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ORIGINAL PAPER

Calcium-Dependent Regulation of the Neuronal Glycine Transporter GlyT2 by M2 Muscarinic Acetylcholine Receptors

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Abstract

The neuronal glycine transporter GlyT2 modulates inhibitory glycinergic neurotransmission and plays a key role in regulating nociceptive signal progression. The cholinergic system acting through muscarinic acetylcholine receptors (mAChRs) also mediates important regulations of nociceptive transmission being the M2 subtype the most abundantly expressed in the spinal cord. Here we studied the effect of M2 mAChRs stimulation on GlyT2 function co-expressed in a heterologous system with negligible levels of muscarinic receptor activity. We found GlyT2 is down-regulated by carbachol in a calcium-dependent manner. Different components involved in cell calcium homeostasis were analysed to establish a role in the mechanism of GlyT2 inhibition. GlyT2 down-regulation by carbachol was increased by thapsigargin and reduced by internal store depletion, although calcium release from endoplasmic reticulum or mitochondria had a minor role on GlyT2 inhibition. Our results are consistent with a GlyT2 sensitivity to intracellular calcium mobilized by M2 mAChRs in the subcortical area of the plasma membrane. A crucial role of the plasma membrane sodium calcium exchanger NCX is proposed.

Keywords Transport · Glycine · Muscarinic receptors · Calcium · NCX · Pain · Hyperekplexia

Introduction

Glycine is the main inhibitory neurotransmitter in caudal areas of the central nervous system (CNS) and controls the processing of motor, sensory and nociceptive information. Glycine receptors activated by synaptic glycine permit chloride influx through the postsynaptic membrane leading to hyperpolarization and decreased propagation of excitatory postsynaptic potentials. Glycinergic neurons present in the dorsal horn of the spinal cord diminish their activity in pathological pain conditions and behave as gate-keepers of the touch-pain circuitry. The reduction of glycinergic inhibitory transmission by application of the prototypical antagonist strychnine produces hyperalgesia [1], while the intrathecal application of glycine prevents it [2]. Synaptic glycine is removed by two specific glycine transporters GlyT1 and GlyT2 that co-transport glycine together with sodium and chloride. GlyT1 is preferentially located in astrocytes and is associated both to the glycinergic and the glutamatergic pathways. However, GlyT2 is exclusively present in glycinergic neurons. The neuronal glycine transporter GlyT2 that co-transports sodium, chloride and glycine (3:1:1) into the neuron, modulates inhibitory glycinergic neurotransmission by controlling the extracellular concentration of synaptic glycine and the supply of neurotransmitter to the presynaptic terminal. The pharmacological blockade of GlyT2 reduces the progression of the painful signal to rostral areas of the CNS by increasing glycine extracellular levels, so it has clear analgesic action. Although there is some role of GlyT1 in analgesia, its inhibition increases excitatory neurotransmission what may promote pro-nociceptive action. In addition, GlyT2 is involved in a pathology of the glycinergic neurotransmission called hyperekplexia or startle disease (OMIM

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149400). Loss of function mutations of the human *SLC6A5* gene encoding GlyT2 cause this sensorimotor disorder potentially lethal in neonates due to apnea episodes [3].

The crucial role of GlyT2 in the inhibitory glycinergic neurotransmission has fostered the study of modulatory factors that regulate its activity, and that might help the success of future therapies. Among several regulatory mechanisms [4], GlyT2 has physical and functional interactions with proteins whose main function is directly or indirectly related to intracellular calcium homeostasis: the Na^+/K^+-ATPase [5], the neuronal plasma membrane calcium ATPase (PMCA) and the ubiquitous Na^+/Ca^{2+} exchanger (NCX1) [6]. Pharmacological inhibition of PMCA activity, as well as specific inhibition of the reverse mode of NCX (NCXrev), led to a marked reduction in GlyT2 activity suggesting that proper Ca^{2+} dynamics in presynaptic terminals is necessary for optimal GlyT2 activity [6]. The role of NCX is crucial in intracellular calcium homeostasis since it can exchange Na^+ and Ca^{2+} in either direction depending on the transmembrane electrochemical gradient of Na^+ [7]. The increase in cytosolic calcium may occur by activation of extracellular calcium entry via Ca^{2+} channels and NCX ($3Na^+$: $1Ca^{2+}$), and by calcium release from intracellular sources. The main pathways to lower internal calcium are the endoplasmic reticulum (ER) Ca^{2+}-ATPase (SERCA), the PMCA and NCX. SERCA has high affinity for calcium and can sequester it into the ER. PMCA is a high affinity low capacity system for calcium extrusion from cells, but NCX is a high capacity low affinity calcium sodium exchanger [6].

The cholinergic system acting through muscarinic acetylcholine receptors (mAChRs) also mediates important regulations of nociceptive transmission in the spinal cord. Muscarinic receptor stimulation and acetylcholinesterase inhibitors produce analgesia at both spinal and supraspinal sites [8]. Pharmacological and receptor knockout studies have proven that some of their antinociceptive effects in the spinal cord are elicited by presynaptic modulation of transmitter release that increases inhibitory and decreases excitatory neurotransmission [9]. Among the five mAChRs (M1–M5, [10, 11]), M2, M3 and M4 are expressed in the spinal cord. M2 receptors represent about 90% of total spinal cord mAChRs being particularly expressed in the superficial dorsal horn, an area of special relevance in pain transmission [8, 12]. The role of the individual receptor subtypes in antinociception has given conflicting results likely due to the limited selectivity of the pharmacological tools available and the opposite actions of the different receptor subtypes in spinal cord [12, 13]. Despite these limitations, M2 receptors have been involved in peripheric [14], spinal [8, 11] and supraspinal [15] antinociception.

It has been shown that ACh increases the release of glycine from inhibitory interneurons in the rat spinal cord with a proposed potentiating effect of the M2 mAChR [16]. Conversely, an inhibitory effect of M2 receptor in the mice glycinergic neurotransmission has been reported [13]. In addition, the individual effects of M2 receptor stimulation on the different synaptic components of the glycinergic neurotransmission are unknown, despite this aspect may give some clues on the action of M2 mAChR on glycinergic neurons. Since GlyT2 is an essential modulator of glycinergic transmission and it can control the strength of the synaptic transmission in spinal cord, in this report we analyzed the effect of M2 mAchR stimulation on GlyT2 function. For this purpose, we used a heterologous system with negligible levels of muscarinic receptors and co-expressed transporter and receptor. Our data show GlyT2 can be inhibited by M2 receptor stimulation in a calcium-dependent manner.

Experimental Procedures

Materials

Wistar rats were bred under standard conditions at the Centro de Biología Molecular Severo Ochoa (CBMSO) in accordance with procedures approved in the Directive 2010/63/EU of the European Union with approval of the Research Ethics Committee of the Universidad Autónoma de Madrid (Comité de Ética de la Investigación UAM, CEI-UAM). M2 acetylcholine receptor cDNA was a gift from Ana Ruíz Gómez (CBMSO, Spain). The intracellular calcium chelator BAPTA-AM (acid 1,2-Bis (2-aminophenoxy) ethane-N, N, N ′, N′-tetraacetic tetrakis (acetoxymethyl ester)) was from Calbiochem. Carbachol (carbamoylcholine), methacholine, atropine, EGTA (ethylene-bis (oxyethylene nitrile) tetraacetic acid tetrasodium), Dantrolene hydrochloride, 2-aminoethoxydiphenyl borate (2-APB), 4-aminopyridine were from Sigma-aldrich. Thapsigargin, CGP37157, KB-R7943 mesylate and tertiapin LQ were from Tocris Bioscience.

