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Author(s)	藤本, 一朗
Citation	大阪大学, 1994, 博士論文
Version Type	VoR
URL	https://doi.org/10.11501/3094139
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Structure of MCD Peptide Receptor Involved in Induction of Long-Term Potentiation of Synaptic Transmission Efficiency, and Its Functional Regulation by MCD Peptide

ICHIRO FUJIMOTO

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ABBREVIATIONS

BCA : bicinchoninic acid

BSA : bovine serum albumin

DMEM : Dulbecco's modified Eagle's medium

DTxI : Dendrotoxin I

EDTA : ethylene-diamine tetraacetic acid

ELISA : Enzyme-linked immunosorvent assay

FITC : fluorescein isothiocyanate

IgG : immunoglobulin G

MCD : mast cell degranulating peptide

NBT : nitroblue tetrazolium

PAGE : polyacrylamide gel electrophoresis

PBS : phosphate-buffered saline

PMSF : phenylmethylsulfonylfluoride

SDS : sodium dodecylsulfate

TBS : Tris-buffered saline

TEMED : N,N,N',N'-tetramethylethylenediamine

TFA : trifluoroacetic acid

X-phosphate : 5-bromo-4-chloro-3-indoryl phosphate

SUMMARY

Long-term potentiation (LTP) is an enhancement of synaptic transmission induced by brief high-frequency stimulation (tetanus) applied to the afferent fibers of the hippocampal formation. Because of its long-lasting, input-specific, cooperative and associative properties, LTP is widely considered to be the cellular basis of learning and memory in the brain. Cherubini et al. (1987) have reported that Mast Cell Degranulating peptide (MCD), purified from bee venom (*Apis mellifera*), induces LTP in the CA1 region of the hippocampus. MCD, which consists of 22 amino acid residues, had been synthesized and this synthetic MCD was also capable of inducing LTP in the CA1 region of the hippocampus. We have been investigating the mechanism by which MCD induces LTP, and found that two different functions of MCD are both required for MCD to induce LTP. These functions are (1) MCD to bind to its high affinity binding sites in rat brain and (2) to activate G proteins. The aim of this study was to purify and characterize the high affinity binding site for MCD in rat brain, and to identify signaling pathway located down stream of the G protein activated by MCD.

(1) Purification and characterization of the high affinity binding sites for MCD in rat brain.

I used an affinity chromatography to purify high affinity binding sites of MCD from rat brain. DTxI was selected as the ligand of choice due to its higher affinity for the MCD binding sites and also its inducibility of LTP in hippocampal slice. DTxI

was purified from the venom of the black mamba *Dendroaspis polylepis polylepis* by three steps column chromatography, including gel filtration on Sephadex G-50 fine, ion exchange HPLC on TSK SP-5PW and reverse-phase chromatography HPLC on COSMOSIL C_{18} . DTxI was coupled to CNBr-activated Sepharose 4B. High affinity binding sites were purified from rat plasma membrane which was solubilized with 4 % Lubrol PX, by using DTx-Sepharose affinity column chromatography and DE-5PW ion exchange HPLC. Purified samples were reconstituted in liposomes and dialyzed for 48 h to remove the detergent. Liposome was then incorporated into the planar bilayer membrane. DTxI binding protein which I purified reconstituted potassium channel. Therefore, it is shown that DTxI binding proteins itself is the DTxI-sensitive voltage-dependent potassium channels. Moreover, I clarified that this channel had a high affinity binding site for MCD and the current produced by this channel was blocked by MCD. The channels reported to be sensitive to DTxI and MCD are RCK1, RCK5 and RCK2. Therefore, to investigate which channel is responsible for mediating LTP induction by MCD in CA1 region of hippocampus, I produced antibodies to RCK1, RCK2 and RCK5 channels for immunohistochemical analysis. The highest level of RCK1 immunoreactivity was found in pyramidal cell layer of CA1 region which was close to that of N-biotinyl-MCD binding sites. RCK2 and RCK5 had less immunoreactivity in the CA1 region of hippocampus. Moreover, strong immunoreactivity for RCK1 in hippocampal primary cultured cells was found in triangular cells, which resembles pyramidal neuron cells morphologically. This locali-

zation was also very close to that of N-biotinyl-MCD binding sites. Immunoreactivity of RCK2 was similar to GFAP staining, which indicates that RCK2 channel is present in glial cell in culture. Immunoreactivity for RCK5 was found in both type of cells, pyramidal cells and glial cells, however it was very weak. These results strongly indicate that RCK1 channel is responsible for MCD binding in pyramidal cells in CA1 region of hippocampus. Current produced by RCK1 channel has been shown to be rapidly activating and non-inactivating current *in vitro* using *Xenopus* oocytes expression system. However, we found that MCD reduced the peak amplitude of fast-inactivating (A-type) current and showed no effect on non-inactivating (K-type) current in hippocampal slice preparation. Therefore, I hypothesized that the A-current suppressed by MCD in the pyramidal cell is mediated by a heteromultimeric channel composed of RCK1 and other potassium channel protein of RCK subfamily. Under immunohistochemical analysis of mouse hippocampal primary culture and paraffin section of rat brain, the staining of anti-RCK4 co-localized with RCK1 and N-biotinyl-MCD binding site. It was reported that RCK4 channel can mediate transient A-type potassium current and also RCK4 could form mixed subunit channels with RCK1 in *Xenopus* oocytes to mediate fast inactivating current. Therefore, the high affinity binding sites for MCD in hippocampal CA1 region is highly likely to be heteromultimeric channel composed of RCK1 and RCK4 proteins.

