spontaneous fusion rate at hippocampal neurons.

In summary, there are studies that investigated the role of cholesterol in presynaptic mechanisms that govern neurotransmission using diverse models such as the neuromuscular junction (Zamir and Charlton, 2006; Petrov et al., 2010), neurons in culture (Wasser et al., 2007; Frank et al., 2008; Linetti et al., 2010; Smith et al., 2010) and rat brain synaptosomes (Gil et al., 2005; Borisova et al., 2009, 2010; Tarasenko et al., 2010). However, to our knowledge, none of them have investigated how acute cholesterol removal by MβCD affects spontaneous and evoked glutamate

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release, intracellular calcium and sodium concentrations and synaptic vesicle exocytosis in isolated nerve terminal in the same experimental condition. To address this question, we therefore used rat brain synaptosomes and fluorimetric assays to investigate the effects of the cholesterol removal agent MβCD on key steps that regulate glutamate release.

Glutamate is the major excitatory neurotransmitter in the mammalian central nervous system and plays a dominant role in fast neurotransmission in the mammalian brain. Due to pathological release of glutamate during brain ischemia and neurodegenerative disorders, many studies have been conducted with the aim to understand the presynaptic mechanisms governing the release of this neurotransmitter. In this work, we have provided new evidences supporting the role of cholesterol as an essential component that control glutamate release machinery, more specifically, by restraining spontaneous vesicular release.

2. Material and methods

2.1. Chemicals

Glutamate dehydrogenase type II (GDH), $NADP^+$, glutamate, fura-2,sucrose, Percoll, sodium dodecyl sulfate (SDS), Ethylene glycol bis(2-aminoethylether)-N,N,N′,N′-tetraacetic acid (EGTA), Hepes, methyl-β-cyclodextrin (MβCD), KN93, Calphostin C and hidroxi-propil-γ-cyclodextrin (HγCD) were obtained from Sigma Aldrich (Labsciences). FM2-10, SBFI-AM and acetoxymethyl ester (Fura2-AM) were purchased from Invitrogen ™. PKI was purchased from Santa Cruz Biotechnology. The Kit LDH Liquiform was obtained from Labtest Diagnóstica S. A. All other reagents were of analytical grade obtained from commercial sources.

2.2. Purification of synaptosome

In our experiments, synaptosomal preparations were obtained from adult Wistar rats of both sexes (180–200 g) which were decapitated and had their cortices removed and homogenized 1:10 (w/v) in 0.32 M sucrose solution containing dithiothreitol (0.25 mM) and EDTA (2 mM). Then, homogenates were submitted to low-speed centrifugation (1000*g* for 10 min) and synaptosomes were purified from the supernatant by discontinuous Percoll-density gradient centrifugation (Dunkley et al., 1988) with small modifications (Romano-Silva et al., 1993). The isolated nerve terminals were ressuspended in Krebs–Ringer-Hepes solution (KRH; 124 mM NaCl, 4 mM KCl, 1.2 mM MgSO4, 10 mM glucose, 25 mM Hepes, pH 7.4) with no $CaCl_2$ to a concentration of 10 mg/ml, divided into aliquots of 200 μl and kept in ice until loaded with fura-2AM, SBFI or used for glutamate release or exocytosis assays. All experimental procedures were approved by the local ethics committee (CETEA) and performed according to their guidelines. In addition, all efforts were made to minimize animal suffering and to reduce the number of animals per experiment.

2.3. Measurement of continuous glutamate release

Glutamate release was accessed by continuous fluorimetric assay described by Nicholls et al. (1987). Briefly, synaptosomes (10 mg/ml) were incubated for 60 min, washed with KRH medium, and transferred to a cuvette (final synaptosomal concentration of 0.5 mg/ml to 1 mg/ml) at 37 °C with constant stirring. At the beginning of each assay, $CaCl_2$ (1 mM), $NADP^+$ (1 mM) and glutamate dehydrogenase (50 units) were added to synaptosomes. By following the increase in fluorescence due to the production of NADPH in the presence of glutamate dehydrogenase and $NADP^+$, we could continuously measure the amount of glutamate released. Fluorescence emission was recorded using a fluorimeter (Shimadzu RF-5301PC) at 450 nm and the excitation wavelength was set at 360 nm. $CaCl_2$ was omitted from experiments performed in the presence of 1.0 mM EGTA for the purpose to assess the calcium-independent glutamate release. Synaptosomes were previously incubated with MβCD (0.05–10 mM) during different time periods (5–30 min) (not shown). We chose the time period of 10 min for all the experiments described in this article. In experiments with HγCD, the synaptosomal suspensions were incubated with the drug for 10 min at concentrations of 1 and 10 mM. Glutamate release was evoked by depolarizing stimuli KCl 33 mM.

2.4. Measurements of intrasynaptosomal free Ca^{2+} concentrations

Intrasynaptosomal free Ca^{2+} concentrations were measured by the fluorescent calcium dye Fura-2 AM using a protocol described by Prado et al. (1996). Synaptosomal suspensions (10 mg ml^{-1}) were incubated for 50 min (37 °C) with fura-2 AM (stock solution 1 mM in DMSO), to a final concentration of 5 μM and then diluted with KRH. After a further 10 min incubation period, Fura-2 labeled synaptosomes were washed, ressuspended in medium (1 mg ml^{-1}) and immediately used for ratiometric quantification of intracellular free calcium ($[Ca^{2+}]i$) in the spectrofluorimeter. Fluorescence emission was recorded at 500 nm at an excitation ratio of 330/380. Calibration of Fura-2 signals and estimation of synaptosomal auto fluorescence were performed as described by Prado et al. (1996). Calcium (2 mM, final concentration) was added to the synaptosomal suspension 30 s after starting each assay. Calcium influx was evoked by depolarizing stimuli KCl 33 mM. Isolated nerve terminals were stirred throughout the experiment in a cuvette maintained at 37 °C. Synaptosomes were previously incubated with MβCD (1 and 10 mM) for 10 min.

