Properties of ω conotoxin MVIIC receptors associated with α1A calcium channel subunits in rat brain

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Abstract Solubilized ^{125}I-ω conotoxin MVIIC receptors from rat cerebellum were immunoprecipitated by antibodies directed against the calcium channel α1A subunit. Anti-α1A antibodies recognized a 240–220, 180 and 160 kDa proteins in immunoblots of cerebellar membranes. Disuccinimidyl suberate cross-linked ^{125}I-ω conotoxin MVIIC to an α2δ-like 200–180 kDa subunit, which migrated at 150–140 kDa after disulfide reduction. These observations are consistent with a heteromeric structure in which high affinity ω conotoxin MVIIC binding sites formed by α1A subunits are located in close proximity to peripheral α2 subunits.

Key words: Calcium channel; ω conotoxin MVIIC receptor; α1A subunit; α2 subunit

1. Introduction

Voltage gated calcium channels in neurons have been classified as L, N, P, Q and T type, on the basis of their electrophysiological and pharmacological properties [1–3]. Calcium channels contain a pore-forming α1 subunit that carries binding sites for channel blockers, and is generally associated with peripheral α2δ and β subunits [4–6]. A family of cDNAs encoding homologous α1 (A, B, C, D, and E) subunits have been isolated from neuronal libraries [7,8]. Important aims at present are to establish a correlation between calcium channel antagonist binding sites expressed in the brain and a given class of α1 subunit and to examine the polypeptide composition of the different calcium channel classes expressed in the brain.

Specific neurotoxins are powerful tools in this field. For example irreversible block by ω conotoxin GVIA (ωGVIA) from the marine mollusc *Conus geographus* [9], is a property unique to N-type channels and ωGVIA receptor sites were subsequently shown to be associated with the α1B subunit in the brain [5,6,10] and in heterologous expression systems [11]. A structurally related peptide from Conus magus, ω conotoxin MVIIC (ωMVIIC), blocks a population of ωGVIA-insensitive calcium channels that are expressed in both the soma and synaptic terminals of central neurones [3,12,13]. Binding studies with rat brain membranes indicate that ωMVIIC and ωGVIA have distinct receptor sites [12,14]. Expression of the α1A subunit in *Xenopus* oocytes supports calcium currents that display sensitivity to sub-micromolar concentrations of ωMVIIC [15], however little is known about the polypeptide composition of the native calcium channels in the mammalian brain that constitute high affinity ωMVIIC receptors. In the present study we have used affinity labeling methods and sequence directed anti-α1 antibodies to address this question.

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Abbreviations: ωGVIA, ω conotoxin GVIA; ωMVIIC, ω conotoxin MVIIC; ωAgaIVA, ω agatoxin IVA; DSS, disuccinimidyl suberate; BSA, bovine serum albumin; TBS, Tris-buffered saline; CHAPS, (3-[(3-Cholamidopropyl)-dimethylammonio]-1-propane-sulfonate); DHP, 1,4-dihydropyridine.

2. Experimental

2.1. Materials

Synthetic ωMVIIC was obtained from Bachem (Bubendorf), ωGVIA from the Peptide Institute (Osaka), and disuccinimidyl suberate (DSS) from Pierce (Interchim, France). Protein A Sepharose CL4B was from Sigma (St. Louis MO) and N-glycosidase F from Boehringer (Mannheim).

2.2. Membrane preparations

Cerebella or cerebra from one-month-old Wistar rats were dissected and homogeneized in a glass/teflon Thomas apparatus in a 0.32 M sucrose, 10 mM HEPES buffer adjusted to pH 7.4 with NaOH, in the presence of a mixture of protease inhibitors: 0.2 mM phenylmethylsulfonyl fluoride, 1 mM iodoacetamide, 2 μM pepstatin A, 1 mM EDTA, 2 μM leupeptine, 0.15 μM aprotinine, and 2 mM benzamidine. After a 10 min centrifugation at 800 × *g*, the supernatant was recovered and spun at 27,000 × *g* for 40 min. Protein was assayed by the BCA method (Pierce) using a BSA standard.