(2) Identification of the signaling pathway following G protein activation by MCD.

During the course of studies on reconstituting potassium channels in a lipid bilay-

er, another channel with a large conductance was found even in the presence of 50 nM MCD. This channel closed after further addition of 100 nM MCD to give final concentration of 150 nM. This concentration of MCD that was required for suppressing a large conductance potassium current was quite similar to the concentration of MCD needed for G protein activation. Thus, the presence of G protein in this fraction was examined. Immunoblot analysis for G protein subunits and ADP-ribosylation by pertussis toxin, IAP, indicated that both Go and Gi were present in the purified fraction in an active form. Therefore, I investigated the effect of G protein activator on the current produced by the purified sample in the lipid bilayer system. Addition of GTP-γ-S to *cis* chamber produced significant change in the current. Therefore, this potassium channel showing a large conductance is suppressed directly by the activated G protein. Thus, I identified that one of the targets located down stream of the G protein activated by MCD is a potassium channel. Many G protein-coupled potassium channels have been found in the brain, however, most of them are inwardly rectifying potassium channel. Therefore, the channel I found was novel, and a future challenge will be to characterize and determine the functional significance of this MCD-sensitive currents.

INTRODUCTION

Repetitive activation of some types of excitatory synapses results in a long-lasting enhancement of synaptic transmission referred to as long-term potentiation (LTP). This use-dependent plasticity provides a useful cellular model for learning and memory. Understanding of cellular mechanisms of LTP comes largely from investigations of the CA1 and dentate regions of the hippocampus (Collingridge and Bliss, 1987; Nicoll et al., 1988) although a similar phenomenon occurs at several synapses in brain (Baranyi and Szente, 1987; Artola and Singer, 1987; Komatsu et al., 1988; Perkins and Teyler, 1988). The current model for the induction of LTP in the CA1 region requires simultaneous N-methyl-D-aspartate (NMDA) receptor activation and postsynaptic depolarization (Collingridge et al., 1983). The depolarization relieves a voltage dependent Mg^{2+} block of the NMDA receptor channel, and entry of Ca^{2+} through the NMDA receptor channel appears to serve as the trigger for LTP. Cherubini et al. (1987) have reported that Mast Cell Degranulating peptide (MCD), purified from bee venom (*Apis mellifera*), induces LTP which is indistinguishable from NMDA receptor-mediated LTP in the CA1 region of the hippocampus (9, Fig 1). MCD consists of 22 amino acid residues and induce epileptiform activity and paroxystic seizures after intracerebroventricular (i.c.v.) injections to rats (Mehraban, F. et al. 1984). MCD produces hyperexitability in mice (Banks et al., 1978; Habermann, 1977), and is reported to bind with high affinity to a single set of sites (Kd=0.15 nM; Bmax=200 fmol/mg of protein) in mouse synaptosomal membranes (Taylor et al.

1984). So far, its basic pharmacological action on neuronal membranes is unknown; however, extensive studies have been conducted on the ability of MCD to induce degranulation of mast cells and it has been concluded that this activity is distinct from its action in the central nervous system (CNS).

During the course of study aimed at clarifying the pathway involved in MCD-induced LTP, we showed three functional aspects of MCD (Kondo et. al. 1992, Table 1).

1: High affinity binding sites for MCD (Kd= 0.15 nM) have been shown to be present in brain membranes and that the binding of ^{125}I-labeled MCD can be inhibited by Dendrotoxin I (DTxI) and vice versa, suggesting a coexistence of MCD and DTxI binding sites in the same protein complex. However, purification studies as well as in situ binding experiments showed that they are not completely identical (Rehm and Lazdunski, 1988; Bidard et al., 1989). DTxI is the most potent member of the Dendrotoxin family known until now. It facilitates transmitter release at peripheral (Harvey and Anderson, 1986) and central (Dolly et al., 1984, 1986) synapses, apparently due to a highly specific blockade of certain subtypes of voltage activated K^+ channel (Paxinos and Watson, 1982; Weller et al., 1985; Benoit and Dubois, 1986; Penner et al., 1986; Stansfeld et al., 1987; Stansfeld and Feltz, 1988). Electric current that is blocked by addition of Dendrotoxin in hippocampus pyramidal neurons has been identified as one of the fast-activating, voltage-dependent, aminopyridine-sensitive potassium current (Halliwell et al., 1986; Anderson and Harvey, 1988). Therefore, the

high affinity binding site for MCD is considered to be one of the potassium channels, however, there was no direct evidence.

2: MCD peptide itself forms voltage-gated and cation-selective channels in lipid bilayers (Ide et al., 1989). The minimum size of the single channel conductance that has been observed is 3.8 pS, which may be the basic unit channel.

3: MCD peptide increases the intracellular calcium concentration of mast cells by activating the pertussis toxin sensitive G-protein and that the initial transient increase in calcium concentration is independent of external calcium, while the latter sustained increase is dependent. MCD peptide directly stimulated the GTPase activity of Go, Gi in a concentration-dependent manner. MCD peptide-induced stimulation of GTPase activity of Go or Gi was completely abolished in G-proteins that had been modified by pertussis toxin-catalyzed ADP-ribosylation .

