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Abstract

The hippocampal mossy fibers contain a substantial quantity of loosely-bound zinc in their glutamatergic presynaptic vesicles, which is released in synaptic transmission processes. Despite the large number of studies about this issue, the zinc changes related to short and long-term forms of potentiation are not totally understood. This work focus on zinc signals associated with chemically induced mossy fiber synaptic plasticity, in particular on postsynaptic zinc signals evoked by KCl depolarization. The signals were detected using the medium affinity fluorescent zinc indicator Newport Green. The application of large concentrations of KCl, 20 mM and 60 mM, in the extracellular medium, evoked zinc potentiations that decreased and remained stable after washout

of the first and the second media, respectively. These short and long-lasting enhancements are considered to be due to zinc entry into postsynaptic neurons. We have also observed that following established zinc potentiation, another application of 60 mM KCl only elicited further enhancement when combined with external zinc. These facts support the idea that the KCl-evoked presynaptic depolarization causes higher zinc release leading to zinc influx into the postsynaptic region.

Keywords: zinc; Newport Green (NG); mossy fiber synapses; hippocampal CA3 area

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Postsynaptic zinc potentiation elicited by KCl depolarization at hippocampal mossy fiber synapses

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Abstract

The hippocampal mossy fibers contain a substantial quantity of loosely-bound zinc in their glutamatergic presynaptic vesicles, which is released in synaptic transmission processes. Despite the large number of studies about this issue, the zinc changes related to short and long-term forms of potentiation are not totally understood. This work focus on zinc signals associated with chemically induced mossy fiber synaptic plasticity, in particular on postsynaptic zinc signals evoked by KCl depolarization. The signals were detected using the medium affinity fluorescent zinc indicator Newport Green. The application of large concentrations of KCl, 20 mM and 60 mM, in the extracellular medium, evoked zinc potentiations that decreased and remained stable after washout of the first and the second media, respectively. These short and long-lasting enhancements are considered to be due to zinc entry into postsynaptic neurons. We have also observed that following established zinc potentiation, another application of 60 mM KCl only elicited further enhancement when combined with external zinc. These facts support the idea that the KCl-evoked presynaptic depolarization causes higher zinc release leading to zinc influx into the postsynaptic region.

Keywords: Postsynaptic zinc, KCl depolarization, Newport Green (NG), mossy fiber synapses, hippocampal CA3 area

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Abbreviations: ACSF, artificial cerebrospinal fluid; AMPA, α-amino-3hydroxy-5-methyl-4-isoxazolepropionic acid; DMSO, dimethyl sulfoxide; KA, kainate; NMDA, N-methyl-D-aspartate;; NG, Newport Green;