Immunofluorescence of Brainstem Primary Neuronal Cultures

Immunofluorescence in neuron-enriched cultures was performed as reported [17]. Brainstem primary cells growing on coverslips were fixed with ice-cold methanol or 4% paraformaldehyde, permeabilized with 0.25% Triton X-100 and nonspecific binding sites were blocked with 10% BSA in PBS. Cells were then incubated with the GlyT2 antibody (generated in house [18]) or M2 mAChR antibody (Alomone Labs. 1/500–1/2000). Secondary antibodies were anti-rabbit coupled to Alexa Fluor® 555 fluorophore for GlyT2, anti-rabbit antibodies coupled to Alexa Fluor® 488 for M2 mAChRs. The cells were visualized by confocal microscopy

on a LSM 510 Confocal Microscope (Zeiss) using a vertical microscope Axio Imager.Z1 M (Zeiss).

Dual Immunofluorescence of Tissue Slices

Adult Wistar rats were deeply anesthetized by intra-peritoneal injection of pentobarbital (100 mg/kg) and transcardially perfused with a fixative solution containing 4% paraformaldehyde in PBS, at a flow rate of 20 ml/min. The brainstem and spinal cord were extracted and maintained overnight in fixative. After washing in PBS, tissue was cut with a vibratome into 50 µm slices that were stored in PBS with 0.02% azide for a maximum of 3 weeks. Dual immunofluorescence in slices was performed as described previously [17] and samples were visualized as above.

Cell Growth and Protein Expression

COS7 cells (American Type Culture Collection) were grown at 37 ºC and 5% CO_2 in high glucose Dulbecco's modified Eagle's medium supplemented with 10% foetal bovine serum supplemented with 1% non-essential amino acids and 1% glutamine. Transient expression in COS7 cells was carried out as previously described [19] using Turbofect Transfection Reagent (Thermo Fisher Scientific, Waltham, MA, USA), following the manufacturer's protocol (2 µl reagent/µg of DNA). Reproducible results were obtained with 60–70% confluent cells on a 100 mm dish using 4 µg of total DNA. Co-expression of GlyT2 transporters and M2 acetylcholine receptors (mAChRs) was performed using 2.5 µg of the transporter cDNA and/or 1.5 µg of the receptor cDNA. Cells were incubated for 48 or 72 h at 37ºC and then used for transport assays. SH-SY5Y cells (American Type Culture Collection) were cultured under the aforementioned conditions and in supplemented DMEMF-12 medium with 10% fetal bovine serum, non-essential amino acids and 2 mM glutamine.

Transport Assays

COS7 cells were washed and incubated at 37 °C in HEPES-buffered saline (HBS, in mM: 150 NaCl, 10 HEPES-Tris, pH 7.4, 1 $CaCl_2$, 5 KCl, 1 $MgSO_4$, 10 glucose) containing 2 µCi/ml [2-^{3}H]glycine (1.6 TBq/mmol; PerkinElmer Life Sciences), at 10 µM final glycine concentration if not otherwise stated [20]. At the end of the desired time (usually 6 min), reactions were washed and terminated by aspiration. Protein concentration (Bradford) and [2-^{3}H]glycine levels (liquid scintillation, LKB 1219 Rackbeta) were determined. Glycine accumulation measured in mock-transfected cells was subtracted from that of the transporter-transfected cells and normalized by the protein concentration. Kinetic analyses were performed by varying glycine concentration in the uptake medium between 0.5 µM and 1 mM.

Pharmacological Treatments

Transfected or mock-transfected cells were washed with HBS and treated for the indicated times with HBS with or without calcium containing 0.01–500 µM carbachol or 0.01-100 µM methacoline minus plus atropine 10 µM. For the compounds indicated in the figure legends, a 15–30 min preincubation before the addition of carbachol or carbachol plus atropine was performed. All the reagents were prepared immediately before the experiment and protected from light. Once the incubation was completed, cells were quick washed and subjected to [^{3}H] glycine transport as above. In the experiments using channel blockers, the treatment was for 5 min and the subsequent glycine transport for 4 min.

Imaging Measurements of Cytosolic Ca^{2+}

COS7 cells growing on coverslips coated with polylysine were washed in HCSS medium (in mM: NaCl, 120; KCl, 5.4; $MgCl_2$, 0.8; Hepes, 25; $NaHCO_3$, 4.2; $CaCl_2$, 1 and glucose, 5), then loaded with 5 µM Fura-2 AM for 40 min at 37 °C in HCSS, and washed rapidly. Then coverslips were placed in a small superfusion chamber on the microscope stage as described earlier [21] and Fura-2 fluorescence was imaged ratiometrically using alternate excitation at 340 and 380 nm and a 510-nm emission filter with a Neofluar 40X/0.75 objective at 37 °C. Additions were made as a bolus, as indicated. Single cell analysis of the changes in $[Ca^{2+}]i$ were expressed as the ratio of fluorescence intensity at 340 (F340, bound calcium) and 380 nm (F380, calcium-free) (F340/F380). Image acquisition and analysis were performed with the Aquacosmos 2.5 software (Hamamatsu).

Data Analysis

Non-linear regression fits of experimental transport data and statistical analyses were performed using GraphPad Prism (GraphPad Software). Kruskal–Wallis test was used to compare multiple groups, with subsequent Dunn's post-hoc test to determine the significant differences between samples. Kolmogorov–Smirnov and Mann–Whitney U tests were used to compare two separate groups. p values are denoted through the text as follows: *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; ****$p < 0.0001$.

Results

Several studies have shown that M2 mAChRs are widely expressed throughout the brain, including all major regions predicted to be involved in pain transmission, modulation, and perception (pons/medulla, midbrain, thalamus, cerebral cortex) [22]. This localization includes regions containing glycinergic neurons where GlyT2 is expressed. However, the comparative distribution of GlyT2 and M2 mAChRs has not been examined. Before analyzing the possible modulation of GlyT2 by M2 mAChRs, we wished to know if receptor and transporter co-localized in spinal cord. For this purpose, we stained spinal cord slices and primary neurons from brain stem and spinal cord with specific antibodies of the two proteins and analyzed their