I analyzed which aspect of MCD is more closely related to the induction of LTP by comparing each aspect of MCD retained in several MCD-related compounds to their ability to induce LTP (Table 1). Among the functions of MCD, binding to the high affinity binding sites seems to be the most important function to induce LTP in hippocampal neurons. Nevertheless, there is a great discrepancy between the dissociation constant (150 pM) of MCD binding to its receptor and the minimum concentration of MCD (500 nM) needed to induce LTP. Therefore, some other functions of MCD may also be necessary for LTP induction. It has been reported that LTP cannot be induced by tetanic stimulation in the CA1 and CA3 regions of the Ptx-treated

hippocampal slice (Goh and Pennefather, 1989). These reports indicate that activation of a Ptx-sensitive G protein plays an important role in the pathway involved in LTP induction. I found that the concentration of MCD required for activation of G protein in mast cells is quite close to that required for LTP induction in the hippocampal slice. Moreover, all the peptides which induced LTP in the hippocampal slice, including DTxI, activated a Ptx-sensitive G protein (Fujimoto et al., 1991; Kondo et al., 1992) (Table 1). Preincubating the hippocampal slice with compound 48/80, a G protein activator, the concentration of MCD needed to induce LTP became lower and closer to the high affinity binding constant (Fig 2, manuscript in preparation). Therefore, there are two functions of MCD acting at different concentrations. These results raise the working hypothesis that activation of a G protein as well as binding to the high affinity binding site is necessary for MCD to induce LTP in the hippocampal neurons. In this sense, G protein may be the low affinity binding sites for MCD in brain, which has been suggested to be present by Bidard, J. N., et.al. (1989). The aim of this study was to purify and characterize the high affinity binding site for MCD in rat brain, and to identify signaling pathway located down stream of the G protein activated by MCD. Since it has been shown that endogenous MCD-like substance is present in rodent brain, the results obtained should add to our understandings of molecular mechanisms underlying synaptic plasticity in brain.

MATERIALS AND METHODS

Materials

All of the chemicals used were the highest purity of commercially available reagents and purchased from Wako Pure Chemical Industries, otherwise noticed.

Purification of Dendrotoxin I (DTxI)

Venom from the black mamba, *Dendroaspis polylepis polylepis*, was obtained from Latoxan (France). DTxI was purified from the crude venom in three steps; (1) gel filtration, (2) ion exchange chromatography on TSK SP-5PW, and (3) reverse-phase chromatography on COSMOSIL C_{18}. Crude venom powder (100 mg) was dissolved in 5 ml of 200 mM NH_4CO_3 and loaded onto the Sephadex G-50 fine (25x600 mm, Pharmacia) gel filtration column which had been packed and equilibrated in 0.2 M NH_4CO_3 to remove small molecules. The sample was eluted with the same buffer at 60 ml/h and 5 ml fractions were collected. Void volume fractions were pooled, lyophilized, and took up in 10 ml distilled water. They were then loaded onto ion exchange HPLC column, TSK SP-5PW (7.5x75 mm, TOSOH) equilibrated with 0.1 M NH_4OAc, pH7.3, and then eluted with a linear gradient between 0.1 M and 1.5 M NH_4OAc. Each peak fraction was collected, lyophilized and hydrolyzed in 6 N HCl. Amino acid composition of the hydrolysate were determined by amino acid

analyzer (L-8500, HITACHI) for primary identification of DTxI. The result of amino acid analysis was; Asp (4.1<calculated 4>), Thr (2.8<3>), Ser (1.8<2>), Glu (7.1<7>), Pro (3.1<3>), Gly (5.0<5>), Ala (1.1<1>), Cys (5.2<6>), Ile (3.6<4>), Leu (2.8<3>), Tyr (2.9<3>), Phe (3.0<3>), Lys (6.4<7>), His (1.1<1>), Trp (0.6<1>), Arg (6.5<7>). Structure of DTxI was further confirmed by sequencing the fragments obtained by digesting DTxI with endoproteinase Lys-C and purifying them on reverse-phase HPLC column COSMOSIL $5C_{18}$-300 (8x250 mm, Nacalai Tesque). The analysis of amino acid sequencer were LCILHRDPGRCYQK, IPAFYYNQK, QCEGFTWSGCGGNSNRFK, TIEECRRTCIRK, and they were consistent with the sequence. The first fragment; ZPLRK could not be detected because the first N-terminal amino acid was pyroglutamate. Minor impurities were eliminated by reverse-phase HPLC chromatography with a linear gradient between 10% and 40% acetonitrile in 0.1% TFA.

Coupling of DTxI to Sepharose

Sepharose 4B was activated by the method of Kohn & Wilchek (1982) using 0.8 mg/ml of CNBr in acetone. After washing to remove excess CNBr and acetone, the resin was resuspended in two times volume of coupling buffer containing 50 mM triethanolamine-HCl, pH 7.8, 0.5 M NaCl and 0.3 mg/ml of DTxI, and reacted for 2 h at room temperature. The resin was then briefly centrifuged and the A_{280} of the super-

natant was measured to determine the amount of DTxI coupled (generally 50-80%). Any free reactive groups remaining were blocked with 0.5 M ethanolamine-HCl, pH 8.5, for 4 h at room temperature. Then the resin was washed sequentially on a sintered glass funnel with 1 liter of washing buffer containing 0.1 mM sodium acetate buffer, pH 5.0, 1 M NaCl. The resin was resuspended in storage buffer containing 0.1 M sodium acetate, pH 6.0, 0.5 M NaCl and 0.05 % (w/v) NaN_3.