Introduction

Zinc is one of the most important divalent cations that are present in the mammalian forebrain (Frederickson *et al.*, 2000; Sensi *et al.*, 2011). Only a small amount of zinc is concentrated in the presynaptic boutons of zinc-containing neurons (Frederickson, 1989), being the larger fraction of zinc found in metalloproteins, which form complexes with zinc with very high-affinity (Jacob *et al.*, 1998). One of the most important zinc releasable pools is found in hippocampal mossy fibers (Choi, *et al.*, 1998), which have large boutons and are located very close to the apical dendrites of CA3 neurons, suggesting that they are part of a uniquely strong synapse (Bischofberger *et al.*, 2006). Mossy fiber synapses sequester, accumulate and release zinc from their glutamatergic presynaptic vesicles that contain the zinc transporter ZnT-3, which pumps zinc into the vesicles and is expressed exclusively in the brain (Palmiter *et al.*, 1996; Frederickson *et al.*, 2005). The depolarization of zinc-containing neurons leads to calcium-dependent glutamate and zinc co-release via the exocytosis of their vesicles (Howell *et al.*, 1984; Perez-Clausell and Danscher, 1986). Large depolarizations, evoked by electrical or chemical stimulation, can result in the formation of long-term potentiation (LTP) (Bliss and Collingridge, 1993; Bortolotto and Collingridge, 1993). This form of synaptic plasticity consists of a long lasting enhancement of synaptic transmission and is considered to be involved in learning and memory processes in the brain (Malenka and Bear, 2004). LTP can be induced by high-frequency stimulation (tetanus) and also by the application of large amounts of extracellular potassium in hippocampal slices (Fleck *et al.*, 1992; Bernard *et al.*, 1994; Roisin *et al.*, 1997) and in dissociated neuronal cultures (Appleby *et al.*, 2011). Potassium-induced LTP shares some properties with tetanus-induced LTP in hippocampal CA1 area (Fleck *et al.*, 1992; Bernard *et al.*, 1994). For example, the population EPSP amplitudes had similar enhancements in both cases (Fleck *et al.*, 1992). Other forms of chemically-evoked LTP include the TEA-LTP (Suzuki and Okada, 2009) and also LTP induced by the application of 4-amino pyridine, mediated by the inhibition of voltage-dependent potassium channels, which causes significant cell depolarization (Bancila *et al.*, 2004). The depolarization associated with chemically-induced LTP may activate simultaneously all potentiable mossy fiber synapses (Zhao *et al.*, 2012). It was observed that the induction of tetanically-evoked mossy fiber LTP in CA3 hippocampal area, is accompanied by

significant zinc release from mossy fibers (Quinta-Ferreira *et al.*, 2004; Qian and Noebels, 2005; Quinta-Ferreira and Matias, 2005; Matias *et al.*, 2010). Thus, intense high-frequency stimulation causes an increase of zinc in the synaptic cleft, that may reach 10-100 μM, and also an enhancement of postsynaptic intracellular zinc (Vogt *et al.*, 2000; Li *et al.*, 2001a,b; Ueno *et al.*, 2002; Paoletti *et al.*, 2009). Potassium-induced depolarization evokes, as well, a postsynaptic zinc increase (Li *et al.*, 2001a,b; Ketterman and Li, 2008), which may, at least in part, be explained by zinc entry through voltage-gated calcium channels and calcium -permeable glutamate receptors, as observed applying exogenous zinc in cell cultures (Sensi *et al.*, 1997; Marin *et al.*, 2000). Cytoplasmic zinc enhancements were also observed in non-neuronal cells, following membrane potassium depolarization (Slepchenko and Li, 2012). In both cortical and non-neuronal cells, there is also evidence that zinc is taken up in intracellular stores upon stimulation, being considered that it could be stored in the endoplasmic reticulum, the Golgi apparatus and mitochondria (Saris and Niva, 1994; Sensi *et al.*, 2000; Stork and Li, 2010; Qin *et al.*, 2011; Sensi *et al.*, 2011). Because of its complexity and the large number of mechanisms involved, the characterization of zinc dynamics associated with chemically-induced synaptic potentiation remains to be clarified.

The aim of this work was to address intracellular zinc changes associated with potassium-evoked mossy fiber synaptic plasticity in CA3 hippocampal area. For this purpose, hippocampal slices were loaded with the permeant form of the zinc selective fluorescent probe Newport Green (NG) (Haugland, 1996) being the cells depolarized with different concentrations of extracellularly applied KCl.

Most of the present findings have been reported in abstract form.