2.5. Measurements of intrasynaptosomal sodium levels

Intracellular sodium measurements were performed according to Massensini et al. (1998). Synaptomes were loaded with SBFI in a similar fashion as described for Fura-2, except that 10 mM SBFI-AM was mixed to Pluronic® (1% final concentration) at 1:1 (v:v) before adding to the synaptosomal suspension. The medium was modified by replacing NaCl with choline chloride and the pH was set to 7.4 by addition of TRIZMA-base (1 M stock). After loading synaptosomes with the dye, the suspension was centrifuged and the pellets were ressuspended in 2.0 ml of KRH and transferred to the cuvette. At the end of each assay, gramicidin D was added to a final concentration of 2 mM to allow equilibration of $[Na^+]_I$ with $[Na^+]o$. Synaptosomes were previously incubated with MβCD (1 and 10 mM) for 10 min in aliquots tests.

2.6. Measurements of synaptic vesicle exocytosis with FM2-10

Measurements of synaptic vesicles exocytosis was performed using FM2-10, a fluorescent styryl dye from the FM family introduced by Betz et al. (1996). Synaptosomal suspension (500 μl) was diluted to 1.0 ml with KRH medium in a stirred cuvette and incubated with FM2-10 (50 μM) and 1.3 mM calcium for 3 min at 37 °C according to De Castro et al. (2008). Synaptosomes were stimulated with KCl 33 mM, washed with two short 10 s spins to remove externally bound dye, and ressuspended in 2 ml of fresh KRH. Labeled vesicles were released during a second round of vesicle cycling, stimulated with KCl (33 mM) in KRH medium. We measured the decrease in fluorescence $[(F-F0)/F0]$ in synaptosomes that were excited at 488 nm and collecting at 570 nm. The aliquots were pre-incubated with MβCD (1 and 10 mM) or HPγCD (1 and 10 mM) before the second round of vesicle cycling and data collection in fluoremeter.