2.3. Conotoxin iodination and binding experiments

Preparation and binding of mono[^{125}I]-iodo ωGVIA were performed as previously described [16,17]. Mono[^{125}I]-iodo ωMVIIC was prepared by the iodogen method using Na^{125}I (New England Nuclear 2,000 Ci/mmol) and purified by HPLC on an analytical C18 column (Beckmann) [12]. The binding of ^{125}I-ωMVIIC to synaptosomal membranes was measured at 30°C in 100 μl Tris-buffered saline: 150 mM NaCl, 25 mM Tris base adjusted to pH 7.4 (TBS), containing 0.1% BSA. Binding was initiated by addition of 20 μg of membrane protein, stopped by rapid filtration on glass fiber filters (GFC-Whatman) pretreated with 0.3% polyethyleneimine and washed three times with 2 ml of ice-cold TBS containing 0.1% BSA, 1.5 mM $CaCl_2$, 0.05% Tween-20.

2.4. Affinity labeling

Synaptosomes (300 μg) were incubated for 2 h at 4°C with 1 nM ^{125}I-ωMVIIC in the presence or absence of 1 μM unlabeled ωMVIIC in 300 μl of 20 mM HEPES, 0.15 M NaCl, adjusted to pH 8.3 with NaOH. DSS solubilized in dimethylsulfoxide was added to a final concentration of 30 μM, and the mixture was incubated for 20 min at 20°C. The synaptosomes were sedimented, washed once with 20 mM HEPES, 0.15 M NaCl, 10 mM $CaCl_2$ adjusted to pH 8.3 with NaOH, and once with the same buffer from which $CaCl_2$ was omitted. Synaptosomes were again incubated with 1 nM ^{125}I-ωMVIIC and the cycle repeated as described above. The labelled membranes were processed for electrophoresis as described below.

2.5. Preparation of antibodies

Polyclonal antisera were produced in rabbits using synthetic peptides

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selected from calcium channel α1 sequences, coupled to keyhole limpet hemocyanin. RbA-1 antibodies were raised against the peptide (C)PSSPERAPGREGPYGRE, and rbA-2 antibodies against the peptide LRQTARPRESARDPDA corresponding to residues 865–881 and 845–860, respectively from the rat brain α1A sequence [18]. Antisera were raised against peptides from the two alternative C-terminal regions of the rabbit brain sequence BI [19], BI-1 antibodies against the peptide (C)RDQRWSRSPSEGRQHTTHRQ and BI-2 antibodies against the peptide (C)GGRRWTASAGKGGGGPRASA, corresponding to BI-1 residues 2,254–2,273 and BI-2 residues 2,402–2,421 respectively. IgG were purified by affinity chromatography on protein A-Sepharose CL4B.

2.6. Immunoprecipitation

Membranes were incubated with 0.5 nM ^{125}I-ωMVIIC overnight at 4°C in binding buffer, pelleted at 10,000 × *g* for 10 min, and solubilized in 1% CHAPS in buffer A (0.32 M sucrose, 10 mM HEPES adjusted to pH 7.4). Protein A-Sepharose CL4B was swollen in TBS containing 0.5% BSA and incubated with antibodies for 1 h at 4°C. Following centrifugation, the pellet was washed with 0.4% CHAPS in buffer A and solubilized membrane proteins containing 3 fmol of ωMVIIC receptor were added in a final volume of 100 μl and incubated for 3 h at 4°C. The pellet was washed once with TBS, 0.5% BSA, and the immunoprecipitated radioactivity was counted.

2.7. Electrophoresis and Western blotting

Proteins were denatured at room temperature for 30 min in 3% SDS, 6% sucrose, in 70 mM Tris-HCl at pH 8.9, in the presence or absence of 25 mM dithiothreitol. Iodoacetamide was then added to a final concentration of 80 mM. SDS-PAGE was performed on 5–12% gradient gels. Proteins were transferred to nitrocellulose membranes (Hybond, Amersham) at 100 mA overnight. The membrane was blocked for 2 h at room temperature with 10% dried milk in TBS and then incubated with antibodies (50 μg/ml) in blocking buffer at 4°C overnight. After extensive washing in 0.05% Tween-20 TBS, detection was performed with anti-IgG peroxidase and the ECL system (Amersham).

2.8. Deglycosylation

Affinity-labeled protein bands were electroeluted from SDS gels in 50 mM Tris, 0.1% SDS, 0.38 M glycine adjusted to pH 8.8 with HCl, diluted five-fold in 0.1 M NaP_i, 0.1% SDS, 0.5% CHAPS, 10 mM EDTA, 1% β-mercaptoethanol, pH 8, and concentrated on Ultrafree TTK24 (Millipore) membranes. N-glycosidase F (2 units) was added and incubated for 24 h at 37°C. Proteins were denatured and separated by SDS PAGE as described.