Materials and Methods

Data were collected in the synaptic system mossy fibers - CA3 pyramidal cells of hippocampal slices obtained from pregnant Wistar rats (10-13 weeks old). The animals were sacrificed by cervical dislocation and the isolated brain was rapidly cooled (5-8° C) in artificial cerebrospinal fluid (ACSF). The slices (400 μm thick) were cut

transversely and transferred to a container with ACSF at room temperature, saturated with a gas mixture (95% O_2, 5 % CO_2). They remained there at least 1 hour before being used in an experiment. The ACSF medium had the following composition (in mM): NaCl 124; KCl 3.5; $NaHCO_3$ 24; NaH_2PO_4 1.25; $MgCl_2$ 2; $CaCl_2$ 2 and D-glucose 10; pH 7.4. The slices were subsequently transferred to the experimental chamber where they were perfused with ACSF, at a rate of 1.5 to 2 ml/min, at temperatures in the range 30-32° C. The KCl solutions consisted of ACSF with higher concentrations of KCl, 20 mM and 60 mM. In some experiments $ZnCl_2$ (1 mM) was added to the 60 mM KCl medium. All media were perfused for periods of 10-30 min.

Experimental setup and optical measurements

The measurement of optical signals was performed using a fluorescence microscope (Zeiss Axioskop) with a transfluorescence arrangement, including a halogen light source (12V, 100 W), a narrow band (10 nm) excitation filter (480 nm) and a high-pass emission filter (> 500 nm). The light was collected by a water immersion lens (40x, N.A. 0.75) and then focused on a photodiode (Hammamatsu, 1 mm^2), passing its signal through a current/voltage converter (I/V) with a 1 GΩ feedback resistance. The signals were digitally processed by means of a 16 bit analog/digital converter, at a frequency of 0.017 Hz and analyzed using the Signal Express™ software from National Instruments. For measuring zinc changes the hippocampal slices were incubated for 1 h in a medium containing the permeant form of the zinc indicator Newport Green (NG) (5 μM). This solution was obtained dissolving 1 mg NG in 250 μl of DMSO and then diluting 5 μl of this mixture (DMSO + NG) in 5 ml of ACSF containing 5 μl of pluronic acid F-127. This indicator has a moderate affinity for zinc (K_d ~ 1 μM) and a relatively low affinity for calcium (K_d > 100 μM (Haughland, 1996). The optical data consist of fluorescence values represented at 1 minute intervals, in ACSF or in a KCl medium. The signals were corrected for the autofluorescence component, evaluated as the average of ten data points obtained from an equivalent region of dye-free slices, perfused with the normal solution. All measurements are presented as mean ± SEM. Statistical significance was evaluated using the Mann-Whitney *U* test ($p<0.05$).

Drugs used were NG, Pluronic acid F-127 (Life technologies, Carlsbad, CA); DMSO (Sigma-Aldrich, Sintra, PT).

All experiments were carried out in accordance with the European Communities Council Directive. All efforts were made to minimize animal suffering and to use only the number of animals necessary to produce reliable scientific data.

Results

The fluorescence signals were collected from the *stratum lucidum* of CA3 hippocampal area, as shown in Fig. 1a. It was observed that dye-free slices have a significant autofluorescence, triggered by 480 nm incident light and detected for wavelengths above 500 nm. In order to evaluate the contribution of autofluorescence to the signals detected from NG-containing slices, both types of data are indicated in Fig 1b. It can be noticed that autofluorescence is a major part of the total fluorescence, representing about 75% of it. Thus, all signals were corrected subtracting the autofluorescence component, that was obtained from non-incubated slices. The remaining fluorescence is due to the formation of the NG-zinc complex (Fig. 2a). Since the permeant form of Newport Green is hydrolyzed in the intracellular medium, becoming charged, it cannot permeate the vesicular membranes and is thus unable to detect presynaptic zinc in the vesicles (Li *et al.*, 2001b). For this reason, it is considered that the corrected optical signals have a postsynaptic origin.

The perfusion of the medium containing 20 mM KCl caused a rise in the zinc signals to 119 ± 5 %, at 35-40 min ($n = 3$, $p<0.05$), that is partially reverted after a 30 min period, upon returning to the initial ACSF solution, as shown in Fig. 2a. However, the medium with a higher concentration of KCl, 60 mM, evoked a zinc potentiation that is maintained following washout. In Fig. 2b it can be observed that the amplitude of the zinc signals obtained in the presence of 60 mM KCl increased to 184 ± 14 %, at 35-40 min ($n = 7$, $p<0.05$). These signals remained stable following the withdrawal of KCl, revealing the establishment of a KCl induced persistent zinc potentiation measuring 181 ± 13 %, at 65-70 min ($n = 7$), with respect to baseline.