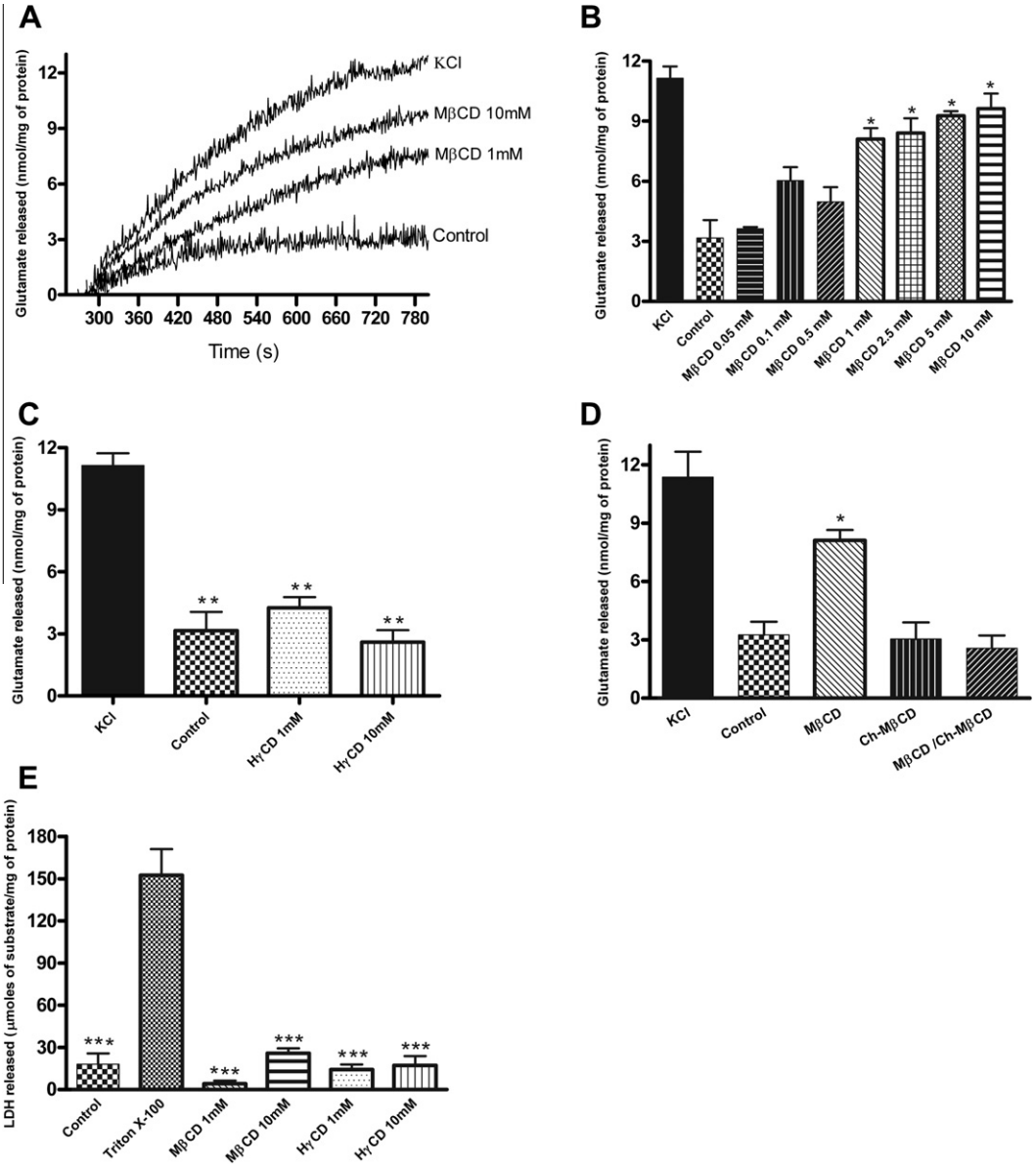

Fig. 1. MβCD increases glutamate release in a reversible, dose-dependent fashion. Synaptosomes were incubated with increasing concentrations of MβCD and the continuous release of glutamate was measured. (A) Representative traces of continuous glutamate release curves after treatment with KCl 33 mM, MβCD 1, or 10 mM. (B) Quantitative analysis of glutamate release induced by MβCD 0.05, 0.1, 0.5, 1, 2.5, 5 and 10 mM ($^{*}p < 0.05$ compared to control). (C) Incubation with HγCD (1 and 10 mM) does not alter glutamate release ($^{**}p < 0.05$ compared to KCl). (D) MβCD effect on glutamate release is reverted by cholesterol replenishment ($^{*}p < 0.05$ compared to control). When synaptosomes were incubated with Ch-MβCD (1 mM), after cholesterol depletion by MβCD (1 mM), glutamate release was similar to that obtained in control conditions. (E) Synaptosomes were incubated with MβCD (1 and 10 mM) or HγCD (1 and 10 mM) for 10 min and the release of lactate dehydrogenase (LDH) was measured. The synaptosomal membrane rupture was obtained by incubating with Triton X-100 0.1%. The control represents the release of LDH in the absence of cyclodextrins and the Triton X-100 ($^{***}p < 0.05$ compared to Triton X-100). The results represent mean ± S.E.M of at least three independent experiments.

FM2-10 can interact with MβCD if they are used in the same solution at the same time so we established a new staining protocol for the experiments described in Fig. 6 based on the one described by Smith et al. (2010). Synaptosomal suspension (500 μl) was diluted to 1.0 ml with KRH medium in a stirred cuvette and incubated with MβCD (1/10 mM) for 10 min at 37 °C. The suspension was washed for 30 s and ressuspended to 1 ml with KRH medium and then incubated with FM2-10 (50 μM) and 1.3 mM calcium for additional 10 min at 37 °C. The synaptosomes were then washed 3 times for 30 s with KRH Medium plus albumin to remove externally bound dye, and ressuspended in 2 ml of fresh KRH. Labeled vesicles were released during a second round of vesicle cycling, stimulated with MβCD (1 mM or 10 mM) in KRH medium. The kinase inhibitors were added just before the second round of vesicle cycling and data collection was performed essentially as described above.

2.7. Lactate dehydrogenase assay

Lactate dehydrogenase (LDH) release was monitored in order to evaluate the integrity of synaptosomal preparations after incubation with MβCD (1 and 10 mM) and HγCD (1 and 10 mM) during 10 min. The LDH activity in the incubation medium and the total LDH activity, which was determined by synaptosomal preparations disruption using 0.1% Triton X-100, were assayed using the LDH Liquiform kit (Labtest Diagnóstica S. A. Brazil). LDH activity was determined by spectrophotometric measurement at 340 nm of NADH (360 μM) oxidation in the presence of excess pyruvate

