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Phosphorylation of synapsin I and MARCKS in nerve terminals is mediated by Ca^{2+} entry via an Aga-GI sensitive Ca^{2+} channel which is coupled to glutamate exocytosis

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Abstract Ca^{2+} entry is a prerequisite for both exocytosis and the phosphorylation of synapsin I and MARCKS proteins in mammalian cerebrocortical synaptosomes. The novel spider toxin Aga-GI completely blocks KCl-evoked glutamate exocytosis but only partially inhibits KCl-evoked cytoplasmic Ca^{2+} elevations, thus revealing at least two pathways for KCl-induced Ca^{2+} entry. Aga-GI completely attenuates KCl-induced phosphorylation of synapsin I and MARCKS proteins. We therefore conclude that both exocytosis and the phosphorylation of synapsin I and MARCKS proteins are specifically coupled to Ca^{2+} entry via a subset of voltage dependent Ca^{2+} channels at the nerve terminal which are sensitive to Aga-GI.

Key words: Glutamate exocytosis; Nerve terminal; Phosphorylation; Calcium channel

1. Introduction

Glutamate exocytosis from mammalian CNS nerve terminals is dependent on localized Ca^{2+} influx through voltage dependent Ca^{2+} channels [1–3]. We have isolated a novel spider toxin, Aga-GI, and established that the Ca^{2+} trigger mediating glutamate exocytosis from mammalian cerebrocortical synaptosomes occurs via voltage dependent Ca^{2+} channels sensitive to Aga-GI, and not via L- or N-type voltage dependent Ca^{2+} channels [4,5]. The action of Aga-GI is distinct from ωAga-IVA in that Aga-GI can completely inhibit glutamate exocytosis under stimulatory conditions which optimize release [6,7]. However, at a concentration of Aga-GI which completely blocks KCl-evoked glutamate exocytosis, only a partial inhibition of KCl-evoked cytoplasmic free Ca^{2+} elevation, $[Ca^{2+}]_c$, is observed [4].

In addition to triggering exocytosis, [8,9], Ca^{2+} initiates many events associated with secretion in nerve terminals including cytoskeletal disassembly [10] and protein phosphorylation/dephosphorylation [3,11–13]. The relationship between protein phosphorylation and exocytotic events in nerve terminals is actively debated [14–18]. The neuron-specific synapsins I and II are localized almost exclusively in regions of the nerve terminal occupied by synaptic vesicles [19,20]. Depolarization-evoked Ca^{2+} entry activates Ca^{2+}-calmodulin-dependent protein kinase II (CaMKII) leading to phosphorylation of the C terminal of synapsin I [21,22] and this has been proposed to facilitate dissociation of synaptic vesicles from the actin cytoskeleton, thus increasing their availability for exocytosis [17,23]. Protein kinase C (PKC) dependent phosphorylation of MARCKS (myristoylated alanine rich C kinase substrate) [13,24,25] is also enhanced by depolarization-stimulated Ca^{2+} entry [26].

Thus Ca^{2+}-activated kinases instigate a sequence of phosphorylation events which occur in parallel with exocytosis. The ion channel responsible for gating the Ca^{2+} influx coupled to phosphorylation has not been identified although recently it has been shown that Ca^{2+} influx via L- or N-type voltage dependent Ca^{2+} channels does not support the phosphorylation of MARCKS in hippocampal slices [27].

Here we ask whether depolarization-evoked Ca^{2+} influx via the subset of Aga-GI sensitive Ca^{2+} channels, which are intimately coupled to glutamate exocytosis [4], is also responsible for the phosphorylation of synapsin I and MARCKS, or whether the portion of the KCl-evoked $[Ca^{2+}]_c$ elevation not blocked by the toxin and not coupled to release is responsible for synapsin I and MARCKS phosphorylation.