The following experiments were designed to study the effect of repeated applications of the KCl media considered before. A second addition of 20 mM KCl caused similar zinc

changes to those induced by the first one, i.e. an enhancement in the presence of that medium followed by a decrease in its absence (Fig. 3a). In the case of the 60 mM KCl solution the repeated perfusion did not induce further potentiation (Fig. 3b). The results in Fig. 3c rule out the possibility of saturation of the indicator (NG) by zinc, since the application of extracellular zinc (1 mM) accompanying KCl (60 mM) resulted in further zinc potentiation that was maintained upon returning to ACSF

Discussion

In this study we observed zinc signals associated with potassium-induced depolarization of hippocampal mossy fibers. It has been shown that zinc is released from these fibers into the extracellular medium when electrical stimuli are delivered (Li *et al.*, 2001a; Quinta-Ferreira *et al.*, 2004, Khan *et al.*, 2014, Vergnano *et al.*, 2014) and that it enters to postsynaptic neurons following intense electrical or chemical stimulation (Vogt *et al.*, 2000; Li *et al.*, 2001a,b; Ueno *et al.*, 2002; Ketterman and Li, 2008). The exposition of the slices to a high concentration of exogenous potassium causes an enhancement of the measured fluorescence signals, considered to be associated with postsynaptic zinc changes (Li *et al.*, 2001b; Ketterman and Li, 2008). The potassium-induced increase in the postsynaptic zinc concentration may be explained by a rise in synaptic activity, caused by the potassium-evoked shift of the presynaptic membrane potential. In the presence of the 20 mM and 60 mM KCl solutions, the resting values increase to about -54 mV and -33 mV, respectively, thus leading to cell depolarization (Bancila *et al.*, 2004). This causes intense co-release of glutamate and zinc, followed by zinc entry into the postsynaptic area, through several types of receptors and channels. The subsequent depolarization of the spine region evoked by glutamate binding to postsynaptic AMPA, NMDA and calcium permeable AMPA/Kainate receptors causes the opening of their channels and also of voltage dependent T- and L-type calcium channels which are located in the same membrane. Except for the AMPA channels, all the others are permeable to zinc, being the permeability ratio P_{Ca} / P_{Zn} for the calcium permeable AMPA/Kainate channels about 1.8 (Weiss and Sensi, 2000; Jia *et al.*, 2002). This allows zinc entry to the postsynaptic region through the mentioned zinc permeant channels, namely L- and T-type VDCCs, NMDA and calcium permeable AMPA/Kainate receptors (Sensi *et al.*, 1997; Sensi *et al.*, 1999; Takeda *et al.*, 2009). There is also experimental evidence that zinc can be released from intracellular stores following the blockade of postsynaptic endoplasmic reticulum calcium pumps (Stork and Li, 2010). In the present work, after removal of the KCl solution, the zinc signals decreased in the 20 mM medium and remained unchanged in the 60 mM one. It was also observed that, after the induction of the long-lasting zinc potentiation, another

application of KCl (60 mM) did not induce further zinc enhancement. However, when KCl (60 mM) was added in combination with extracellular zinc (1 mM), a second zinc potentiation was elicited, with similar magnitude. The mossy fiber boutons contain a huge amount of synaptic vesicles (~16,000), with about 20 active zones, being up to 1400 vesicles ready to undergo exocytosis (Hallermann *et al.*, 2003; Rollenhagen and Lubke, 2010). However, the inexistence of the second potentiation in the absence of exogenous zinc might be due to the lack of additional ready releasable vesicles, caused by the previous intense release. Overall the results suggest that the evoked zinc potentiations are due to zinc entry in the postsynaptic area.