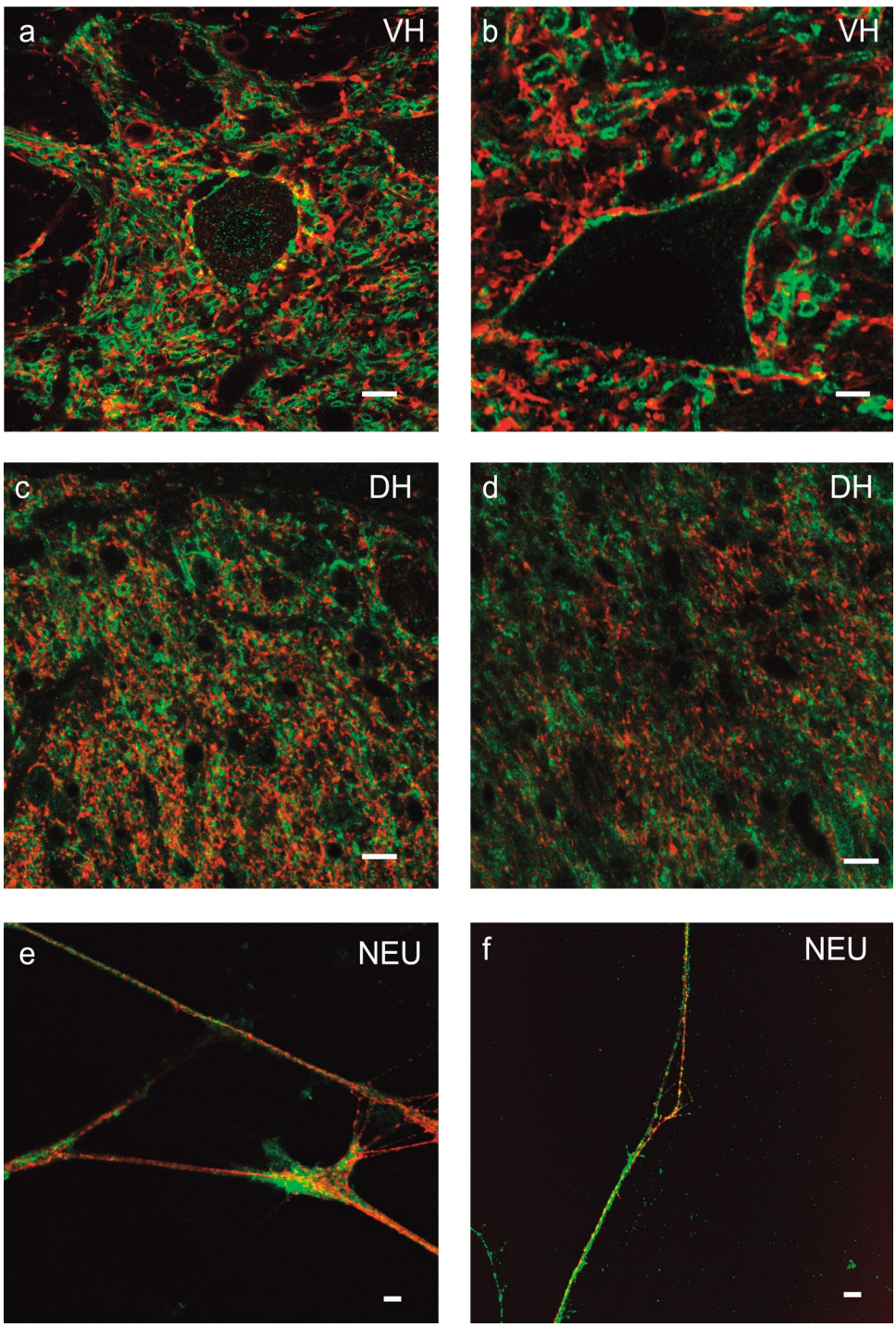

Fig. 1 Immunolocalization of GlyT2 and M2 mAChRs in spinal cord. **a**–**d** Dual immunohistochemical detection for GlyT2 (red) and M2 mAChR (green) in 50 µm rat spinal cord slices was performed as indicated in Experimental Procedures. **a**, **b** Ventral horn slices (twofold magnification of 63 × objective). **c**, **d** Dorsal horn slices. Scale bars, 20 µm. **e**, **f** Localization of GlyT2 and M2 mAChRs in brainstem primary neuronal cultures. 13 DIV grown brainstem/spinal cord primary cells were fixed and subjected to dual immunostaining as described in Experimental Procedures. Single channels for M2 mAChRs and GlyT2 are shown in green and red respectively. A merge of the two channels is presented. Scale bars, 10 µm

distribution by immunofluorescence (Fig. 1). As shown in Fig. 1, there is partial overlap of the two protein distributions in the ventral and the dorsal horn of the spinal cord and also in primary neurons obtained from these regions, suggesting there are neurons where the transporter could be modulated by the receptor.

In order to dissect the consequences of M2 mAChR stimulation on GlyT2, we chose the COS7 cell line where the endogenous expression of mAChRs was not detected [23] and consequently the cells do not respond to Ach [24]. M2 mAChR was co-expressed together with GlyT2 in COS7 cells and stimulated with carbachol before measuring [^{3}H] glycine transport by GlyT2 (Fig. 2). Carbachol stimulation inhibited GlyT2 transport activity in a dose–response manner with an $EC_{50} = 2.6 \pm 0.7$ µM. The inhibition was prevented by the general AchR antagonist atropine and also by the preferential muscarinic-selective antagonist metoctramine (Fig. 2a). The inhibition was not observed in cells expressing the GlyT2 transporter alone, indicating it was triggered by the M2 mAChR heterologously expressed and not by an endogenous receptor (Fig. 2b). Glycine transport inhibition by carbachol was rapid being almost maximal in about 5 min and it was maintained at least during 30 min (Fig. 2c). Kinetics analysis showed carbachol treatment mainly affected the Km of glycine transport promoting about fourfold increase and a much smaller (about 30%) reduction of the Vmax, strongly suggesting M2 mAChRs modulates the transport activity of the transporters present at the plasma membrane (Fig. 2d). To confirm the inhibitory effect of M2 mAChRs on GlyT2, we used the SH-SY5Y human neuroblastoma cell line, devoid of GlyT2, but endogenously expressing predominantly the M3 mAChR subtype (approximately 74% of total) and also significant amounts of M1 mAChRs and M2 mAChRs [25]. Carbachol treatment of SH-SY5Y cells transiently expressing GlyT2 significantly increased [^{3}H]glycine transport, suggesting M3 (and perhaps M1) activate GlyT2. This is in agreement with previous reports that measured opposite regulatory actions of the different mAChRs subtypes [12, 26]. However, the overexpression of M2 mAChRs together with GlyT2 in SH-SY5Y cells promoted an inhibition of GlyT2 transport similar to that observed in COS7 cells (Fig. 2e,f). These data robustly indicate GlyT2 transport activity can be down-regulated by M2 mAChR stimulation.

Next, we addressed the mechanism behind GlyT2 inhibition by M2 mAChRs. Classical signaling of this receptor couples through the inhibitory G protein, Gi/o and inhibits formation of cAMP through inhibition of adenylate cyclases [10]. We used several pharmacological agents interfering with this signalling pathway by increasing cAMP levels (forskolin, IBMX) or by inhibiting the cAMP activated protein kinase A (H89) and found no alteration of carbachol-induced GlyT2 inhibition (not shown). We, thus, investigated the role of calcium since M2 mAChR and muscarinic receptor activation can induce increases in cytosolic calcium [27–30]. During agonist stimulation, the intracellular calcium concentration reached reflects a balance between sustained calcium entry from the extracellular space by channels and transporters, NCX in reverse mode (NCXrev) and calcium clearance from the cytoplasmic compartment. Removal of calcium occurs through sequestration into intracellular stores or calcium extrusion by the PMCA and the NCX (forward mode) [31]. We repeated the carbachol treatment of COS7 cells co-expressing M2 mAChRs together with GlyT2 but this time in the presence of the intracellular calcium chelator BAPTA-AM. In this condition, the inhibition of glycine transport by M2 mAChR stimulation was completely abolished (Fig. 3a). As BAPTA-AM treatment has been reported to increase the rate of NCXrev transport (calcium entry mode), [32, 33], a condition compatible with optimal GlyT2 activity [6], this result suggested GlyT2 down-regulation by the M2 mAChR was mediated by intracellular Ca^{2+}.