3. Results and discussion

3.1. ^{125}I-ω conotoxin MVIIC binding to cerebellar membranes

Specific binding of 0.5 nM ^{125}I-ωMVIIC to cerebellar membranes reached equilibrium in about 30 min at 30°C and pH 7.4 (Fig. 1A). In these conditions non-specific binding determined in the presence of 0.1 μM unlabeled ωMVIIC, accounted for approximately 50% of the total bound radioactivity. The addition at equilibrium of a large excess of unlabeled ωMVIIC initiated dissociation with 50% release occuring at about 10 min (Fig. 1B). Semi-logarithmic plots of the association and dissociation kinetics (insets to Fig. 1A,B) allowed measurement of the apparent association rate constant (k_{app}) = 7.7×10^{-4} s^{-1} and the dissociation rate constant (k_{-1}) = 5.5×10^{-4} s^{-1}, and subsequent calculation of the association rate constant k_1 = 0.5×10^6 $s^{-1} \cdot M^{-1}$. The equilibrium dissociation constant was therefore ($K_D = k_{-1}/k_1$) = 1.8 nM, which was in good agreement with results obtained from displacement of ^{125}I-ωMVIIC by unlabeled ωMVIIC (K_D = 1 nM) at equilibrium (not shown). Direct receptor saturation experiments were not performed. However from experiments carried out with a ligand concentration = K_D, a total receptor capacity of approximately 0.9 pmol/mg protein

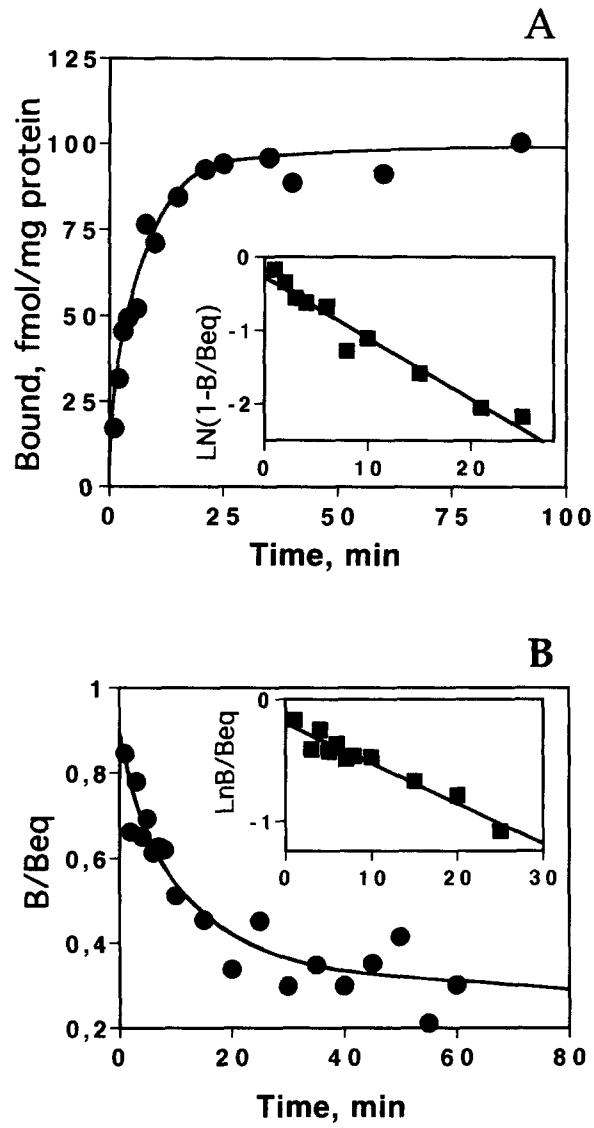

Fig. 1. Binding kinetics of ^{125}I-ω conotoxin MVIIC to rat cerebellar membranes. (A) Association kinetics: membranes were incubated for the indicated time with 0.5 nM ^{125}I-ωMVIIC, in the presence (non-specific binding) or absence (total binding) of 0.3 μM unlabeled ωMVIIC. The specific binding component was determined by subtraction. B, Dissociation kinetics: at equilibrium, unlabeled ωMVIIC was added to obtain a final concentration of 0.3 μM and initiate release. The remaining specifically bound radioactivity was then measured at the indicated times. Insets A and B: linear semi-logarithmic plots. B = specifically bound, Beq = specifically bound at equilibrium.

in cerebellar synaptosomes and 0.4 pmol/mg protein in cerebral synaptosomes was estimated. ^{125}I-ωMVIIC binding was not displaced by unlabeled ωGVIA, or ω agatoxin IVA (ωAgaIVA) but was inhibited by Ca^{2+} with a K_i < 1 mM (Table 1).