It was previously shown that KCl depolarization induces LTP in CA1 hippocampal area (Fleck *et al.*, 1992; Bernard *et al.*, 1994; Roisin *et al.*, 1997). That potentiation may be evoked by an enhancement of the glutamate release process or be due to persistent modifications of postsynaptic channels permeabilities or an increase in the number of AMPA receptors in the hippocampal neurons (Malenka and Bear, 2004). Thus, the potassium-induced long-lasting potentiation, that is a form of LTP, may be expressed pre- or postsynaptically. There are a large number of studies that characterize mossy fiber LTP as presynaptically expressed, being mediated by enhanced glutamate release (Johnston *et al.*, 1992; Malenka and Bear, 2004). However, some studies are in favor of the hypothesis of a postsynaptic *locus* for mossy fiber LTP expression (Yamamoto *et al.*, 1992; Yeckel *et al.*, 1999; Quinta-Ferreira *et al.*, 2004, Suzuki and Okada, 2009). The main argument in favor of the presynaptic nature for mossy fiber LTP is the reduction of the paired-pulse ratio (the ratio of the amplitude of the second excitatory postsynaptic response to that of the first in two consecutive pulses), i.e. of paired-pulse facilitation, which is inversely correlated with the transmitter release probability (Zalutsky and Nicoll, 1990; Zucker and Regehr, 2002). However, changes in paired-pulse ratio are not exclusively mediated by modifications of the presynaptic release probability. For example, they can be influenced by postsynaptic receptor desensitization and lateral diffusion (Frischknecht *et al.*, 2009). Further support for the presynaptic *locus* of mossy fiber LTP, comes from quantal analysis, since the failure rate is negatively correlated with the average release probability. Thus, a lower failure rate after LTP induction means a higher probability of glutamate release (Malinow and Tsien, 1990). However, that conclusion can only be achieved assuming a constant number of synapses. The discovery of postsynaptically silent synapses provided an explanation for the mentioned lower failure rate after LTP (Isaac *et al.*, 1995). More

experimental evidence in favor of the presynaptic hypothesis for the expression of mossy fiber LTP, is the effect of cAMP which mediates presynaptic mossy fiber LTP processes (Tong *et al.*, 1996). Assuming a purely presynaptic *locus* for mossy fiber LTP, the zinc released from mossy fibers should rise after electrically- or chemically-induced depolarization, since it is generally accepted that zinc is co-released with glutamate. However, there are experimental results showing that zinc release is not enhanced after high-frequency mossy fiber stimulation (Budde *et al.*, 1997; Quinta-Ferreira *et al.*, 2004) and also following exposure to high-potassium concentrations (Ketterman and Li, 2008). Thus, the lack of enhancement of zinc release after LTP induction argues in favor of the contribution of postsynaptic mechanisms for the expression of mossy fiber LTP. Furthermore, the fact that the blockade of postsynaptic T-type VDCCs prevents the expression of this form of LTP is another strong argument in line with the postsynaptic hypothesis (Suzuki and Okada, 2009). As expected, in CA1 hippocampal area, it was already shown that the potassium-induced LTP is mainly mediated by postsynaptic mechanisms (Roisin *et al.*, 1997). The possible postsynaptic expression of mossy fiber LTP might be mediated by zinc influxes into postsynaptic neurons. However, there is still controversy about the role of zinc in mossy fiber LTP, existing studies in favour (Lu *et al.*, 2000; Li et *al.*, 2001a) and against it (Vogt *et al.*, 2000; Matias *et al.*, 2006). The reason for these different results may be the variety of experimental approaches used that may lead to different intracellular zinc availability and metal/chelator complexes, some of which are potentially toxic (Armstrong *et al.*, 2001). Another possible explanation is that the chelators used may be neuroprotective or neurotoxic, in pathological or normal situations (Cuajungco and Lees, 1997; Armstrong *et al.*, 2001). Further support for the role of zinc in mossy fiber LTP comes from the existence of signal transduction pathways that are modulated by zinc (Frederickson and Bush, 2001). Our results support the idea that the zinc signals are due to the formation of postsynaptic zinc-NG complexes, since they increase with extracellular zinc that may permeate the postsynaptic membrane. They also suggest that the zinc potentiation associated with a long-term enhancement of synaptic activity is expressed postsynaptically.