Purification of DTxI Binding Protein

The synaptic plasma membranes (200 mg) were prepared from rat brain obtained immediately after slaughter, and resuspended (10 mg/ml) in extraction buffer containing 62.5 mM imidazole-HCl, pH 7.4, 250 mM KCl, 2.5 mM EDTA, 25μg/ml soybean trypsin inhibitor, 50μg/ml bacitracin, 0.25 mM benzamidine, 0.5 mM PMSF, and 4 % (w/v) Lubrol PX (Bennett et al., 1986). After dilution and centrifugation, 215 ml of crude extract was loaded at a flow rate of 30-40 ml/h onto DTxI-Sepharose column (150x550 mm) equilibrated with 25 mM imidazole-HCl, pH 8.2, 100 mM KCl, 0.2 mM benzamidine, 0.5mM PMSF, and 0.2 % (w/v) Lubrol PX (buffer A). The column was then washed with 200 ml of buffer A at 100 ml/h and eluted at 5 ml/h by the addition of 10 mM DTT in buffer A. The adsorbed fractions collected (2.5ml) were directly applied to an HPLC ion exchanging column DE-5PW TSK-GEL (5.0 x 50 mm, TOSOH) equilibrated with 25 mM imidazole-HCl (pH 8.2), 100 mM KCl, 1 mM

EDTA, 0.2 mM benzamidine, 0.2mM PMSF, and 0.05% (w/v) Tween 80 (buffer B) at flow rate of 0.8 ml/min. After washing with buffer B to remove DTT, the sample was eluted with 300 mM KCl in buffer B at 0.8 ml/min.

Protein Assay

Concentration of protein was determined using Micro BCA Protein Assay Reagent (PIERCE) according to the supplier's protocol. Samples were diluted against distilled water before mixing with an equal volume of BCA solution containing 2 % bicinchoninic acid (BCA) and 0.02 % cupric sulfate. After incubation at 60 °C for 1 h, the absorbance at 562 nm was measured. Bovine serum albumin (BSA) was used as a standard.

SDS-Polyacrylamide Gel Electrophoresis

Electrophoresis on a SDS-polyacrylamide gel was carried out by the method of Laemmli (1970). The separating gel containing 0.375 M Tris-HCl, pH 8.6, 0.1 % (w/v) SDS, 17.5 % (w/v) acrylamide, 0.58 % (w/v) N,N'-methylene-bisacrylamide, 0.05 % (w/v) ammonium persulfate and 4 µl TEMED in a final volume of 10 ml. The stacking gel containing a mixture of 0.125 M Tris-HCl, pH 6.8, 0.1 % SDS, 5 % acrylamide, 0.17 % N,N'-methylene-bisacrylamide, 0.05 % ammonium persulfate and

2 μl TEMED in a total volume of 5 ml. Electrophoresis was carried out at 20 mA per gel slab at room temperature in a running buffer containing 25 mM Tris, 192 mM glycine and 0.1 % SDS. After electrophoresis, the gel slab was silver stained using Silver Stain II Kit (Wako).

Binding Assay of Purified Protein

Membrane-bound acceptor was assayed by a filtration method using ^{125}I-MCD, ^{125}I-DTxI as described (Bruns et al. 1983). The binding assay mixture in a total incubation volume of 250 μl contained 15 mM imidazole-HCl, pH 7.4, 0.6 mM EDTA, 60 mM KCl, 0.1-1 % detergent, 3 nM ^{125}I-MCD or ^{125}I-DTxI, 100 μg/ml cytochrome C and 50 μl sample. After incubating 30 min on ice, they were diluted to a volume of 1 ml by addition of washing buffer [15 mM imidazole-HCl, pH 7.4, 0.6 mM EDTA, 60 mM KCl, 0.2 % Triton X-100] immediately before filtration. GF/C filter discs (Whatman) were soaked in 0.3 % (w/v) polyethyleneimine in washing buffer before use. The tubes and filters were then rapidly washed three times with 5 ml of the buffer, and the radioactivity retained on the filters were counted using γ-radiation counter (ARC-501, Aloka).

Reconstitution of the Purified Protein

The purified DTx-I binding proteins were diluted with 25 mM imidazole-HCl, pH 8.2, 100 mM KCl, 1 mM EDTA, 0.1 % Tween80 and lipids (13:6:1:2, phosphatidylcholine, phosphatidylethanolamine, phosphatidylserine, ergosterol). Phospholipids were obtained from Avanti Polar Lipid, Inc. (Birmingham, AL). The mixture was dialyzed for 48 h at 4 °C against 20 mM Na-Hepes, pH 7.2, 120 mM KCl and 1 mM EDTA, and changed six times every 8 h. The resulting liposomes with the reconstituted purified protein were stored in liquid nitrogen. Nystatin was added to the liposome-vesicle mixture to a final concentration of 0.06 mg/ml, and the mixture was frozen, thawed, and sonicated for 15-20 s.