2. Materials and methods

2.1. Materials

Glutamate dehydrogenase (E.C. 1.4.1.3) was from Sigma Chemical Co. (Poole, UK). The enzyme was dialyzed under pressure to remove contaminating glutamate. Fura-2 acetoxymethylester was from Calbiochem Novabiochem (Nottingham, UK). ^{32}P was from Amersham International, Amersham, UK. *Agelenopsis aperta* venom was obtained from Spider Pharm, Feasterville, USA and Aga-GI purified as previously described [4]. Omega-conotoxin GVIA (ωCgTx GVIA) was obtained from RBI Research Biochemicals Inc, Semat Technical, UK. All other reagents were from Sigma Chemical Co. and BDH Laboratory Supplies (Poole, UK).

2.2. Synaptosomal preparation

Synaptosomes from the cerebrocortices of 6 week old male Wistar rats were purified as described by [28], with minor modifications [13]. Protein was determined by the Bradford method [29]. The final synaptosomal pellet was resuspended in 'HEPES-buffered medium' (HBM) consisting of 140 mM NaCl, 5 mM KCl, 5 mM $NaHCO_3$, 1 mM $MgCl_2$, 10 mM glucose, 10 mM HEPES and 1.2 mM NaH_2PO_4 (pH 7.4).

2.3. Glutamate release

Glutamate release was assayed by on-line fluorimetry as described previously [6]. Synaptosomal pellets (0.25 mg protein/ml) were incubated in HBM at 37°C in the presence of $NADP^+$ (1 mM), glutamate dehydrogenase (50 units/ml) and $CaCl_2$ (1 mM). After 10 min at 37°C, synaptosomes were depolarized by the addition of 30 mM KCl. Aga-GI (500 nM final concentration) was added 30 s prior to depolarization where indicated. Fluorescence was monitored in a Perkin-Elmer LS5B

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spectrofluorometer at 340 nm (excitation) and 460 nm (emission), and data were accumulated at 2 s intervals. A standard of exogenous glutamate (2.5 nmol) was added at the end of each experiment. The value of the fluorescence change produced by the standard addition was used to calculate the released glutamate as nmol glutamate release/mg of synaptosomal protein.

2.4. Cytosolic Ca^{2+} measurements

Synaptosomes (0.25 mg/ml) were incubated in HBM as described above. At 5 min, 0.1 mM $CaCl_2$ and 5 μM fura-2-acetoxymethyl ester were added; at 30 min, the synaptosomes were centrifuged in a microfuge for 15 s and resuspended in fresh HBM. $CaCl_2$ (1 mM) was added after 5 min, and further additions were made as detailed in the legends to figures. Fluorescence data were accumulated at excitation wavelengths of 340 and 380 nm with an emission wavelength of 505 nm at intervals of 3.4 s. Cytoplasmic free Ca^{2+}, $[Ca^{2+}]_c$, was calculated according to [30].

2.5. Synaptosomal phosphorylation

Synaptosomes (0.5 mg/ml) were prelabelled with [^{32}P]orthophosphate (1 mCi/mg) for 60 min in phosphate free HBM. Extra-synaptosomal [^{32}P]orthophosphate was removed by centrifugation of the synaptosomes in a microfuge at $10{,}000 \times g_{max}$ for 15 s. Synaptosomes were then resuspended to 0.5 mg/ml in HBM + phosphate and incubated for 10 min at 37°C in the presence of $CaCl_2$ (1 mM).

The labeled synaptosomes were incubated with Aga-GI (500 nM) or control buffer for 30 s prior to depolarization with 30 mM KCl as indicated in the legends. The final synaptosomal protein concentration was 0.3 mg/ml. Incubations were stopped by the addition of stop buffer to give a final concentration of 1% SDS, 6.25 mM Tris (pH 6.8), 5% (v/v) 2-mercaptoethanol, 10% (v/v) glycerol and 0.001% Bromophenol blue. Samples were boiled for 10 min and run on 7.5% SDS polyacrylamide gels. Gels were stained with Coomassie blue and destained. Where appropriate, the 75 kDa band containing both the MARCKS protein and the synapsins was excised from the wet gel, proteolyzed with *Staphylococcus aureus* V8 protease and rerun on a 15% SDS-polyacrylamide gel according to Wang and Greengard 1988, [13]. ^{32}P-labeled proteins were visualized by autoradiography or phosphorimaging (for the proteolytic fragments). Radioactivity in the latter was quantified by scanning the phosphorimages using a Molecular Dynamics program (Sunnyvale, CA, USA).