Next we used thapsigargin, a specific and irreversible inhibitor of the SERCA pump that blocks the pumping of Ca^{2+} into the ER [31, 34]. Experimentally, thapsigargin has been extensively used to mobilize calcium from the ER and, if replenishment is not allowed (i.e. in the absence of external calcium), it can finally deplete the calcium stores [35–37]. We first observed that the addition of thapsigargin by itself to COS7 cells expressing GlyT2 produced a decrease in glycine transport activity, and this decrease was greater in the absence of external Ca^{2+} (Fig. 3b). This could indicate a GlyT2 sensitivity to raises in cytosolic calcium but also a GlyT2 inhibition by inward depolarizing currents that are triggered by thapsigargin upon ER calcium depletion [38]. In addition, thapsigargin treatment has an inhibitory effect on the NCXrev [32, 33], that may further reduce GlyT2 transport activity [6]. Furthermore, when we incubated cells expressing GlyT2 and M2 mAChR simultaneously with thapsigargin and carbachol, the inhibition of GlyT2 was increased when the treatment was performed in the absence but not in the presence of external Ca^{2+} (Fig. 3b), suggesting an action of the replenishment currents triggered by thapsigargin. However, if the cells were preincubated with thapsigargin in the absence of calcium before the addition of carbachol, the reduction of GlyT2 transport was much reduced and become not significant, indicating carbachol action is quite dependent on filled calcium internal stores. Interestingly, BAPTA-AM prevented carbachol inhibition independently of the presence or absence of thapsigargin, in agreement with the reported thapsigargin inhibition of NCXrev only in the absence of BAPTA-AM [32, 33].

To confirm the involvement of calcium transients in the molecular mechanisms elicited by M2 mAChRs, we analyzed the intracellular calcium responses after M2 mAChRs stimulation in Fura-2AM-loaded COS7 cells expressing

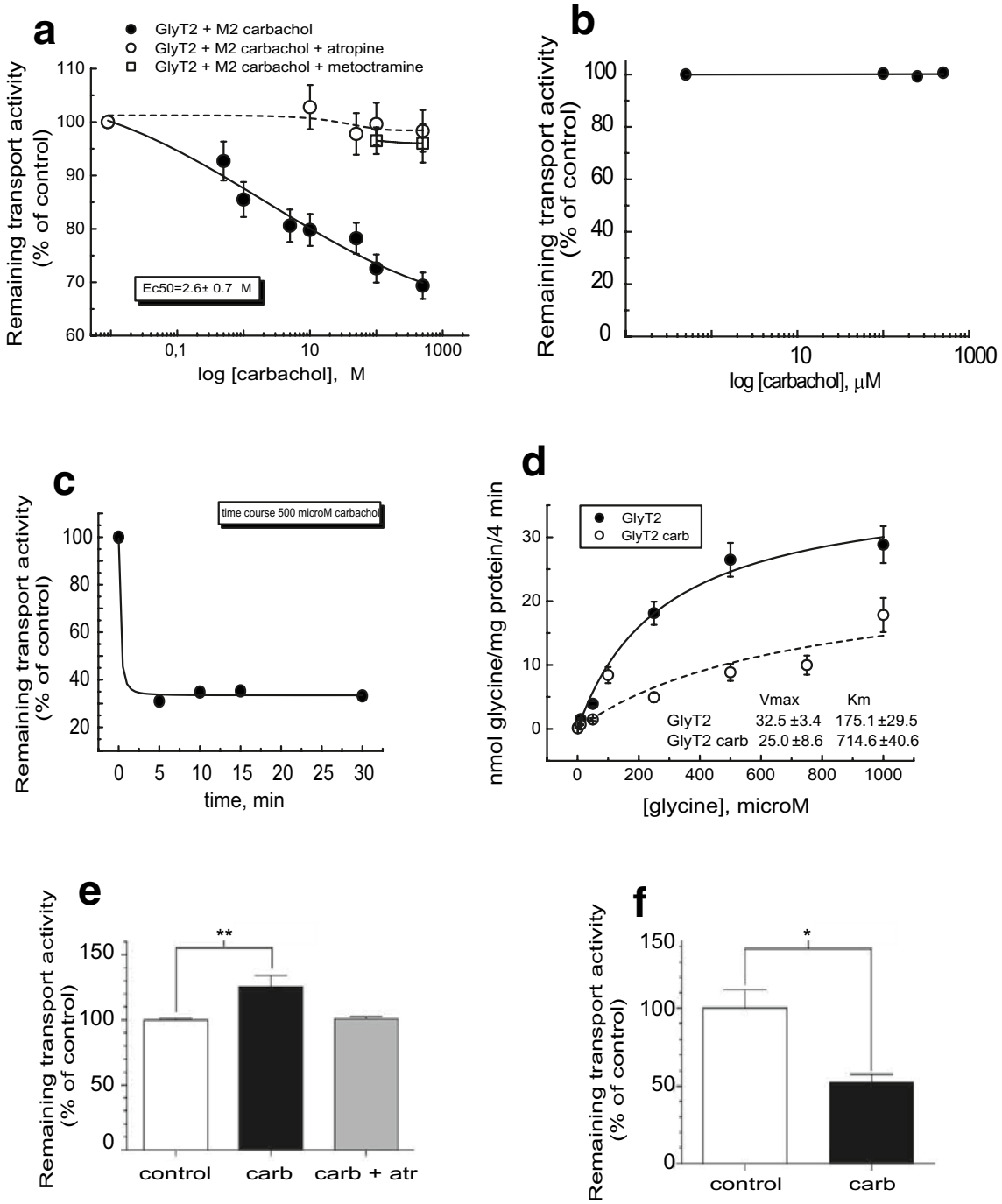

Fig. 2 Effect of M2 mAChR activation on glycine uptake by GlyT2. **a**, **b**, **d** COS7 cells co-expressing M2 mAChRs and GlyT2 (**a**–**d**) or GlyT2 and vector (pcDNA3, **b**) were incubated for 30 min at 37ºC with increasing concentrations of carbachol (**a**, **b**) in the absence or presence of the antagonist atropine (10 μM) or methoctramine (20 μM) (**a**). **c** Time course of carbachol inhibition at 500 μM carbachol. **d** Kinetics of glycine transport in the absence or presence of 250 μM carbachol. After washing, glycine transport activity was measured at 6 min or the indicated times. **e**, **f** SH-SY5Y cells expressing GlyT2 (**e**) or co-expressing M2 mAChRs and GlyT2 (**f**) were treated with 250 μM carbachol minus-plus 10 μM atropine (atr). After washing, glycine transport activity was measured during 10 min. *Significantly different from vehicle, *$p < 0.05$ and ** $p < 0.01$ by Student´s t test

GlyT2 and the M2 mAChRs and compared it with the responses by untransfected cells (Fig. 4). The addition of carbachol to cells expressing M2 mAChRs promoted an immediate calcium response measured by microfluorimetry that lasted for some min (Fig. 4B) and could not be observed in untransfected cells (Fig. 4A,C). The response