Planar Lipid Bilayer Measurement

The purified DTxI binding protein in liposome was incorporated into planar bilayer membrane. Planar lipid bilayer was formed from phosphatidylcholine (10 mg/ml in decane). The liposome (5μl) was added to one chamber, designated *cis*, composed of 300 mM KCl in 10 mM Tris-Hepes, pH 7.2. The other chamber, designated *trans*, contained 100 mM KCl in 10 mM Tris-Hepes, pH 7.2. Baths were voltage clamped using a voltage clamp amplifier (3900A, DANGAN) and bypassed with a digital pulse code modulation (PCM-501ES, SONY). All signals were filtered with a low-pass filter with cutoff frequency 500 Hz on dual channel programmable filter syncro-

scope (3624, NF Electronic), and stored on videotapes (F81, SANYO), or printed out by a pen recorder (TA240 easy graf, GOULD). All experiments were performed at room temperature.

Distribution of N-biotinyl-MCD Binding in Hippocampal slices

Adult rats weighing 300-400 g were used. After decapitation, hippocampus was immediately removed and slices (500 μm thick) were made by a Microslicer (DTK-3000, D.S.K.). The obtained slices were placed in an incubation chamber in a medium containing 124 mM NaCl, 5.0 mM KCl, 1.25 mM NaH_2PO_4, 2.0 mM $MgSO_4$, 2.5 mM $CaCl_2$, 22.0 mM $NaHCO_3$ and 10.0 mM glucose. They were kept at 29-31 °C and equilibrated with a gas mixture of 5 % CO_2 in O_2. The slices were incubated for 5 min with 1 μM N-biotinyl-MCD followed by incubation for 30 min in the same medium without N-biotinyl-MCD. The slices were fixed with 2 % paraformaldehyde, 0.2 % glutaraldehyde in phosphate-buffered saline (PBS) [137 mM NaCl, 2.7 mM KCl, 4.3 mM Na_2HPO_4 and 1.4 mM KH_2PO_4, pH 7.4] for 16 h at 4 °C. The slices were then embedded in paraffin after dehydration in a graded alcohol series. Paraffin-embedded samples were cut into 6 μm thick sections, which were then deparaffinized and equilibrated in PBS. The sections were incubated in PBS containing 0.5 % skimmilk (DIFCO) for 30 min followed by incubation in 25 μg/ml FITC-conjugated avidin in PBS containing 0.5 % skimmilk for 30 min at room temperature. After several wash-

ing with PBS, the sections were examined by laser scanning confocal microscopy (MRC-500, Bio Rad).

Cell Culture

Primary cultures of dissociated hippocampal cells were prepared from the brains of 18-day fetal mouse (ICR). Tissue fragment of the hippocampal areas were dissect from embryonic mouse brains. Dissection was carried out under a dissecting-microscope (SZH-ILLD, OLYMPUS). Tissue fragments were incubated in 5 ml of Ca^{2+}, Mg^{2+}-free PBS containing 0.25 % trypsin and DNase I (100 units, Boehringer-Mannheim) for 15 min at 37 °C. After mechanical dissociation by pipeting, the cells were resuspended in serum-containing medium [10 % (v/v) heat-inactivated fetal calf serum (Gibco) in Dulbecco's modified Eagle's medium (DMEM) (Gibco)]. The dissociated cells were plated at a final cell density of $5x10^5$ cells/cm^2 on a polyethyleneimine-coated cover slips (2 cm^2 of culture surface area, Matsunami). After 1 day of culture in a humidified CO_2 incubator under 5 % CO_2 atmosphere, the medium was changed to serum-free chemically defined medium [1 mg/ml bovine serum albumin (BSA), 0.01 mg/ml insulin, 0.1 mg/ml transferin, 30 nM selenium, 0.1 mg/ml streptomycin, 100 units/ml penicillin and 0.1 nM thyroxine in DMEM]. The medium of cultures was changed every 3-4 days.

Distribution of N-biotinyl-MCD Binding in Hippocampal Primary Cell Culture

The cells were incubated for 10 min with 1 μM N-biotinyl-MCD in culture medium followed by incubation for 30 min in culture medium without N-biotinyl-MCD. The cells were fixed with 4 % paraformaldehyde in Tris-buffered saline (TBS; 20 mM Tris-HCl, pH 7.5, 150 mM NaCl) on ice for 40 min. After pre-incubation with TBS containing 2 % (v/v) normal goat serum and 3 % skimmilk for 2 h at room temperature, cells were incubated with alkaline phosphatase conjugated streptoavidin (1:5000) for 30 min at room temperature. After washing 2 times with 0.3 % skimmilk in TBS and two times with TBM buffer [100 mM Tris-HCl, pH 9.5, 150 mM NaCl and 50 mM $MgCl_2$] N-biotinyl-MCD bound was visualized by adding 22.5 μl NBT solution and 17.5 μl X-phosphate solution dissolved in 5 ml TBM buffer.

Peptide Synthesis

Peptides corresponding to rat RCK1, RCK4 and RCK5 S1-S2 region were synthesized by the Fmoc(9-fluorenylmethyloxycarbonyl)-polyamide solid phase method using Biosearch 9050 Peptide Synthesizer (MilliGen). The peptides were released from the resin by 95 % trifluoroacetic acid (TFA) for 1.5 h at room temperature. Scavenger (2.5 % phenol, 2.5 % ethandithiol, and 2.5 % thioanisole) was also added to prevent side reactions. The peptide was precipitated immediately by adding equal

volume of diethyl ether, and centrifuged 3,000 rpm for 5 min to remove ether by decantation. After washing six times with ether, the peptide was dried up under high vacuum and dissolved in 0.1 N acetic acid. The peptide was lyophilized and further purified using reverse-phase chromatography on a μBondasphere C_8 (19x150 mm, Waters) with a linear gradient between 15 % and 25 % acetonitrile in 0.1 % TFA. Confirmation of the peptide structure were performed using 477A Protein Sequencer (Applied Biosystems).