3.2. Immunoprecipitation of ω conotoxin MVIIC receptors

RbA-1 antibodies were raised against a peptide from the cytoplasmic loop between domains II and III that is specific to the rat brain α1A subunit sequence [18]. The ability of these antibodies to recognize the ωMVIIC receptor was tested. The addition of protein A-Sepharose CL4B beads coated with increasing amounts of rbA-1 antibodies to CHAPS solubilized

^{125}I-ωMVIIC receptors resulted in concentration-dependent immunoprecipitation (Fig. 2A). After subtraction of background precipitation determined with non-immune rabbit IgG, approximately 50% of ωMVIIC receptors were specifically recognized at the plateau level. Immunoprecipitation was shown to be specific by two other criteria: block of recognition by the synthetic peptide used to raise rbA-1 antibodies, and lack of immunoprecipitation of solubilized ^{125}I-ωGVIA receptors (Fig. 2B). These results indicate that high affinity receptors for ^{125}I-ωMVIIC are associated with the α1A subunit.

BI-1 and BI-2 antibodies were designed to distinguish between two α1A splice variants expressed in rabbit brain that contain alternative C-terminal segments [19]. The peptide used to raise BI-1 displayed 85% identity with the rat brain α1A C-terminus [18]. Specific immunoprecipitation of about 15% of ^{125}I-ωMVIIC receptors was achieved with BI-1 antibody, but no reaction was detected with the BI-2 antibody (Fig. 2B). Some of the α1A subunits that constitute the ωMVIIC sensitive calcium channel in the cerebellum thus contain the variant BI-1 C-terminus.

3.3. Detection of α1A polypeptides by immunoblotting

In order to detect the α1A subunits expressed in the brain, immunoblots were probed with rbA-2 antibodies. RbA-2 antibodies were raised against a second peptide specific to the loop linking domains II and III of α1A. Although this antibody specifically immunoprecipitated a smaller fraction (approximately 35%) of ωMVIIC sensitive calcium channels than rbA-1, it was found to be more sensitive for staining of immunoblots. RbA-2 reacted with several bands in cerebellar synaptosomes (Fig. 3, lane 1). Specificity of recognition was ascertained by parallel experiments in which the cognate peptide was used to block antibody binding (Fig. 3, lane 2). A 240–220 kDa doublet that sometimes appeared as a single band, and two polypeptides of 180 and 160 kDa were identified, with the 180 kDa band producing the most intense reaction. Similar experiments with cerebral synaptosomes (Fig. 3, lanes 3 and 4), produced much lower levels of immunostaining, and only the 180 kDa band was consistently detected.

These results suggest that the 240–220 kDa doublet represents α1A subunits whereas the 180 and 160 kDa size forms may result from post-translational cleavage of the larger polypeptides. The reduction of disulfide bridges did not produce any change in electrophoretic mobility of these proteins (not shown).

3.4. Affinity labeling of ^{125}I-ω conotoxin MVIIC receptors

A homobifunctional reagent DSS was used to crosslink

Table 1
Effects of neurotoxins and Ca^{2+} on ^{125}I-ω conotoxin MVIIC binding

Assay conditions	Binding (%)
Control	100
0.3 μM ω conotoxin GVIA	103
0.3 μM ω agatoxin IVA	93
0.3 mM $CaCl_2$	64
1 mM $CaCl_2$	32

Assays were performed with 0.5 nM ^{125}I-ωMVIIC in the presence and absence of 0.1 μM unlabeled ωMVIIC. Specific binding was calculated by subtraction and represented as a % of the control. Results are the mean of duplicate determinations from two independent experiments.