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449 **Figure legends:**

450

451 Fig. 1 – Diagram of the hippocampal slice, autofluorescence and basal fluorescence. (a)
452 Schematic representation of the hippocampal slice. The circle indicates the region from where
453 the optical signals were recorded. mf – mossy fibers, DG – dentate gyrus. (b) Fluorescence from
454 non-incubated and from Newport Green containing slices. Autofluorescence (open symbols)
455 and fluorescence signals from slices incubated with 5 μM of the zinc indicator Newport Green
456 DCF (closed symbols) (n = 16). The points represent the mean ± SEM.

457

458 Fig. 2- Pooled data of KCl induced zinc changes obtained with Newport Green. (**a**) Application
459 of 20 mM KCl evoked a rise in the NG fluorescence that was reverted upon washout (n = 3,
460 $p<0.05$). (**b**) Similar to a, but for 60 mM KCl (n = 7, $p<0.05$) (**c**). All values were normalized by
461 the average of the first 10 responses and represent the mean ± SEM.

462

463 Fig. 3- Zinc signals during consecutive applications of KCl media. (a) Repeated perfusion of 20
464 mM KCl induced similar transient potentiations.(n = 3, $p<0.05$) . (b) Subjecting the slices a
465 second time to 60 mM KCl caused no further zinc enhancement. (n = 3, $p>0.1$). **c**. Subsequent
466 zinc potentiations in slices exposed first to KCl (60 mM) and then to a mixture of KCl (60 mM)
467 and $ZnCl_2$ (1 mM) (n = 2, $p<0.05$). All values were normalized by the mean of the first 10
468 responses and represent the mean ± SEM.

469

470

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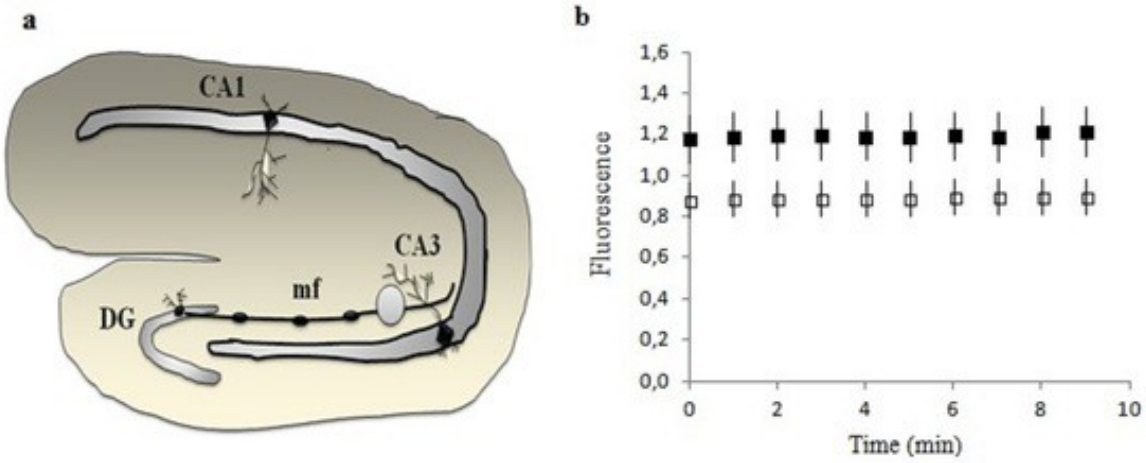