3. Results

At a concentration of 500 nM, Aga-GI totally inhibits the Ca^{2+}-dependent KCl evoked glutamate release from rat cerebrocortical synaptosomes (Fig. 1A). This is the first report that this toxin is able to block glutamate release from nerve endings derived from rat brain; previous reports have shown this toxin blocks exocytosis from guinea pig cerebrocortical synaptosomes [4]. At the same concentration, Aga-GI blocks 48.15 ± 1.26% of the KCl-evoked $[Ca^{2+}]_c$ elevation whether added before or after KCl (Fig. 1B). It should be noted that it is the plateau component of the fura-2 response which is coupled to glutamate exocytosis [1]. The Ca^{2+} elevation induced by the Ca^{2+} ionophore ionomycin is not blocked by Aga-GI (Fig. 1B); this is consistent with the locus of action of the toxin being at a voltage-dependent Ca^{2+} channel. These results resolve the KCl-evoked Ca^{2+} influx into two components; an Aga-GI sensitive component coupled to glutamate exocytosis and an Aga-GI insensitive component which is not coupled to release.

Fig. 2A shows a representative 7.5% SDS-polyacrylamide gel electrophoresis depicting the influence of KCl and Aga-GI on the phosphorylation profile of rat cerebrocortical synaptosomal proteins. It is qualitatively evident that the depolariza-