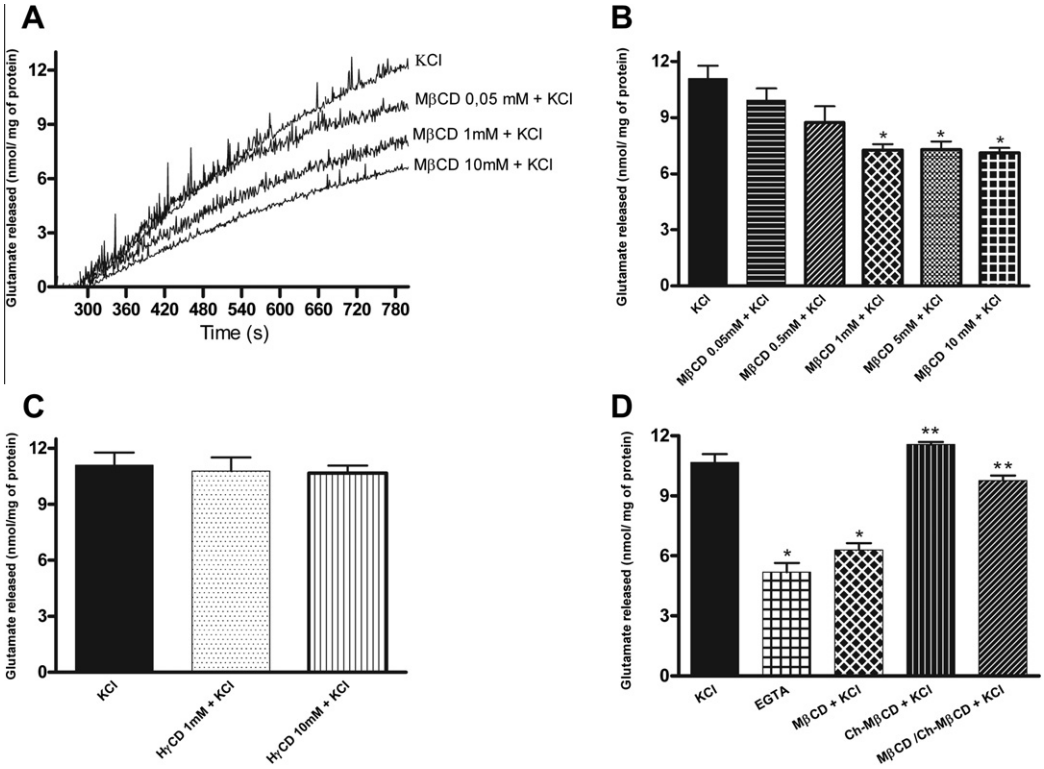

Fig. 2. MβCD inhibits glutamate release evoked by KCl depolarization. Synaptosomes were depolarized with 33 mM KCl and the continuous release of glutamate was followed. (A) Representative traces of continuous glutamate release curves after treatment with MβCD 0.05, 1 and 10 mM in the presence of KCl 33 mM. (B) Quantitative analysis of KCl-evoked glutamate release when synaptosomes were incubated with MβCD 0.05, 0.5, 1, 5 and 10 mM (*$p < 0.05$ compared to KCl). (C) Incubation with HγCD 1 and 10 mM does not interfere with KCl-evoked glutamate release. (D) MβCD effect on evoked glutamate release can be reverted by cholesterol replenishment. Incubation with MβCD (1 mM) decreases the evoked glutamate release (*$p < 0.05$ compared to KCl). When membrane cholesterol was replaced by Ch-MβCD (1 mM) after cholesterol depletion by MβCD (1 mM), glutamate release was reestablished to levels similar to that obtained by KCl alone (**$p < 0.05$ compared to MβCD + KCl). The results represent mean ± S.E.M of at least three independent experiments.

(6 mM) at 37 °C for 1 min. The control represents LDH release in the absence of cyclodextrins or Triton X-100.

2.8. Statistical analysis

Results shown in this work represent the mean of at least three independent experiments. Statistical significance was accessed by analysis of variance (ANOVA). A value of $p < 0.05$ was considered to be statistically significant.

3. Results

It is well established that proteins involved in vesicular neurotransmitter release, such as SNARE proteins and voltage-gated calcium channels, are found in membrane rafts (Gil et al., 2005; Taverna et al., 2004). These data prompted us to investigate the effect of MβCD on continuous glutamate release from rat brain synaptosomes. Initially, we tested if different concentrations of MβCD, a cholesterol scavenger agent that remove cholesterol from the plasma membrane, interfere with spontaneous glutamate release measured by a fluorimetric assay (see Section 2). When synaptosomes were incubated with increasing concentrations of MβCD (1, 2.5, 5 and 10 mM), we observed an increase in glutamate release (Fig. 1A and B). We next performed two control experiments to test if the glutamate release induced by MβCD was due to cholesterol removal from the plasma membrane. First, synaptosomes were incubated with HγCD, a cyclodextrin with low affinity for cholesterol and Fig. 1C shows that HγCD (1 and 10 mM) had no significant effect ($p > 0.05$) on spontaneous glutamate release. We next treated the cholesterol depleted synaptosomal preparation with cholesterol conjugated with MβCD (Ch-MβCD-1 mM). Fig. 1D shows that cholesterol removal with MβCD (1 mM) was reversible. We also evaluated the integrity of synaptosomal preparations after incubation with MßCD (1 and 10 mM) and HγCD (1 and 10 mM) during 10 min. The graph in Fig. 1E shows that the spontaneous glutamate release induced by removal of cholesterol occurred not by a non-specific leakage, since the release of LDH induced by MβCD was very low and not statistically different from control.

We next investigated the effects of MβCD on glutamate release induced by depolarizing stimuli. Synaptosomes were treated with MβCD (1, 5 and 10 mM) and then depolarized with KCl (33 mM). Fig. 2A and B show that MβCD (1, 5 and 10 mM) inhibited the KCl-evoked glutamate release. By contrast, incubation with HγCD 1 and 10 mM, had no significant effect on glutamate release evoked by depolarizing stimulus (Fig. 2C). The MβCD effect on evoked glutamate release could be reverted by cholesterol replenishment (Fig. 2D).

Thiele et al. (2000) have pointed out that MβCD (3.8 mM) caused a 60–70% cholesterol depletion in PC12 cells and that higher concentrations resulted in changes in cell morphology that could account for unspecific effects of the drug. Therefore, to minimize secondary effects, for the next set of experiments, we chose two doses of MβCD (1 and 10 mM) and compared the effects of both.

Since voltage-gated calcium channels are concentrated in membrane rafts (Taverna et al., 2004), we next investigated if the MβCD