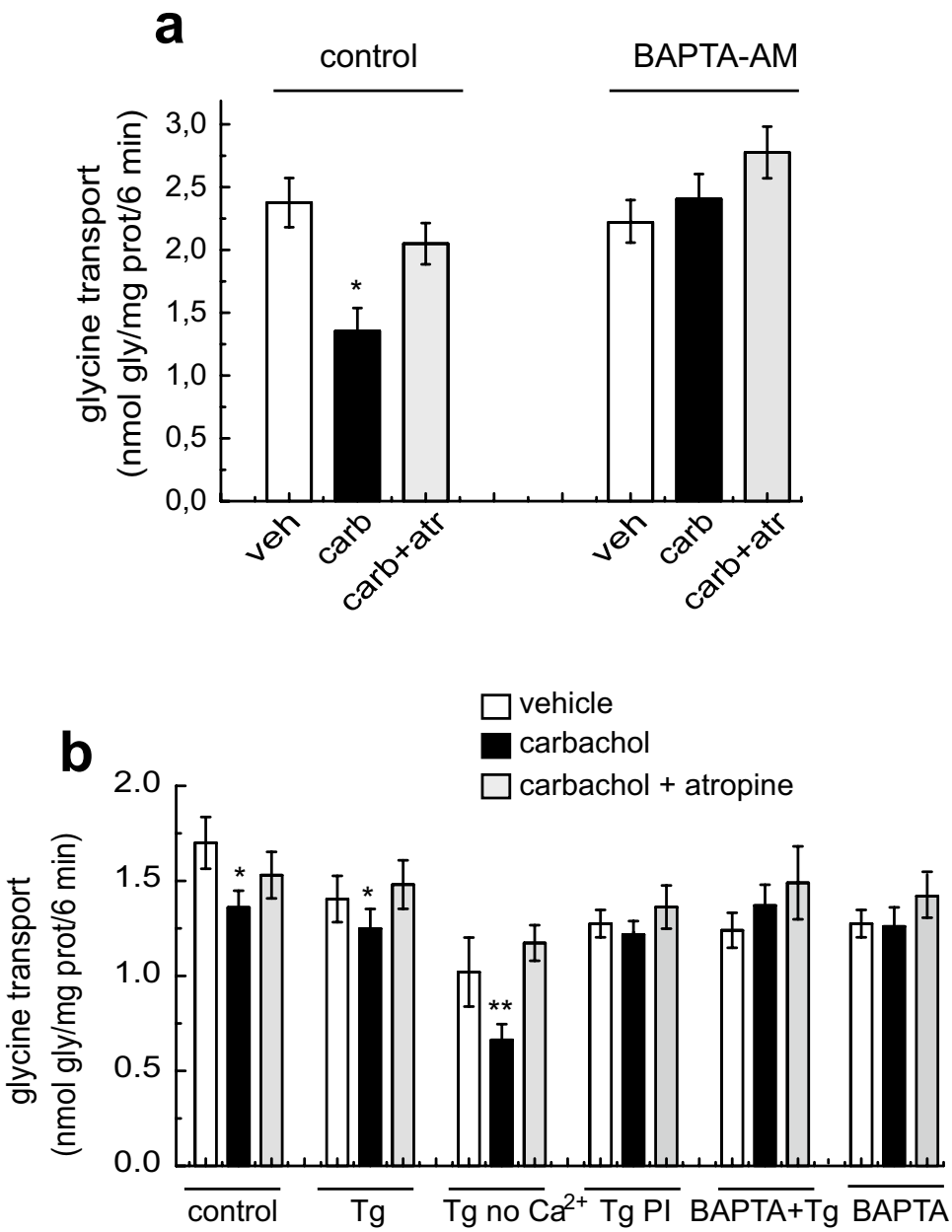

Fig. 3 Effect of BAPTA-AM and thapsigargin on glycine transport inhibition by carbachol. COS7 cells co-expressing M2 mAChRs and GlyT2 were incubated for 15 min at 37ºC with vehicle or 50 µM BAPTA-AM (**a**, **b**), 5 µM thapsigargin in HBS or HBS without calcium (**b**) or BAPTA-AM plus thapsigargin (**b**) and then incubated for 15 min with 250 µM of carbachol in the absence or presence of 10 µM atropine. In Tg PI condition 5 µM thapsigargin was added in a preincubation of 30 min in HBS without calcium before carbachol or carbachol plus atropine. After washing, glycine transport activity was measured during 6 min. *Significantly different from vehicle, *$p < 0.05$ and ** $p < 0.01$ by Student´s t test

was sensitive to atropine, so that the maximal amplitude of the response was reduced to basal levels after atropine treatment (Fig. 4d, e). These data confirmed the M2 mAChRs promote intracellular calcium changes in COS-7 in the presence of carbachol.

In order to know if GlyT2 was sensitive to calcium released from the ER and this had a role in the inhibition by carbachol, we addressed the main ER pathways for the release of Ca^{2+}: the inositol triphosphate (IP3) pathway, which can be activated by some G-protein-coupled receptors including M1 and M3 mAChR subtypes, and the calcium-induced calcium release (CICR), in which ryanodine receptors (RyRs) are involved [31]. The two type of ER calcium channels (IP3R and RyRs) are endogenously present in COS7 cells and several reports indicate they can be activated by M2 mAChR in different cell systems [27, 29, 39–41]. Hence, we treated the cells with blockers of the two ER channels (Fig. 5). The IP3R blocker 2-aminoethoxydiphenyl borate (2-APB, [42, 43]) did slightly reduce the inhibition of GlyT2 by carbachol (Fig. 4a), but this was only significant at high concentrations of the compound (100 µM), suggesting no or minimal involvement of the IP3R pathway in GlyT2 inhibition. In fact, concentrations of 2-APB around 100 µM have been shown to inhibit store operated calcium channels (SOC, see below). Moreover, the ryanodine receptor antagonist dantrolene