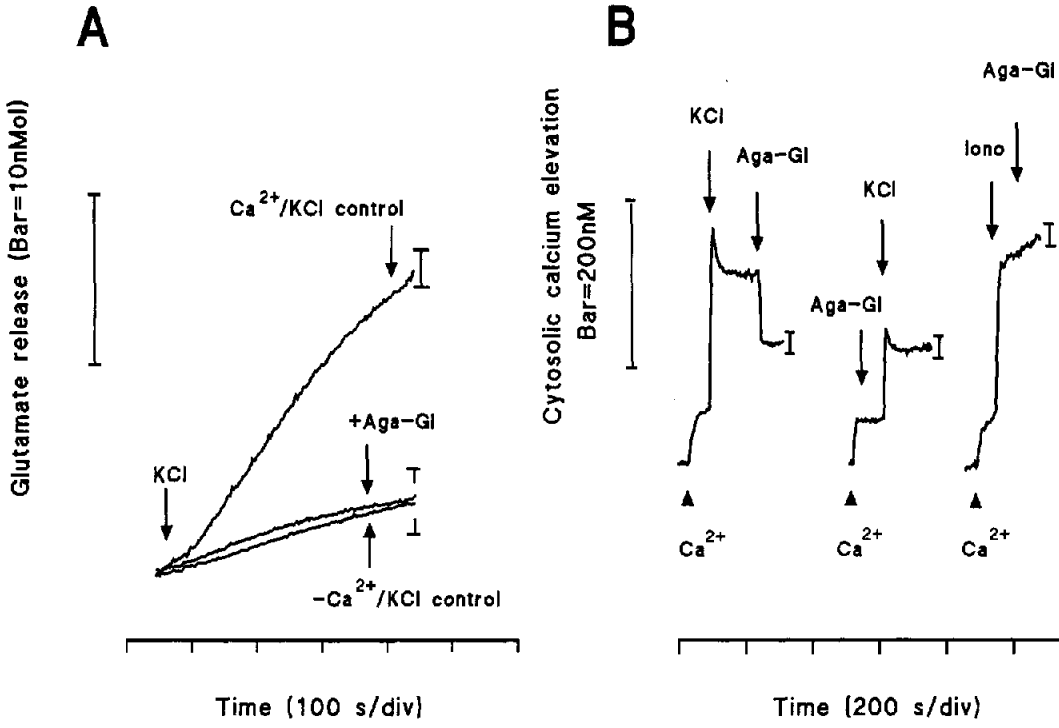

Fig. 1. Aga-GI blocks KCl-evoked glutamate exocytosis and $[Ca^{+}]_c$ elevations in rat cerebrocortical synaptosomes. (A) Glutamate release, assayed by on-line fluorimetry, was evoked by the addition of 30 mM KCl in the presence of 1 mM Ca^{2+} (Ca^{2+}/KCl control) or absence of external Ca^{2+} ($-Ca^{2+}$/KCl control). Preincubation with 500 nM Aga-GI for 30 s before KCl addition in the presence of 1 mM Ca^{2+} inhibited glutamate release to that produced in the absence of Ca^{2+}. Data are the means of 3 replicates ± S.E.M. (bar to side of each trace). (B) Increased $[Ca^{2+}]_c$ following 30 mM KCl stimulation was assessed with fura-2. 500 nM Aga-GI added before KCl (i) or after (ii) attenuated a maximum of 48% of the plateau $[Ca^{2+}]_c$ elevation. 0.25 μM ionomycin-induced $[Ca^{2+}]_c$ elevations are not attenuated by Aga-GI (iii). Data are the means of 3 replicates ± S.E.M. (bar to side of each trace).

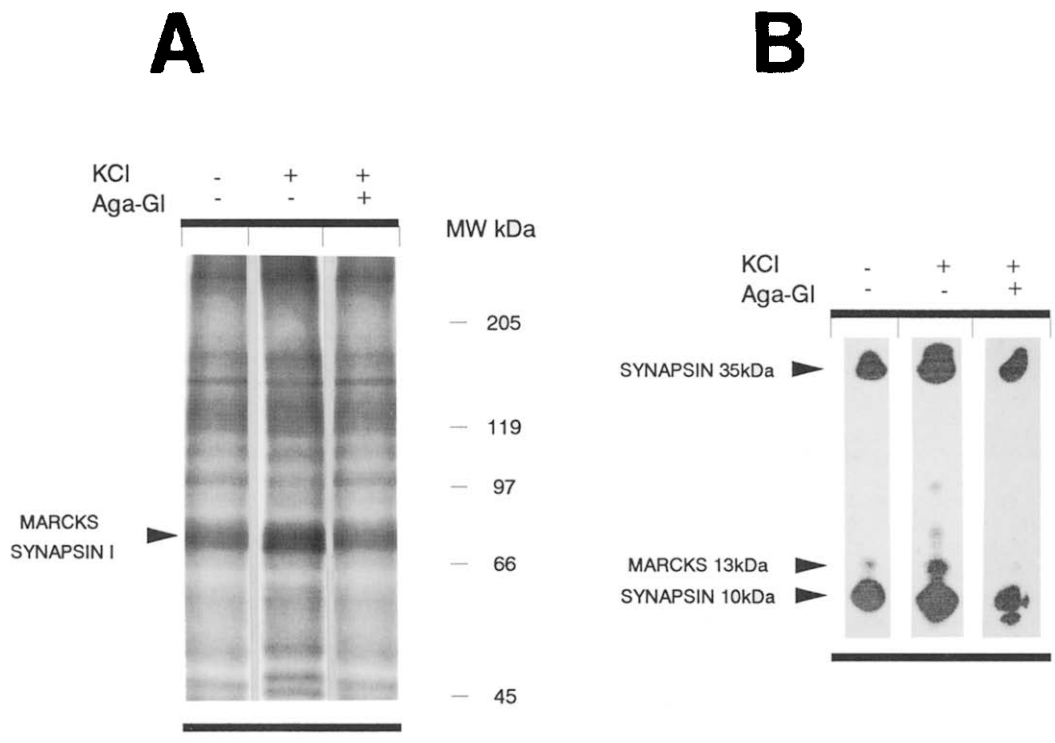

Fig. 2. Phosphorylation profile of rat cerebrocortical synaptosomal proteins on depolarization with KCl ± Aga-GI. (A) A representative autoradiogram of synaptosomal phosphoproteins following 7.5% polyacrylamide gel electrophoresis of ^{32}P-prelabeled synaptosomes incubated in the presence of 30 mM KCl, 1 mM Ca^{2+} ± 500 nM Aga-GI. Aga-GI and KCl were added 45 s and 15 s respectively prior to quenching. (B) A representative autoradiogram of phosphopeptides contained in the 75 kDa MARCKS/synapsin I band excised from a 7.5% polyacrylamide gel processed according to (A), digested with limited *S. aureus* V8 protease treatment and separated on a 15% polyacrylamide gel. This allows quantification of the 35 kDa and 10 kDa synapsin I peptides and the 13 kDa MARCKS protein.