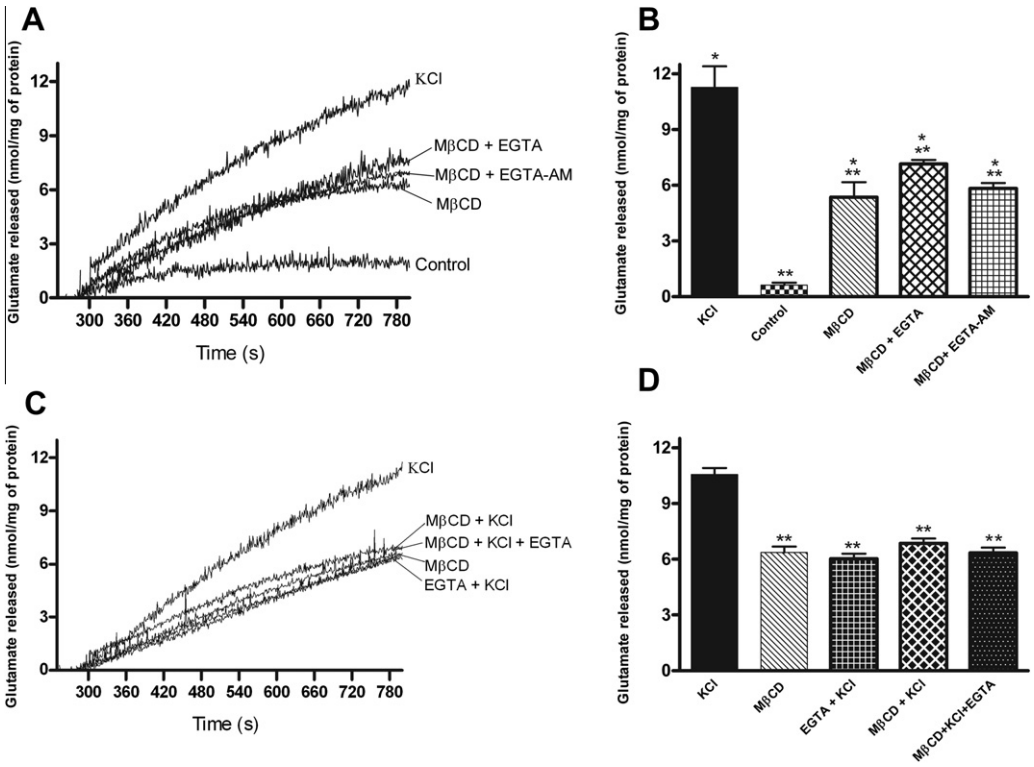

Fig. 3. MβCD effects on glutamate release in the absence of calcium. (A) Representative traces of continuous glutamate release curves after treatment with MβCD 1 mM in the presence of EGTA (2 mM) or EGTA-AM (50 μM). (B) EGTA (2 mM) or EGTA-AM (50 μM) did not change glutamate release induced by MβCD 1 mM (*$p < 0.05$ compared to control; **$p < 0.05$ compared to KCl). (C) Representative traces of continuous glutamate release curves after treatment with MβCD 1 mM in the presence of EGTA (2 mM) and KCl (33 mM). (D) EGTA inhibited KCl-evoked glutamate release and treatment with MβCD (1 mM) did not induce any further inhibition (**$p < 0.05$ compared to KCl). The results represent mean ± S.E.M of at least three independent experiments.

effects on spontaneous and evoked glutamate release were calcium dependent. Synaptosomes were incubated with EGTA (2 mM) or EGTA-AM (50 μM), an extracellular and intracellular calcium chelator respectively. The glutamate release induced by MβCD (1 mM) was not changed by treatment with EGTA nor with EGTA-AM (Fig. 3A and B).

We next asked if MβCD inhibition of KCl-evoked glutamate release was calcium-dependent. Fig. 3C and D shows that EGTA and MβCD inhibited in approximately 42% and 35% respectively the calcium dependent component of KCl-evoked glutamate release. Interestingly, the KCl-evoked glutamate release was no further inhibited when synaptosomes were incubated with MβCD (1 mM) and EGTA (Fig. 3D). These findings suggested that cholesterol removal by MβCD interferes with the exocytotic (calcium-dependent) component of KCl-evoked glutamate release without interfering with the transporter-mediated (calcium-independent) component.

We measured intrasynaptosomal calcium concentration using the fluorescent probe Fura 2-AM. Fig. 4A shows representative traces of changes in intracellular calcium concentration. Synaptosomes labeled with Fura 2-AM were exposed to MβCD (1 or 10 mM) and these treatments did not induce significant changes in basal or KCl-evoked increase in intrasynaptosomal calcium concentration (Fig. 4A and B). These data suggest that the MβCD actions on spontaneous or evoked glutamate release might not be due to an effect on voltage-gated calcium channels.

Since sodium influx through voltage-activated sodium channels is an essential step to neurotransmission, we investigated whether cholesterol removal by MβCD induced changes in intrasynaptosomal sodium levels Fig. 4C shows that treatment with MβCD (1/10 mM) did not induce significant changes in basal sodium levels. Veratridine, an alkaloid that activates sodium channels, triggered an increase in intrasynaptosomal sodium levels compared to basal (Fig. 4C). The sodium levels increase evoked by veratridine did not change during treatment with MβCD (1 or 10 mM) (Fig. 4C and D). These results suggest that the inhibitory effect of cholesterol removal on glutamate release evoked by KCl might be upstream to sodium and calcium entry through voltage-activated channels.

We next investigated if MβCD would have a direct effect on synaptic vesicle exocytosis that would bypass sodium and calcium influx through voltage-gated channels. Rat brain synaptosomes were stained with the fluorescent dye FM2-10 and then incubated with MβCD (see material and methods). Fig. 5A shows representative FM2-10 destaining curves. The control curve reflects exocytosis of synaptic vesicles that are fusing spontaneously and we observed that cholesterol removal by MβCD (1 mM) induced spontaneous synaptic vesicles exocytosis that was not statistically different from control (Fig. 5A and B). By contrast, exocytosis evoked by KCl (33 mM) alone was inhibited in approximately 26% inhibited when synaptosomes were pre-treated with MβCD (1 mM) (Fig. 5B). Taken together, these data suggested that cholesterol removal by MβCD (1 mM) inhibits synaptic vesicle exocytosis evoked by depolarizing stimuli and that the effect of MβCD on glutamate release might be due, at least in part, to an inhibition on exocytosis of glutamatergic vesicles.

Lastly, we performed experiments as an attempt to identify the mechanism by which MβCD increases spontaneous exocytosis. We first established a new FM2-10 staining protocol to measure exocytosis of synaptic vesicles that were labeled spontaneously after cholesterol removal. Fig. 6 A shows the destaining curves for synaptosomal preparations that were stained with FM2-10 following an incubation period in the presence of MβCD. The fluorescence decay evoked by MβCD was more pronounced when synaptic vesicles were labeled with FM2-10 spontaneously (after cholesterol