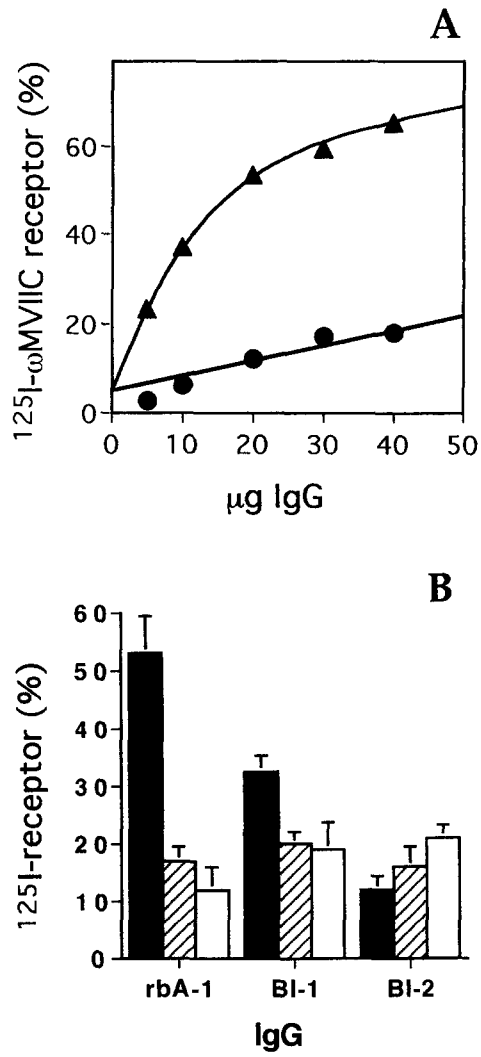

Fig. 2. Immunoprecipitation of ^{125}I-ω conotoxin MVIIC receptors. (A) Solubilised ^{125}I-ωMVIIC-labeled receptors were incubated with protein A-Sepharose CL4B beads coated with rbA-1 (triangles) or control IgG (circles). Labeled immune complexes were recovered by centrifugation and counted. (B) Solubilised ^{125}I-ωMVIIC receptors (solid and hatched bars) or ^{125}I-ωGVIA receptors (open bars) were incubated as in A with anti-α1A antibodies alone (solid and open bars) or after preincubation with the synthetic peptide used to raise the antibody (hatched bars).

^{125}I-ωMVIIC to membrane polypeptides in proximity to the binding site (Fig. 4). When electrophoresis was performed in non-reducing conditions, labeled proteins of 200 and 180 kDa were detected by autoradiography (Fig. 4A, lane 1). However after disulfide reduction with dithiothreitol (Fig. 4A, lane 2), these proteins migrated as a poorly resolved 150–140 kDa doublet. Labeling was blocked by the presence of 0.1 μM ωMVIIC (Fig. 4A lanes 3 and 4), but not by 0.1 μM ωGVIA (not shown).

Treatment with dithiothreitol, which had no effect on the mobility of the immunostained α1A polypeptides, thus apparently dissociated an unlabeled disulfide-linked polypeptide of 40–50 kDa from both the 200 and the 180 kDa proteins. The change in electrophoretic mobility of these proteins upon reduction is similar to that of the α2 subunit of the skeletal muscle L type calcium channel, which is disulfide linked to a δ polypeptide, and displays extensive N-glycosylation [4]. Electroelution

and N-glycosidase F treatment of the proteins cross-linked to ^{125}I-ωMVIIC confirmed the presence of N-linked carbohydrate and indicated a core polypeptide mass of about 100 kDa (not shown), which is in good agreement with the electrophoretic mobility of deglycosylated α2 subunits from skeletal muscle calcium channels [4]. Control incubations in the absence of N-glycosidase F did not affect migration. Alpha 2 and δ polypeptides are encoded by a single gene [20], of which a single splice variant α2b is expressed in the nervous system [21].

Two neurotoxins, ωAgaIVA and ωMVIIC, are currently used to dissect ωGVIA-insensitive, DHP-insensitive calcium channel currents in central neurons and heterologous expression systems [22]. Low nanomolar concentrations of ωAgaIVA [23], but significantly higher concentrations of ωMVIIC [12], block P type channels, which are predominant in cerebellar Purkinje neurons. Preferential expression of class A transcripts in the cerebellum [18,19] has thus suggested that P type channels contain α1A subunits. However expression of α1A, α2 and β subunits in *Xenopus* oocytes induces currents with reversed sensitivity that are strongly blocked by ωMVIIC but only partially inhibited at high concentrations of ωAgaIVA [15]. Subsequently a native calcium current with similar properties has been identified in cerebellar granule cells and designated as the Q channel [3]. Evidence therefore suggests that α1A subunits support both P and Q type channels, although little is known about the heteromeric composition of native channels.