tion-evoked Ca^{2+}-dependent phosphorylation of a number of proteins is inhibited by prior treatment with Aga-GI. In particular, Aga-GI inhibits the phosphorylation of a group of phosphoproteins migrating as a 75 kDa band on SDS-PAGE. Since synapsins I and II, and MARCKS are contained in this group, this broad band was further dissected by limited proteolysis as described in section 2. Three major bands were obtained by this treatment (Fig. 2B) which have been shown to correspond, respectively, to a 35 kDa peptide encompassing the two CaMK phosphorylation sites of synapsin I, a 13 kDa MARCKS-derived peptide and a 10 kDa band which is a composite of a MARCKS peptide and the PKA/CaMKI site of synapsin I [13].

The data from multiple replicate phosphorylation experiments are expressed quantitatively in Fig. 3. It is clear that depolarization-enhanced phosphorylation of MARCKS and synapsin I is totally inhibited. The toxin also inhibited depolarization-enhanced phosphorylation of synapsin 35 kDa and 10 kDa proteins from guinea pig cerebrocortical synaptosomes to 1.43 ± 27.14% and 17.91 ± 14.93% of control KCl-induced phosphorylation. This in turn implies that the kinases responsible for the phosphorylation of MARCKS, i.e. PKC, and synapsin I (Ca^{2+}-calmodulin-dependent protein kinase I, CaMKII and protein kinase A [17] are not activated in the presence of the toxin. The L-type voltage dependent Ca^{2+} channel antagonist, nifedipine (1 μM), the N-type voltage dependent Ca^{2+} channel antagonist ωCgTx GVIA (1 μM), and the P-type voltage dependent Ca^{2+} channel antagonist ωAgaIVA had no significant effects on synapsin I and MARCKS phosphorylation following KCl stimulation (data not shown).

4. Discussion

At a concentration of 500 nM, Aga-GI completely attenuates glutamate exocytosis from rat cerebrocortical synaptosomes but only blocks a maximum of 48% of the plateau KCl-evoked $[Ca^{2+}]_c$ elevation. The toxin also completely blocks the KCl-evoked phosphorylation of synapsin and MARCKS proteins.

The total inhibition of phosphorylation in the presence of Aga-GI is not due to a reduction of the bulk cytoplasmic free Ca^{2+} concentration below a critical threshold for activation of the kinases. Thus suboptimal depolarization of rat cortical synaptosomes with 10 mM KCl, sufficient to cause a 60% decrease in the maximal release of glutamate and a similar decrease in the fura-2 plateau, still allows a proportionate phosphorylation of the MARCKS/synapsin band to be observed [3]. This in turn amplifies the previous conclusion [3], that the pathway of Ca^{2+} entry is critical for the phosphorylation of these proteins.

While structural data on presynaptic Ca^{2+} channels are almost non-existent, pharmacological studies suggest that multiple channel sub-types might co-exist on individual terminals to control Ca^{2+} entry [31], and neurotransmitter release [7,32–34]. Under the present conditions of stimulation, no modulation of glutamate release is observed with L, N or P type voltage dependent Ca^{2+} channel antagonists [4,7]. Thus the channel coupled to release and kinase activation is a specific subtype