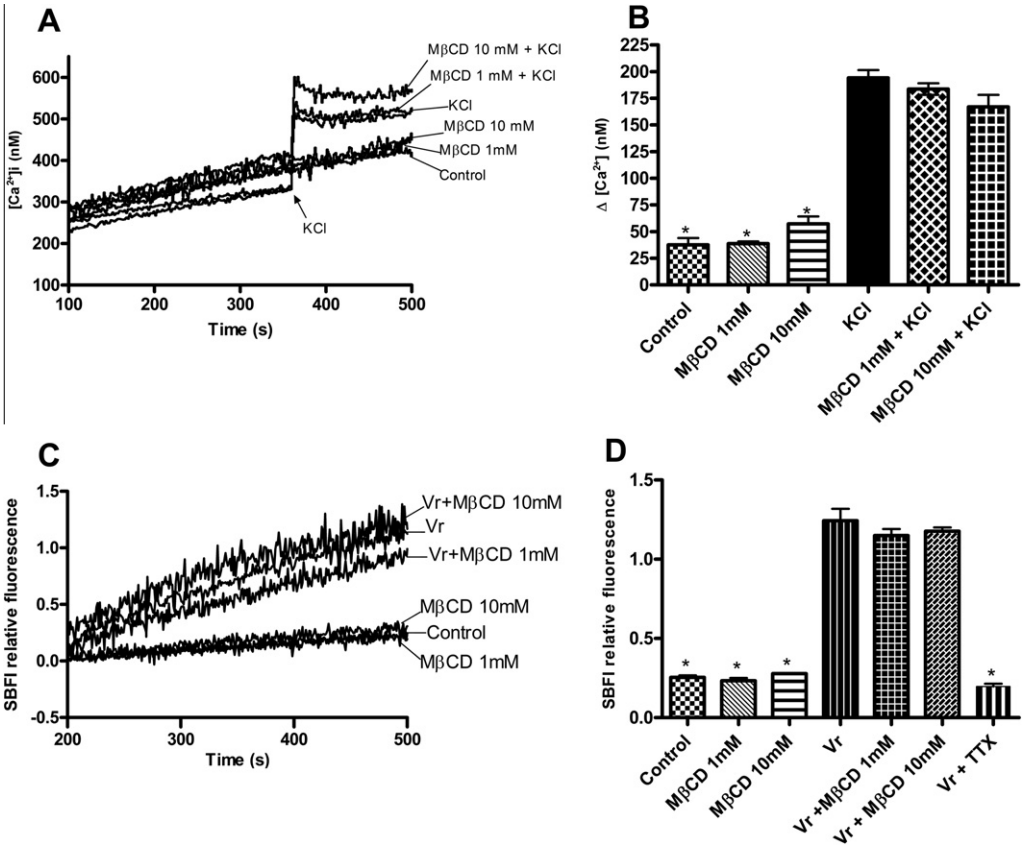

Fig. 4. Intrasynaptosomal sodium and calcium levels in the presence of MβCD. Synaptosomes were incubated with Fura 2-AM, followed by treatment with MβCD (1 and 10 mM). (A) Representative intracellular calcium concentration curves in the presence of MβCD (1 and 10 mM) alone or with depolarizing stimuli (KCl 33 mM). (B) Quantitative analysis of intrasynaptosomal calcium concentration for the conditions indicated (*$p < 0.05$ compared to KCl). The results represent mean ± S.E.M of at least three independent experiments. (C) Synaptosomes were incubated with the sodium indicator SBFI (10 μM). MβCD 1 and 10 mM were added to synaptosomal suspension and intrasynatopsomal sodium variation was obtained in the presence and absence of Veratridine 10 μM. Representative curves of increase in SBFI fluorescence in the presence of MβCD (1 and 10 mM). (D) Quantitative analysis of intrasynaptosomal sodium levels for the conditions indicated (*$p < 0.05$ compared to Vr). The results represent mean ± S.E.M of at least three independent experiments.

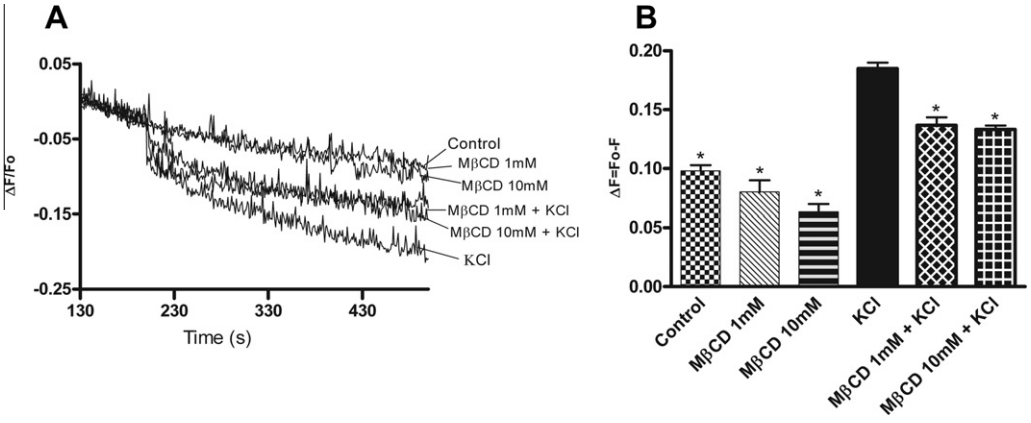

Fig. 5. MβCD effects on synaptic vesicles exocytosis. Synaptosomes were labeled with FM2-10 (see Methods) and then incubated with MβCD for 10 min. The release of FM2-10 was monitored in the presence and absence of KCl (33 mM). (A) Representative FM2-10 destaining curves for control, MβCD (1 and 10 mM), KCl 33 mM, MβCD 1 mM + KCl 33 mM and MβCD 10 mM + KCl 33 mM). (B) Quantitative analysis of synaptic vesicle exocytosis for the conditions indicated. $\Delta F/F0$, normalized fluorescence (*$p < 0.05$ compared to KCl). The results represent mean ± S.E.M of at least three independent experiments.

removal by MβCD) (compare Fig. 5A and Fig. 6A). Considering that protein kinases are key signaling proteins that modulated synaptic response, we used several selective protein kinase inhibitors, to determine whether kinases regulating neurotransmitter release were activated by cholesterol depletion. To determine the identity of kinases mediating the increase in spontaneous exocytosis we assessed the ability of selective PKC, PKA and calcium/calmodulin kinase II (CaMKII) inhibitors to block the spontaneous exocytosis induced by cholesterol removal by MβCD. Pretreatment with the PKC inhibitor Calphostin C (1 μM) for 10 min prevented the spontaneous exocytosis induced by MβCD treatment (1 or 10 mM, Fig. 6A). Calphostin C by itself did not cause a significant increase