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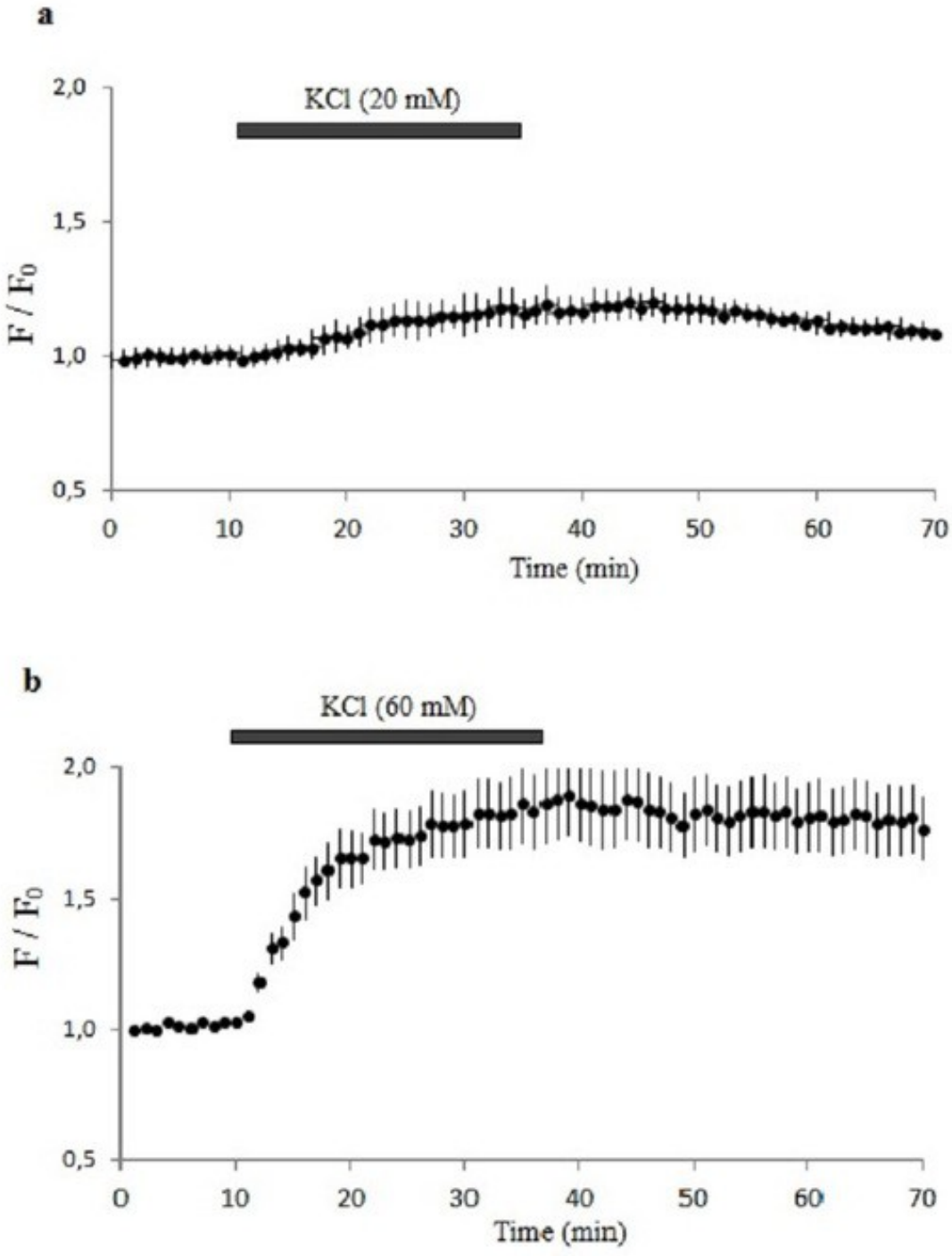

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