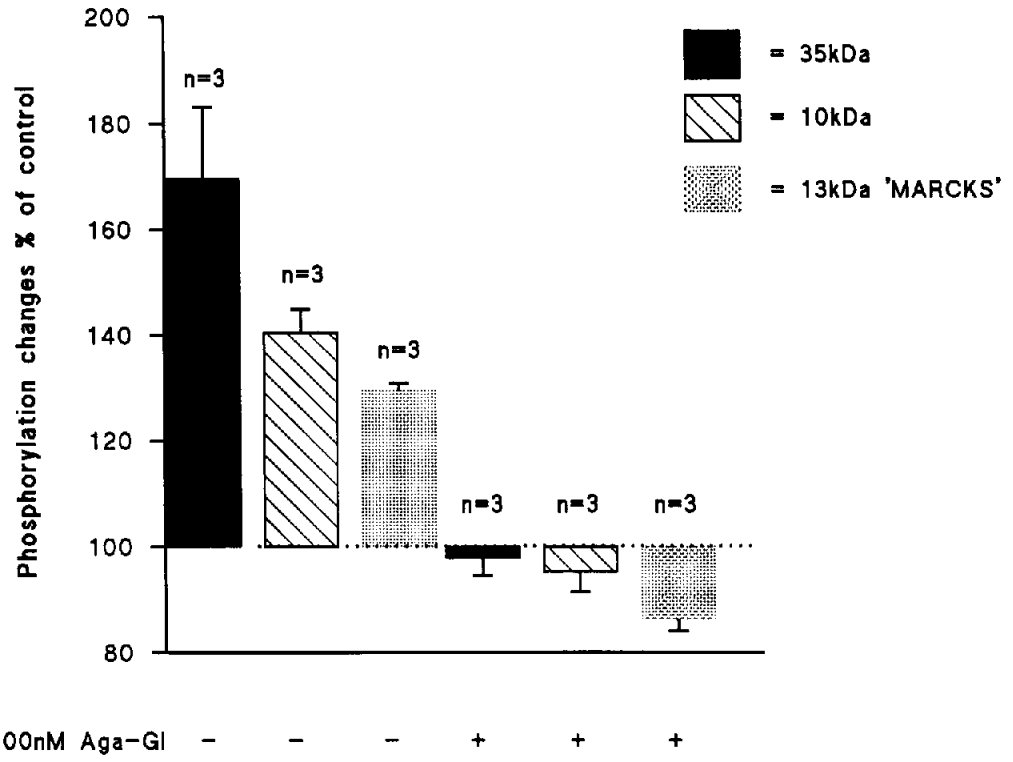

Fig. 3. Aga-GI blocks the depolarization evoked phosphorylation of synapsin I and MARCKS Meaned data of replicate experiments performed as described in Fig. 2A,B depicting the 35 kDa and 10 kDa Ca^{2+}/calmodulin (CaMKI and II) and PKA phosphorylated synapsin I peptides and the 13 kDa PKC phosphorylated MARCKS peptide are shown. Data are expressed as a percent of the control phosphorylation obtained in the absence of a KCl stimulus ($n = 3 \pm$ S.E.M.).

which can be blocked by Aga-GI. Ca^{2+} influx via L- or N-type voltage dependent Ca^{2+} channels failed to influence the phosphorylation of MARCKS in hippocampal slices [27]. Since P- and N-type channels in hippocampal slices are able to modulate glutamate exocytosis [34], it is of interest that ωCgTx GVIA had no effect on phosphorylation events.

The complete block by Aga-GI of the KCl-evoked glutamate exocytosis and the phosphorylation events described above suggests that there is a tight coupling between Ca^{2+} entry via this channel and the phosphorylation of synapsin I and MARCKS. This may be a result of either colocalization of synapsin I and MARCKS proteins with Aga-GI sensitive Ca^{2+} channels, or colocalization of synapsin I and MARCKS specific Ca^{2+} dependent kinases with these Ca^{2+} channels.

This is the first report to identify a specific voltage-dependent Ca^{2+} channel which modulates both glutamate exocytosis and the phosphorylation of synapsin I and MARCKS. Although we have shown that phosphorylation events which occur in cerebrocortical synaptosomes are not a primary requirement for KCl-stimulated glutamate exocytosis [16,35,36], recent evidence suggests a modulatory role for synapsins in synaptogenesis [37] or short term plasticity [38].

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