Conjugation of Peptide to Bovine Serum Albumin

Each RCK peptide (20mg), BSA (20 mg), and 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (20 mg) were dissolved in PBS at 4°C. The reaction was allowed to proceed overnight by stirring. Undissolved materials were removed by centrifugation and the supernatant was applied to a column of Sephadex G-50 (superfine) equilibrated with 50 mM ammonium acetate to remove uncoupled peptide or coupling agent. Fractions of the void volume were collected and lyophilized.

Immunization of Rabbits

Japanese White rabbits (JWY-NIBS) were initially injected subcutaneously along the back side of animals with peptide-BSA conjugate in Freund complete adjuvant

(1:1). Approximately 1 mg of peptide conjugate was injected three times at different sites. After two weeks, the rabbits were injected again along the back side of animals similarly. Four weeks after the first immunization, another 1 mg of the peptide conjugate in Freund incomplete adjuvant (1:1) was injected to the rabbit, and a week later the rabbit was bled. Blood was collected in a glass centrifugal tube, incubated at 37 °C for 1 h, and standed still at 4 °C overnight. After centrifuging 3,000 rpm for 15 min, the supernatant was used as an antiserum and stored at 4 °C.

ELISA

The wells of a 96-well microtitre plate (Nunc) were coated with the purified synthesized peptides (2 μg/well in 50 mM sodium carbonate buffer, pH 9.5) and the plate was stored in a humid atmosphere at 4 °C overnight. Then unattached antigen was washed out with 0.1 M sodium phosphate buffer, pH 7.2, containing 0.2 M NaCl and 0.05 % Tween 20. Blocking solution containing 1 % skimmilk in 0.1 M sodium phosphate buffer, pH 8.2, was added and the plate was incubated at 4 °C for 1 h. Various concentrations of each antiserum were added and the plate was incubated at room temperature for 30 min. Then the plate was washed with washing buffer and 50 μl of biotinylated anti-(rabbit IgG) antibody (1:200) (Vectastain) was added. After incubation at room temperature for 30 min, the plate was washed with washing buffer, and 50 μl of avidin-conjugated peroxidase (1:200) (Vectastain) was added. The plate

was incubated and then washed as described above. Finally, 100 μl of the substrate solution containing 6 mM H_2O_2 and 40 mM o-phenylenediamine dihydrochloride in PBS was added. The reaction was stopped by the addition of 50 μl of 8 M H_2SO_4, and the absorbance at 492 nm was measured with Micro Plate Reader (Toyosoda).

Western Blot Analysis.

Rat brain membranes were homogenized in distilled water, followed by addition of equal volume of 2x SDS buffer (50 mM Tris-HCl, pH 6.8, 10 % β-mercaptoethanol, 4 % SDS and 5 % glycerol) and sheared with a 22 G needle. Ten micro grams of the samples were fractionated by SDS-PAGE. They were transferred to a Hybond ECL nitrocellulose filter (Amersham) using semi-dry electroblotter (Sartorius). The filter was incubated with 5 % skimmilk (DIFCO) in PBS containing 0.2 % Tween 20 (PBS-T) at 4 °C overnight for blocking of non-specific bindings and incubated with the anti-RCK peptide antibody for 5 h at room temperature. After washing with PBS-T, the filter was incubated with biotinylated goat anti-rabbit IgG (1:50) (Vectastain)in for 1 h at room temperature. The immunoreactive products were visualized by developing in 4-chloro-1-naphtol or by ECL system (Amersham).

Immunohistochemistry of Cultured Hippocampal Neurons

Primary cultured hippocampal cells were fixed in 4 % paraformaldehyde or ethanol containing 5 % acetic acid, on ice for 20 min. After pre-incubation with PBS containing 2 % (v/v) normal goat serum and 0.3 % (v/v) Triton X-100, for 1 h at room temperature, cells were incubated with anti-RCK peptide antibody (1:100) for 1h at 4 °C. After washing three times for 5 min, cells were incubated with FITC-conjugated goat anti-rabbit IgG (1:200) (Cappel) for 30 min at room temperature. After washing several times with PBS, the cover slips were mounted in Perma-Fluor (Lipshaw Immunon). Visualization was carried out by fluorescence microscopy (Axiovert 35, ZEISS).

Immunohistochemistry of Rat Brain

Rats were perfused with 4 % paraformaldehyde in 0.1 M phosphate buffer, pH 7.4. Brains were dissected out and post-fixed overnight in the same fixative at 4°C. After paraffin embedding, serial sagittal sections (6 μm thick) were prepared on a microtome. After deparaffinization they were rinsed with PBS-T and incubated with 0.5 % solution of skimmilk in PBS-T for blocking of non-specific bindings. Tissue sections were incubated overnight at 4 °C with rabbit anti-RCK peptide antibody (1:50). After washing with PBS-T three times for 10 min each, they were incubated with FITC-conjugated anti-rabbit IgG (1:200) (Cappel) at 37 °C for 30 min. After several wash-

ing with PBS-T, the sections were mounted in PermaFluor (Lipshaw Immunon) and examined by laser-scanning confocal microscopy system (LSM-GB200, OLYMPUS).