Our data suggests that high affinity ωMVIIC binding sites in the cerebellum, which may correspond to Q type calcium channels, are associated with α1A subunits. Although we have not directly addressed the interaction of β-subunits with ωMVIIC receptors, heterologous expression of α1A has indicated that the generation of robust currents requires co-expression of a β subunit [15,19]. Co-expression of a β subunit with the cardiac α1C subunit has been shown to increase the binding affinity for dihydropyridines [24]. By analogy it is possible that β subunits may play a role in defining the sensitivity of α1A subunits to ωMVIIC and ωAgaIVA.

Chemical cross-linking suggests proximity of an α2-like subunit to receptor-bound ωMVIIC, although conotoxin binding sites are thought to be formed by α1 subunits. This is not without precedent as cross-linking of ωGVIA to an α2-like subunit in chick but not rat brain membranes has been reported [25], although the α1B subunit is now known to constitute the binding site. Subtle differences in the spatial relationships of homologous conotoxin binding sites to the α2 protein may therefore exist between different species and channel classes. Negative charges at the surface of these highly glycosylated subunits may conceivably affect the access of different toxins to their pharmacological sites. Consequently we may speculate that heterogeneity in the glycosylation of α2 subunits may also be relevant to differences in the potency of ωMVIIC and ωAgaIVA in blocking P and Q type currents supported by the α1A subunit.

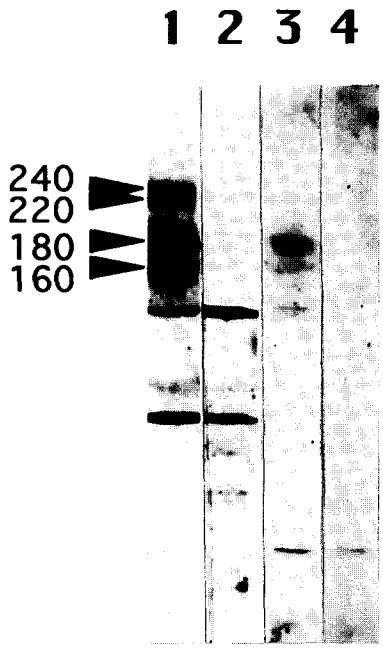

Fig. 3. Immunoblot detection of α1A subunits in rat brain membranes. Immunoblots of cerebellar (lanes 1 and 2) or cerebral (lanes 3 and 4) membranes were probed with rbA-2 antibodies alone (1,3) or with rbA-2 antibodies that had been preincubated with the rbA-2 peptide (2,4). Immunoreactive proteins were revealed by incubation with anti-IgG peroxidase and chemiluminescent detection.

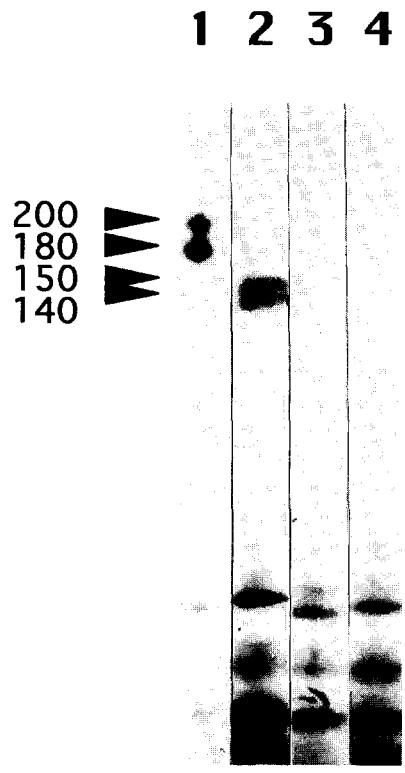

Fig. 4. Covalent cross-linking of ^{125}I-ω conotoxin MVIIC to receptor associated proteins. Cerebellar membranes were incubated with ^{125}I-ωMVIIC in the presence (lanes 3 and 4) or in the absence (lanes 1 and 2) of 0.1 μM unlabeled ωMVIIC. Disuccinimidyl suberate was added to cross-link the ligand to receptor proteins. Samples were prepared for SDS PAGE after alkylation (lanes 1 and 3) or after disulfide bridge reduction followed by alkylation (lanes 2 and 4). After SDS PAGE labeled polypeptides were detected by autoradiography.

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