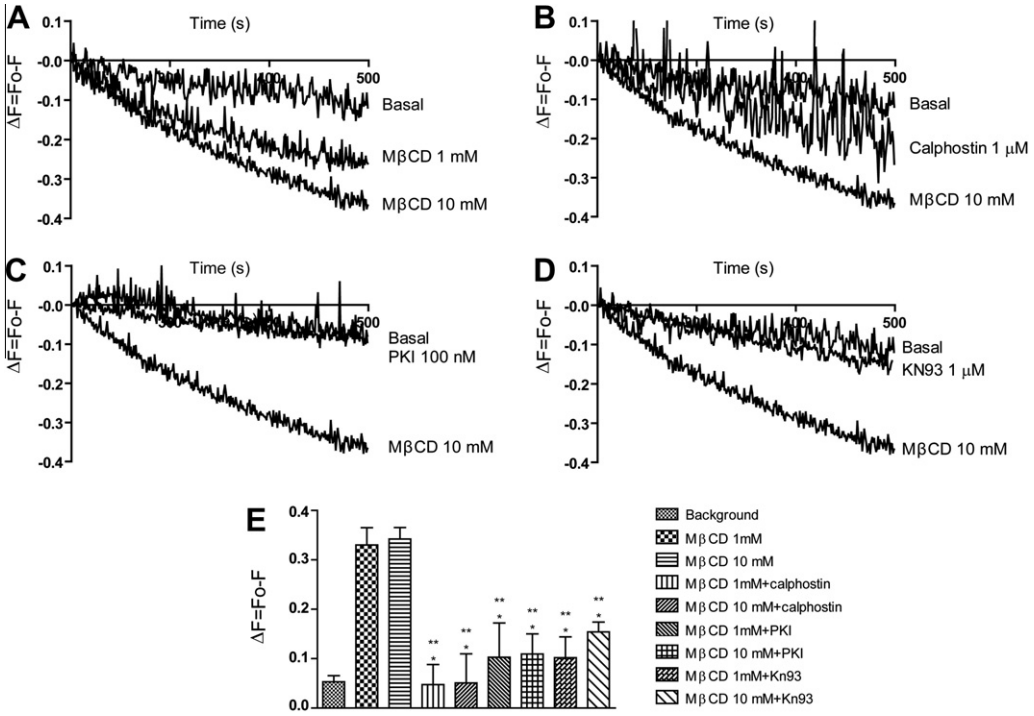

Fig. 6. Protein kinase inhibition affects the spontaneous exocytosis evoked by MβCD. Synaptosomes were incubated with MβCD for 10 min and then labeled with FM2-10 (see Section 2). (A) Representative FM2-10 destaining curves for control, MβCD (1 and 10 mM). (B–D) Representative FM2-10 destaining curves for control, MβCD (10 mM), Calphostin C (1 μM), PKI (100 nM) or KN93 (1 μM). (E) Quantitative analysis of synaptic vesicle exocytosis evoked by control, MβCD (1/10 mM) alone or in the presence of kinase inhibitors as indicated. ΔF/F0, normalized fluorescence ($^{*}p < 0.05$ compared to MBCD 1 mM and $^{**}p < 0.05$ compared to MBCD 10 mM). The results represent mean ± S.E.M of at least five independent experiments.

in exocytosis (Fig. 6B). Pretreatment with the PKA selective inhibitor PKI (100 nM) or with the CAMKII selective inhibitor KN93 (1 μM) significantly reduced the extent of the increase in exocytosis observed following cholesterol extraction when compared with control experiments performed in parallel (Fig. 6E). The kinase inhibitors PKI and KN93 did not cause a significant increase in spontaneous exocytosis by their selves (Fig. 6C and D).

Our results therefore demonstrate that spontaneous exocytosis induced by MβCD is dependent on protein kinase activation and we suggest that the effects of cholesterol depletion on synaptic transmission can be partly explained by increased presynaptic kinase activity.

4. Discussion

In the present study, we aimed to investigate, using the same experimental conditions, the effect of cholesterol removal on continuous glutamate release, intracellular sodium and calcium levels and synaptic vesicles exocytosis in rat brain synaptosomes.

Using MβCD we found that cholesterol removal from plasma membrane induced spontaneous glutamate release and reduced KCl-evoked release (Figs. 1 and 2). Such dual effect of MβCD is in accordance with the study performed by Zamir and Charlton (2006) showing that removal of cholesterol with MβCD increased the frequency of MEPPS (mini excitatory postsynaptic potentials) and also blocked the conductance of the action potential at the glutamatergic neuromuscular junction of crayfish. Moreover, Wasser et al. (2007) observed an increase in the frequency of spontaneous fusion events and a decrease in vesicular fusion, triggered by stimulation in hippocampal neuronal cultures treated with MβCD.