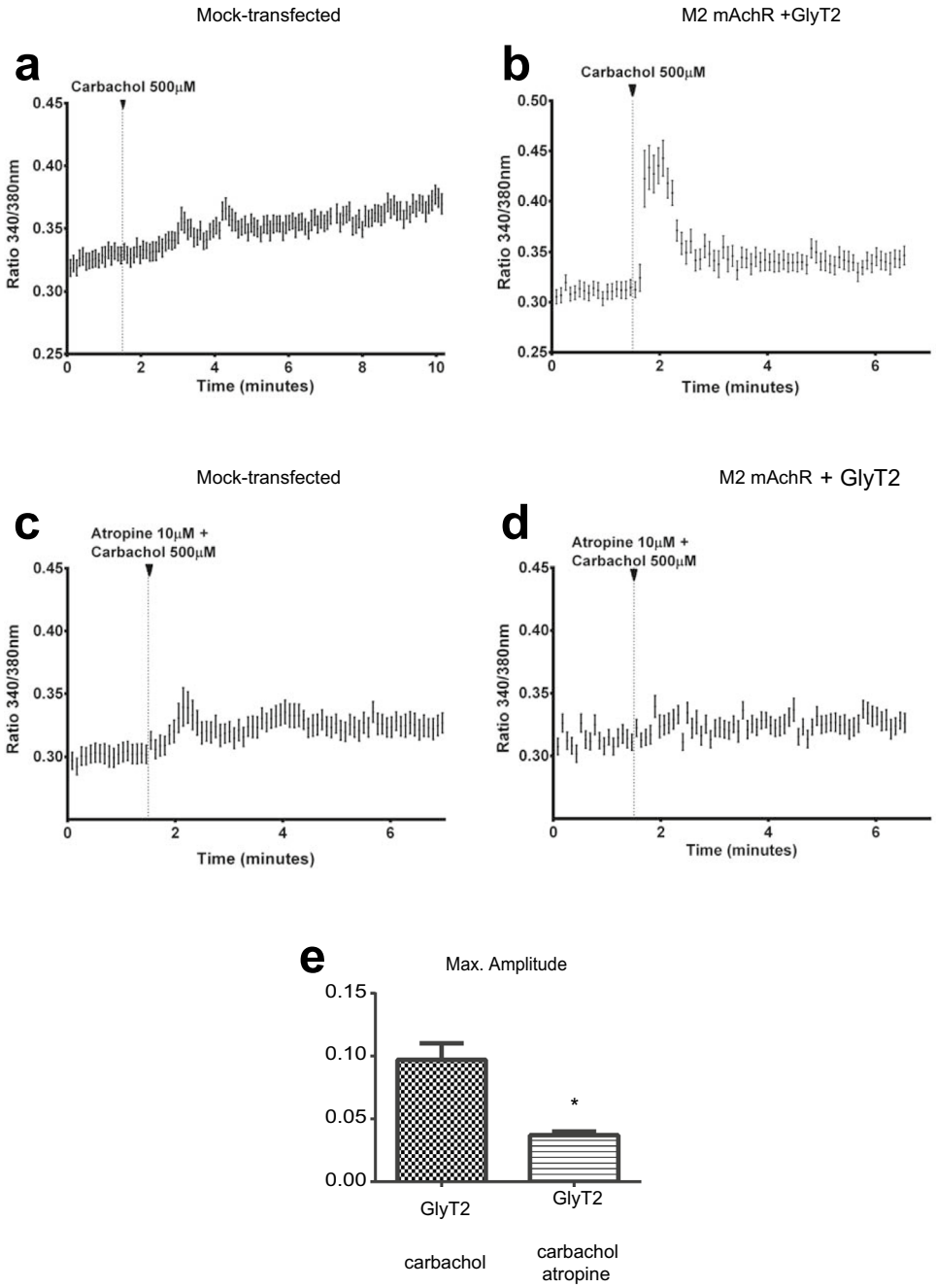

Fig. 4 Carbachol-induced Ca^{2+} influx into COS7 cells expressing M2 mAChRs. Cytosolic calcium responses in Fura-2AM-loaded mock-transfected COS7 cells (**a**, **c**) or COS7 cells expressing M2 mAchRs + GlyT2 (**b**, **d**), exposed to 500 μM carbachol (**a**, **b**) or 500 μM carbachol plus 10 μM atropine (c,d) added at the arrows. In **a**–**d** each trace represents the average the $[Ca^{2+}]$i of 18–27 cells. The increases in the fluorescence ratio (F340/F380) were calculated as described in the Experimental Procedures. **e** Maximum amplitude of the calcium responses induced by carbachol and prevented by atropine. *p < 0.05 by Student´s t test

[44] was also ineffective on the action of carbachol, discarding the CICR pathway involving RyRs as the target of regulation (Fig. 5b). Since mitochondria have the ability to take up, store and release Ca^{2+} from the ER, and the release of mitochondrial calcium to the cytosol can take place by the action of mitochondrial Na^{+}/Ca^{2+} exchangers (MNCX) [41], we also used the specific MNCX membrane-permeant blocker CGP37157 on carbachol action [45]. CGP37157 did not alter the inhibition of GlyT2 by carbachol, suggesting in this system, GlyT2 is not sensitive to cytosolic calcium of mitochondrial origin (Fig. 5b).

From the above data we concluded inhibition of GlyT2 by carbachol was not caused by cytosolic calcium released from ER or mitochondria. We, thus, focused our attention to plasma-membrane channel-mediated events. We first wished to know whether the inhibition of GlyT2 by carbachol was sensitive to plasma membrane depolarization. Therefore, we treated the cells co-expressing GlyT2 and M2 mAChRs with carbachol in the presence

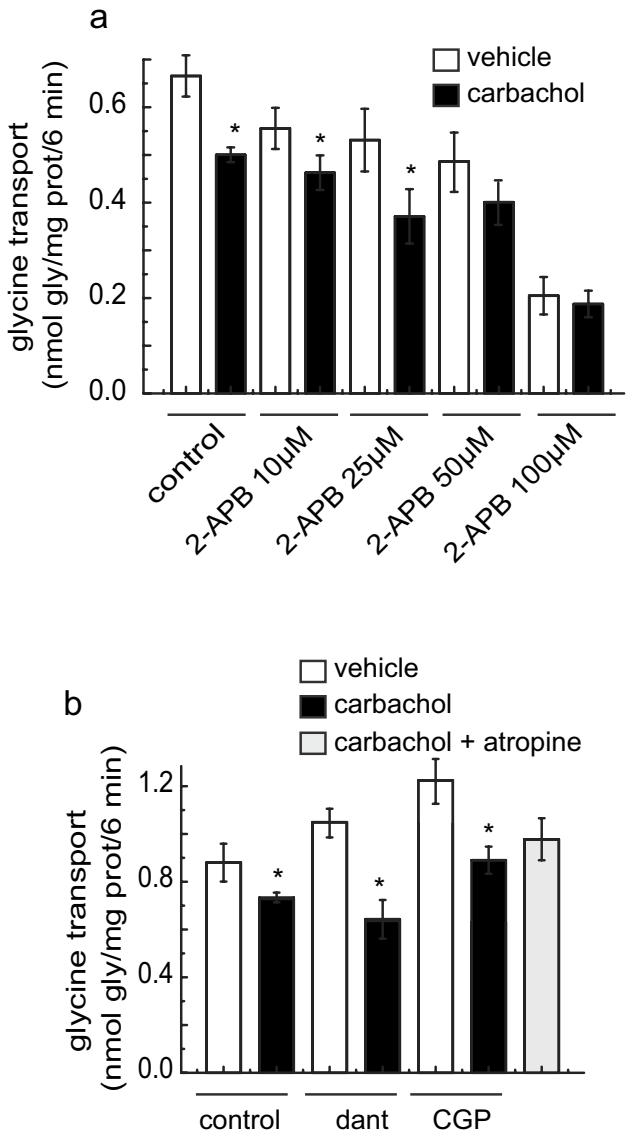

Fig. 5 Effect of inhibitors of IP3Rs and RyRs on glycine transport down-regulation by carbachol. COS7 cells co-expressing M2 mAChRs and GlyT2 were incubated for 15 min at 37ºC with vehicle or the indicated concentrations of 2-APB (HBS without calcium) (**a**), or 10 μM dantrolene (dant) (**b**) or 15 μM CGP37157 (CGP) (**b**) and then incubated for 15 min with 250 μM carbachol in the absence or presence of 10 μM atropine. After washing, glycine transport activity was measured during 6 min. *Significantly different from vehicle, *$p < 0.05$ by Student´s t test

of depolarizing treatments such as 4-aminopyridine/Ca^{2+} or high external KCl (Fig. 6a). These two treatments indeed prevented the inhibition, opening the possibility that GlyT2 down-regulation by carbachol may involve a voltage-dependent event. Furthermore, GlyT2 transport, as expected, was sensitive to the magnitude of the depolarization, which was reported to be lower for 4-aminopyridine/Ca^{2+} than for 30 mM KCl treatment [46]. One possible mechanism to generate a depolarizing current is the blocking of potassium channels. M2 receptors may couple to GIRK potassium channels opening [47] or even closing [48]. Since these channels are blocked by 4AP and some of the carbachol-induced GlyT2 inhibition is prevented by 4AP, we tested the involvement of GIRK channels using a selective GIRK channel blocker. However, although the presence of GIRK channels seems to be confirmed in kidney [49], we did not detect any effect of GIRK inhibition using specific tertiapin blocker in the action of M2 mAChRs on GlyT2 (Fig. 6b). Finally, the calcium-dependent BK channels present in epithelial cells [50], are frequently inhibited by M2 mAChRs through a Gβγ-mediated mechanism [51]. We performed the carbachol treatment in the presence of the general K-channel blocker tetraetylammonium (TEA) that inhibits BK channels [52], but it did not prevent GlyT2 down-regulation (Fig. 6c). Therefore, TEA treatment made us discard BK channel as target of carbachol action, besides this treatment indicated M2 mAChRs were not inactivated by depolarization [53].