RESULTS

Localization of MCD Binding Sites in a Hippocampal Slice and in Hippocampal Primary Culture

Binding sites for MCD have been investigated using ^{125}I-MCD (Bidard et al., 1989). However, the concentration of labeled MCD used in previous experiments (38 pM) was insufficient to induce LTP. N-biotinyl-MCD was synthesized in our laboratory and was shown to induce LTP in CA1 region of hippocampus (Kondo et al. 1990). One millimolar of N-biotinyl-MCD, which is sufficient to induce LTP, was added to hippocampal slices for 5 min, and the slices were kept incubating without N-biotinyl-MCD for another 30 min, during which time synaptic transmission should have been fully enhanced. N-biotinyl-MCD bound to the slices was then detected histochemically using FITC-conjugated avidin after fixation of the tissue by 2% paraformaldehyde and 0.2% glutaraldehyde in PBS. In the CA1 region, as shown in Fig 3, N-biotinyl-MCD was found mainly in the cell body and stem of the dendrite of a pyramidal cell. Very little signals for N-biotinyl-MCD were detected in the molecular layers of pyramidal cells or granular cells, where the high-affinity binding sites for MCD were reported to be enriched (Bidard et al., 1989).

To investigate the cellular distribution in detail, primary cultured hippocampal cells were used. Primary cultures of dissociated hippocampal cells were prepared from the brains of 18-day fetal mouse. Tissue fragment of the hippocampal areas were dissect from embryonic mouse (ICR) brains. The dissociated cells were plated at a

final cell density of $5x10^5$ cells/cm^2 on a polyethyleneimine-coated cover slips. After 1 day cultivation in 10 % fetal calf serum-containing medium, medium was changed to a serum-free chemically defined medium, and used after 14 days *in vitro*. Localization of MCD binding sites in hippocampal primary cultured cells were detected using 1 μM N-biotinyl-MCD, followed by visualization using alkaline phosphatase-conjugated streptoavidin. MCD binding sites were concentrated on large triangular cells, which morphologically resembled hippocampal pyramidal neurons. The staining were observed both in the cell body and in the stem of neurites (Fig 4). The other types of cells were not stained.

-Characterization of High Affinity Binding Sites for MCD-

Pharmacological Characterization and Solubilization of MCD Binding Protein

MCD has high affinity and low affinity binding sites in brain membrane (Rehm and Lazdunski, 1988). Replacement of ^{125}I-MCD bound to the membrane by DTxI and MCD gave half-replacement value (IC_{50}) of 0.1 nM for DTxI, and 1 nM for MCD. No replacement was observed by apamin, mastoparan and D-MCD at a concentration of 10 nM (Fig 5). The secondary structure of MCD (Kobayashi et al., 1991) resembles that of apamin, another bee venom peptide, which blocks one subtype of Ca^{2+}-dependent K^+ channel (Banks et al., 1979; Hughes et al. 1982; Fosset et al., 1984). Nevertheless, no interaction between MCD and apamin binding sites in brain membranes was detected (Fig 5). Another mast cell degranulating agent, compound

48/80 was also without effect on the high affinity binding of the two toxins, MCD and DTxI (data not shown). The results described above are in good agreement with these previous findings and confirm that the binding site is highly specific for MCD and DTxI. Since the high affinity binding site is an integral membrane protein (Black et al., 1988), its solubilization in biologically active form required extraction of the plasma membranes with non-ionic or zwitterionic detergents. Rat synaptic plasma membranes were solubilized with 4 % (w/v) Lubrol PX. In initial studies, Triton X-100 was used at an optimal concentration of 4 % (w/v). However, the half-life of the acceptor activity in this medium was only 20-30 h, which was impractical for the purification procedures. Most importantly, Lubrol PX increased the stability significantly to a satisfactory level (half-life at 4 °C > 7days) (Parcej and Dolly, 1989).

Purification of MCD and DTxI High Affinity Binding Proteins

A number of purification methods including chromatography on Sepharose 6B and phenyl-Sepharose, have been reported, however, little enrichment of the acceptor was obtained (Parcej and Dolly, 1989). Thus, use of an affinity chromatography seemed to be suitable for purification. DTxI was selected as the ligand of choice due to its higher affinity for the MCD binding sites (Fig 5) and also its inducibility of LTP in hippocampal slice (Table 1). DTxI was purified from the venom of the black mamba *Dendroaspis polylepis polylepis* by three steps column chromatography, including gel filtration on Sephadex G-50 fine, ion exchange HPLC on TSK SP-5PW

and reverse-phase HPLC chromatography on COSMOSIL C_{18} . It is a 60 amino acid polypeptide with 6 cysteines forming three disulfide bridges. Its primary structure is indicated below.