The dual effects of MβCD on glutamate release in synaptosomes described here suggest that cholesterol removal might interfere with the calcium-dependent (exocytotic) and/or calcium independent (transporter mediated) components of release (Fig. 2). We tested this paradigm by performing experiments in the presence or absence of calcium and measured glutamate release in synpatosomes pre-treated with MβCD (Fig. 3). We observed that the glutamate release induced by MβCD was not calcium-dependent, suggesting a putative effect on the glutamate transporter (Fig. 3A and B). Indeed, Borisova et al. (2009), (2010) described an increase in extracellular L-[^{14}C] glutamate radioactivity in synaptosomes treated with high concentrations of MβCD (15 mM). These authors suggested that this effect might be due in part to a significant reduction in the rate of glutamate uptake by synaptosomes. However, when we investigated the calcium-dependence of the inhibitory effect of MβCD on KCl-evoked glutamate release, we observed that only the transporter mediated component remained after such treatment (Fig. 3C and D) suggesting an effect exclusively in the exocytotic component of release. In fact, this was confirmed by experiments where we measured synaptic vesicle exocytosis by FM2-10 destaining (Fig. 6) and this increase in exocytosis by cholesterol reduction has also been previously shown in neurons in culture (Wasser et al., 2007; Linetti et al., 2010; Smith et al., 2010). Therefore, our data shows that the same might be applied for synaptosomes and that the discrepancy between our data and the one from Borisova et al. (2009), (2010) may be due to differences in the MβCD concentration (ten times lower in our study) and the technique to measure glutamate release. This inhibitory effect of cholesterol is essential for neurotransmission, since exceeding spontaneous fusion of synaptic function may affect the long-term synaptic function (Wasser et al., 2007). Moreover, unregulated release of excitatory neurotransmitters may lead to damage and neuronal death. High levels of glutamate are involved in several degenerative diseases such as Alzheimer's and Huntington's disease (Nishizawa, 2001; Hynd et al., 2004).

Since voltage-gated calcium channels are concentrated in membrane microdomains rich in cholesterol (Lang et al., 2001; Taverna et al., 2004) the alterations in glutamatergic release after cholesterol removal could be triggered by changes in the functioning and location of these channels. However, our results indicate no difference in intrasynaptosomal calcium concentration when cholesterol was removed by MβCD (Fig. 4). Zamir and Charlton (2006), using crayfish neuromuscular junctions as model, also reported that the increase in spontaneous exocytosis dependent MβCD detected by them is independent of calcium. In addition, Wasser et al. (2007) and Krysanova et al. (2007) showed that exocytotic release of aspartate and glutamate evoked by the calcium ionophores A23187 and ionomycin was also lower after the removal of cholesterol. Together these data indicate that the effects of MβCD cannot be explained by changes in calcium influx to the presynaptic terminal.

Proteins related to vesicular fusion with the plasma membrane, known as SNARES, are concentrated in membrane microdomains rich in cholesterol (Churchward et al., 2005; Gil et al., 2005; Lang et al., 2001; Taverna et al., 2004). Thus, alterations in spatial organization and/or physiology of these compounds can cause changes in neurotransmitter release in cholesterol deficiency. Our data, as well as several works mentioned above, indicate an increase in spontaneous neurotransmission during cholesterol deficiency. However, the increase in spontaneous release of glutamate detected by fluorescence (Fig. 1) was not accompanied by a major decrease in the fluorescence of FM2-10 in synaptosomes treated with MβCD, which may indicate an increase in spontaneous exocytosis of synaptic vesicles (Fig. 5B third column). At first glance, this fact may suggest that the increased spontaneous release by cholesterol removal might not have vesicular origin. However, Wasser et al. (2007) also demonstrated that in hippocampal neuronal cultures, the number of vesicles per synapse is increased during spontaneous release of neurotransmitters when cells were treated with MβCD, which suggests that there is an increase in fusion and recycling of synaptic vesicles after removal of cholesterol. In this direction, Fredj and Burrone (2009) demonstrated that the vesicular pool responsible for the spontaneous release of neurotransmitters is different from the one released after stimulation. Since the FM2-10, because of the intrinsic characteristics of this probe, stains only the vesicular pools that are effectively recycling in the terminal, the pool responsible for the spontaneous glutamate release, called ‘‘resting pool’’ by the these authors, would not be stained by the probe. To test this hypothesis, synaptosomes were treated with MβCD and then exposed to FM2-10. In this condition, the amount of destaining evoked by MβCD was higher than that obtained when KCl was used to label synaptic vesicles with the fluorescent dye, reinforcing the idea that a different pool of synaptic vesicles is recruited during spontaneous or evoked synaptic vesicles release (compare Fig. 5A and Fig. 6A). Therefore, we suggest that the resting pool could be one of the factors responsible for the increase in spontaneous glutamate release not accompanied by augmentation in spontaneous FM2-10 destaining (Fig. 5B, second column).

In many synapses, spontaneous transmitter release is controlled in part by various protein kinases and the activity of some of those kinases might be sensitive to changes of membrane cholesterol content (Burgos et al., 2004; Cabrera-Poch et al., 2004). Here we provided evidences that spontaneous exocytosis activity depends on PKA, PKC and CAMKII (Fig. 6E). Our results is in accordance with previous data from Charlton’s group (Smith et al., 2010) showing that in cerebellar neurons in culture, the rate of spontaneous neurotransmitter release is increased following cholesterol depletion by a mechanism requiring activation of presynaptic protein kinases. In addition, previous data obtained in different cell culture models also corroborate the hypothesis that cholesterol removal by MβCD might facilitate protein kinases activation. For example, in MDCK cells MβCD treatment activates PKA without increasing cAMP levels, possibly by disrupting inhibitory complexes localized to lipid rafts (Burgos et al., 2004). Activation of PKCε and src in MβCD-treated PC12 cells is also thought to be a consequence of alterations to localized lipid domain interactions (Cabrera-Poch et al., 2004). The precise nature of the phosphorylation event (s) linking cholesterol depletion to increased spontaneous exocytosis is not fully understood but the most plausible mechanistic explanation is that lipid raft disruption allows active kinases access to some component of the release apparatus and subsequent phosphorylation increases release probability.

5. Conclusion

In conclusion, this work shows the importance of membrane cholesterol for efficient neurotransmission. Acute removal of cholesterol not only alters the structure of the plasma membrane, but also acts on the functional state of synaptic vesicles therefore interfering with the neurotransmitter release machinery. Moreover, our results give additional evidences that cholesterol appears to be essential for the balance between spontaneous and evoked release and this involves protein kinase activation.

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