Addressing the possibility that carbachol inhibition may involve plasma membrane depolarization, we reasoned that in COS7 cells, as in other systems, carbachol (and thapsigargin) could trigger cationic currents that depolarize the plasma membrane. These currents are generated upon mobilization of internal calcium that elicits the influx of extracellular calcium for store replenishment [36, 38]. Several channels and transporters may allow the entry of calcium through the plasma membrane. L-type calcium channels of the Cav type, which are endogenously present in COS7 cells [54], are frequently coupled to ER depletion [36, 38]. The selective inhibition of L-type calcium channels by nifedipine and low lanthanum concentrations did not alter the GlyT2 down-regulation by carbachol in the cells expressing receptor and transporter both in the absence or presence of external calcium (Fig. 6c). This observation suggested that the inhibition of GlyT2 by carbachol is not a direct consequence of plasma membrane depolarization but may involve an event itself controlled by plasma membrane voltage. On the contrary, we found that the inhibition of GlyT2 by carbachol was extremely dependent on the external sodium concentration and was abolished when external sodium was reduced (replaced by equimolar N-methyl-D-glucamine) below 100 mM (Fig. 7a). This observation suggests that store operated calcium channels (SOC), a ubiquitous Ca^{2+} entry pathway that is activated in response to stimulation of plasma membrane receptors which mobilize internal calcium stores [55–57], is required for carbachol inhibition. Finally, the inhibition by carbachol of GlyT2 activity in cells co-expressing the transporter and M2 mAChRs was prevented in the presence of KB-R7943 mesylate an inhibitor of the NCXrev (Fig. 7b, [58]). Inhibition by KB-R7943 mesylate,

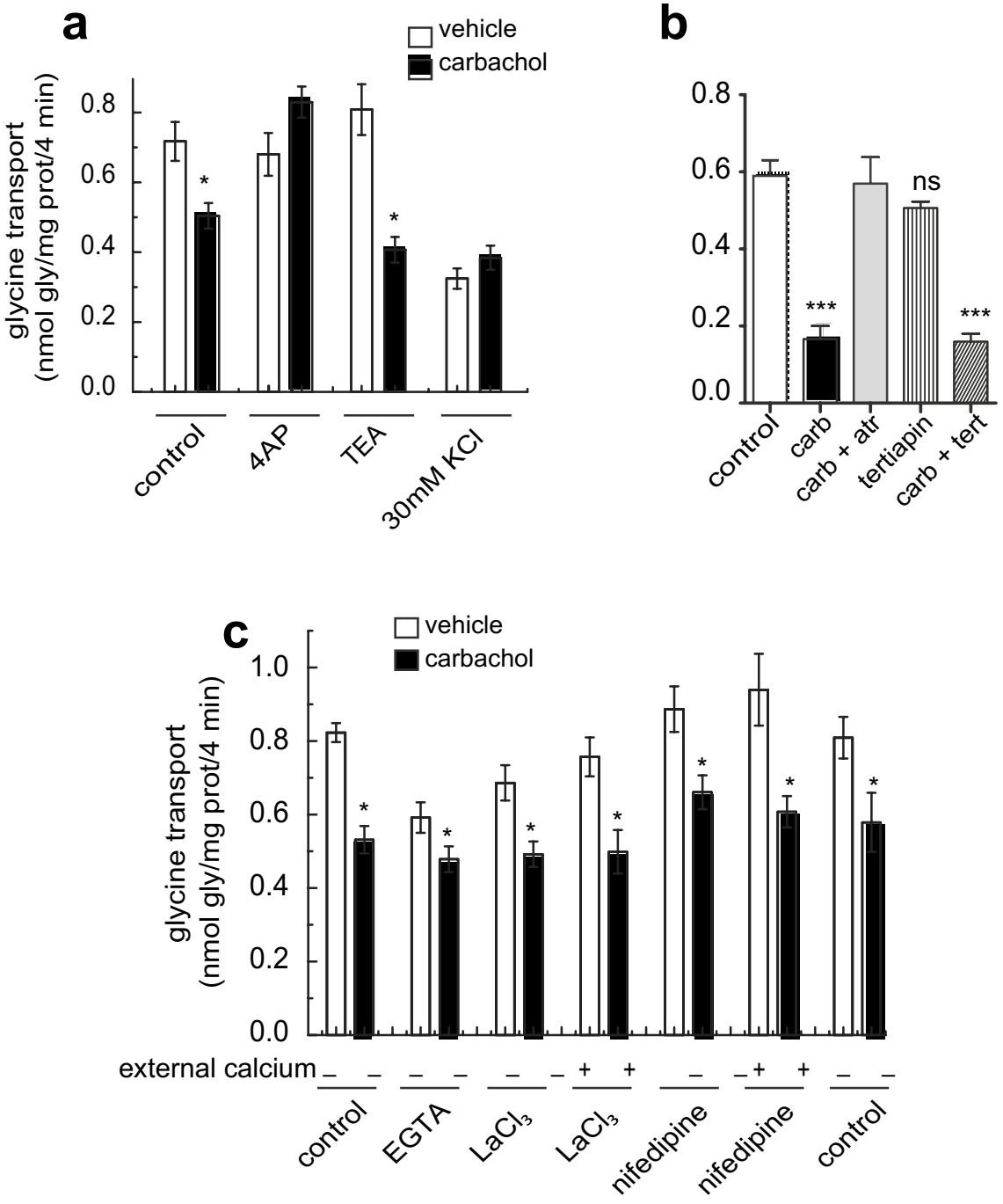

Fig. 6 Effect of plasma membrane channel blockers on glycine transport down-regulation by carbachol. COS7 cells co-expressing M2 mAChRs and GlyT2 were incubated with vehicle or 250 µM carbachol for 5 min at 37ºC in the absence or presence of 1 mM 4-aminopyridine, 10 mM TEA or 30 mM KCl (**a**) or 2 mM EGTA, 10 µM $LaCl_3$ 10 µM or 1 µM nifedipine in HBS or HBS without calcium (**c**). COS7 cells co-expressing M2 mAChRs and GlyT2 were treated with 500 µM carbachol or 10 µM atropine and 500 µM carbachol with or without 10 µM Tertiapin-LQ (**b**). Glycine transport activity was measured during 4 min in the same medium. *Significantly different from vehicle, *$p < 0.05$ by Student´s t test

might reduce the subcortical calcium concentration by allowing calcium extrusion by NCX forward mode and may compensate carbachol-induced calcium raise.

Discussion

In this work we have studied the down-regulation of GlyT2 exerted by the major mAChR subtype in the spinal dorsal

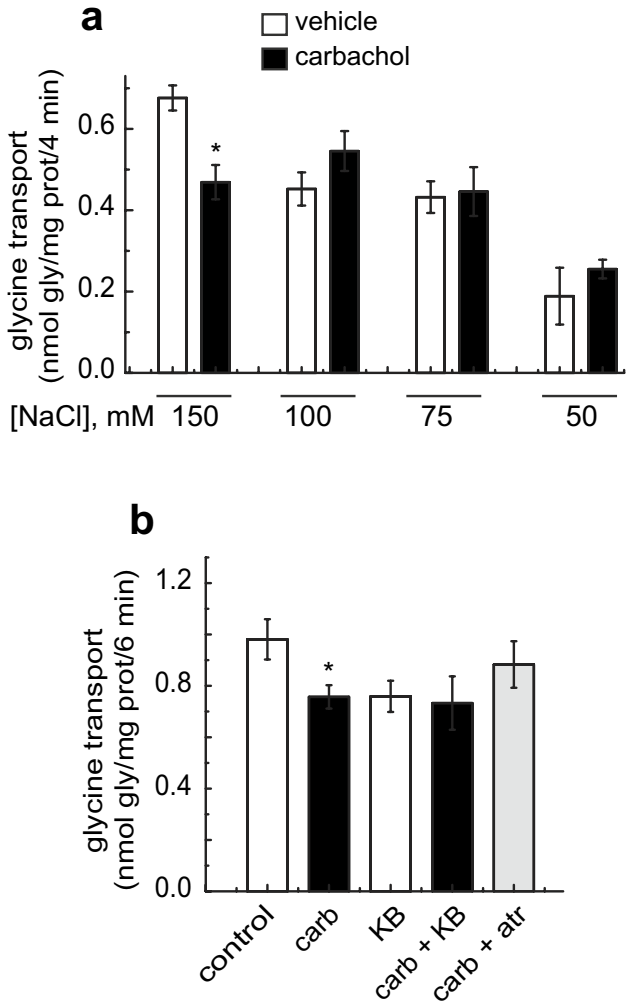

Fig. 7 Effect of low external sodium and inhibition of NCX on glycine transport down-regulation by carbachol. **a** COS7 cells co-expressing M2 mAChRs and GlyT2 were incubated for 5 min at 37ºC with vehicle or 250 µM carbachol in HBS containing the indicated NaCl concentrations (N-methylglucamine substitution). Glycine transport activity was measured during 4 min in the same medium. *Significantly different from vehicle, *p < 0.05 by Student´s t test. **b** COS7 cells co-expressing M2 mAChRs and GlyT2 were incubated for 15 min at 37ºC with vehicle or 250 µM carbachol in the absence or presence of 10 µM KB-R7943 mesylate. *Significantly different from vehicle, *p < 0.05 by Student´s t test

horn, the M2 mAChR, co-expressed with the transporter in COS7 cells. This heterologous system devoid of endogenous muscarinic receptor activity allows to focus on M2 mAChRs effects difficult to examine in brain-derived preparations, where the actions of the different mAChRs subtypes are compensatory [12–14]. We have demonstrated that GlyT2 and M2 mAChRs can be present in the same cells of the spinal cord and, therefore, M2 mAChRs can regulate GlyT2. We have looked for several possible mechanisms to explain the inhibitory action of M2 mAChRs and, using calcium imaging measurements, we found M2 mAChRs induce calcium transients in COS7 cells and these trigger the inhibition of GlyT2. As GlyT2 has been previously shown to be modulated by several proteins involved in calcium homeostasis such as the Na^+/K^+-ATPase [5], the PMCA and the ubiquitous NCX1 [6], it represents a good candidate for direct regulation by calcium raises produced by M2 mAChRs [41]. There are several signaling pathways triggered by M2 receptors including inhibition of adenylyl cyclase activity [10, 12], but many others involve the control of plasma membrane conductances [51, 52] or intracellular calcium mobilization [26–30].

Our first analysis of GlyT2 inhibition, revealed carbachol treatment mainly affects the Km of glycine transport suggesting M2 mAChRs modulates the transport activity of the transporters present at the plasma membrane. Since inhibition of ER calcium exit pathways do have only a minor effect, the inhibitory mechanism seems to get restricted to the plasma membrane. The blocking of many of the potassium channels sometimes coupled to M2 mAChRs signaling including GIRK, did not alter GlyT2 inhibition by M2 mAChRs. Calcium channels were also not involved in the inhibitory mechanism, and these observations suggest the inhibition of GlyT2 by carbachol is not a direct consequence of plasma membrane depolarization but may involve an event itself controlled by plasma membrane voltage. For this reason, we turned to the subcortical area and found that the inhibition of GlyT2 by carbachol was extremely dependent on the external sodium concentration and was abolished when external sodium was reduced and in the presence of KB-R7943 mesylate an inhibitor of the NCXrev. Inhibition by KB-R7943 mesylate, might reduce the subcortical calcium concentration by allowing calcium extrusion by NCX forward mode and may compensate carbachol-induced calcium raise. In summary, the results presented in this report are consistent with a GlyT2 sensitivity to intracellular calcium mobilized by M2 mAChRs in the subcortical area of the plasma membrane.

NCX is a critical molecule for the control of intracellular calcium that has been shown to modulate the increases in cytosolic calcium promoted by carbachol in other systems [30, 55]. In fact, the increases in cytosolic calcium by carbachol have been shown to be significantly attenuated by NCX inhibition in tracheal smooth muscle [30]. GlyT2 is sensitive to calcium but also to sodium present in the restricted plasma membrane-ER junctional space [6], the NCX, coupled to GlyT2 activity, and with high capacity for exchange, may modulate the carbachol regulation. Many of the experimental conditions we tested on the down-regulation of GlyT2 exerted by carbachol, have been reported to increase cytosolic calcium, a condition in which NCX is in its forward mode (calcium extrusion). The NCX forward mode might be inhibitory for GlyT2 since it accumulated sodium in the

submembranous space [6]. Sodium influx into the restricted subcortical space may inhibit GlyT2 by reducing the electrochemical sodium gradient necessary for glycine uptake or causing membrane depolarization [6]. Depolarization (and Na^+ load) are conditions that promote the reversal of NCX [30, 56]. We propose carbachol increases internal calcium what may promote NCX forward mode [35–37], without discarding carbachol treatment may require an active NCX [30, 55, 56] and its inhibition may block GlyT2 down-regulation. M2 mAChRs pharmacological and receptor knockout studies have proven that some of their antinociceptive effects in the spinal cord are elicited by presynaptic modulation of transmitter release that increases inhibitory and decreases excitatory neurotransmission [9]. The possible inhibition of GlyT2, a presynaptic glycinergic protein, by a mechanism involving intracellular calcium raises, may exert a proper coordination of neurotransmitter uptake and release that may trigger analgesic actions mediated by M2 mAChRs.

Future Directions

The regulation of GlyT2 by calcium is not fully understood. Whether GlyT2 directly detects intracellular calcium rises deserves future attention since this might be a mechanism with the potential of coordinating the presynaptic ionic composition during antinociception mediated by M2 mAChRs in the spinal cord. The investigation of M2 mAChRs in brain-derived preparations using siRNA experimental tools for selective mAChRs down-regulation will shed light not only in the role of calcium in GlyT2 regulation but in the role of M2 mAChRs in glycinergic neurotransmission and pain modulation.

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Author Contributions Conceived the work: BL-C performed the experiments: EJ, AF, RF and EN analyzed the data: BL-C and CA. Wrote the paper: BL-C.

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Declarations

Conflict of interest None.

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