ZPLRKLCILHRNPGRCYQKIPAFYYNQKKK-

QCEGFTWSGCGGNSNRFKTIEECRRTCIRK

To identify the purified protein as DTxI, I analyzed amino acid composition and sequenced the fragments obtained after endoproteinase Lys-C treatment. Coupling of DTxI to CNBr-activated Sepharose 4B proved to be most successful for acceptor purification. Rat plasma membrane was solubilized with 4 % Lubrol PX and loaded onto DTx-Sepharose column and eluted with 0.3 M KCl. Despite using such an improved affinity resin, elution with high concentrations of K^+ yielded a complex polypeptide pattern, and purification efficiency was low. To improve the purification procedure, I used DTT for elution. DTxI has the same backbone conformation as bovine pancreatic trypsin inhibitor; disulphides linking Cys7 to 57, 16 to 40 and 32 to 53, and no others (Creighton, 1977; Hollecker and Creighton, 1983). One of the disulfide bonds, 32 to 53, has been shown to be easily reduced by a low concentration of DTT, and alter the conformation of DTxI (Creighton, 1983). This reduced DTxI looses its ability to bind to its high affinity binding sites. The optimized conditions for such biospecific elution are summarized in the Materials and Methods. The DTxI-Sepharose routinely used normally adsorbed more than 80% of DTxI binding activity while more than 99% of the protein passed though. The eluate was directly applied to

DE-5PW. The presence of DTT and detergent precluded the direct measurement of the low amounts of protein present in the eluate fraction. Therefore, purification step was monitored by SDS-PAGE followed by silver staining (Fig 6). Binding activity was measured after DE-5PW chromatography, however, overall recovery was low (less than 1 %) and the specific activity only increased for 5-10 fold. In contrast to the numerous bands observed in the crude extract and flow through fractions from affinity column, blurring bands were seen in the DTT eluate, with Mr values of 70K, 60K and 31K with some minor contaminants. The eluate from the DE-5PW column shows essentially the same pattern as that seen in the DTxI-Sepharose eluate (Fig 6). Fig 7 shows an inhibition of ^{125}I-DTxI binding to its acceptor by non-labeled DTxI using a Lubrol PX membrane extract or affinity purified fraction. ^{125}I-DTxI bound to its acceptor was replaced by nonlabeled DTxI at the same IC_{50} value of 0.1 nM in both fractions, indicating that the high affinity binding sites were enriched from the rat brain. A similar result was obtained using ^{125}I-MCD (data not shown).

Reconstitution of the Purified MCD High Affinity Binding Proteins into Planar Lipid Bilayers

DTxI selectively inhibits a family of voltage-sensitive, fast-activating potassium channels that is sensitive to aminopyridine in mammalian (Dolly et al., 1984; Halliwell et al., 1986; Penner et al., 1986; Stansfeld et al., 1987; Anderson and Harvey, 1988) and amphibian (Benoit and Dubois, 1986) neuronal preparations. However, it

was not known whether the DTxI binding proteins itself is the DTxI-sensitive voltage-dependent potassium channels. Moreover, there were no information about the effects of MCD on the potassium current produced by DTxI-sensitive potassium channel.

MCD/DTxI binding proteins were purified in the presence of 0.05-0.2% Lubrol PX. Occasionally, this Lubrol PX itself formed a channel in a planar lipid bilayer system, thus making analysis of the results complicating. Purified samples were, therefore, reconstituted in liposomes and dialyzed for 48 h to remove the detergent. Liposome was then incorporated into the planar bilayer membrane consisting of phosphatidylcholine by adding to one chamber, designated *cis*, containing 300 mM KCl. The other chamber, designated *trans*, contained 100 mM KCl to make KCl gradient between the chambers. Baths were voltage clamped and bypassed with a digital pulse code modulation. All signals were filtered with a low-pass filter with cutoff frequency of 500 Hz. When the liposomes were added to the *cis* chamber, current fluctuations were observed after about 1 min, but almost no fluctuation was observed when the acceptor protein was added in the absence of KCl. Fig 8 illustrates the outward currents mediated by reconstituted channel at holding potential -100 mV. While there are several subconductance states, the current-voltage (I-V) relationship of the most frequently observed amplitude revealed a slope conductance of 16.2 pS and a reversal potential of -11 mV (Fig 9). Therefore, DTxI binding protein which I purified reconstituted potassium channel in the planar lipid bilayer. This channel seemed to be reconstituted in an inverse way, because the conductance plots were at

negative stage. When 50 nM MCD was added to the *cis* chamber, open current probability decreased gradually (Fig 8, 1→2→3). Therefore, this channel had a high affinity binding site for MCD and the current produced by this channel was blocked by MCD at a concentration that is lower than that required for LTP induction.

Use of antibodies directed against synthetic peptides for characterization of high affinity binding sites

Vertebrate genes encoding several distinct voltage-dependent potassium channels have recently been identified and reported (Table 2). The channels reported to be sensitive to DTxI and MCD are Kv1.1 (RCK1), Kv1.2 (RCK5) and Kv1.6 (RCK2) (Stuhmer et al., 1989; Kirsch et al., 1991). RCK1, RCK2 and RCK5 are member of RCK subfamily cloned from rat cortex (Stuhmer et al., 1989). To investigate which channel is responsible for mediating LTP induction by MCD in CA1 region of hippocampus, I produced antibodies to RCK1, RCK2 and RCK5 channels for immunohistochemical analysis.

The RCK proteins include six hydrophobic, possible membrane-spanning segments. They have been designated S1-S6 as indicated in Fig 10 by analogy to the proposed models for the voltage-dependent sodium (Noda et al., 1984) and the *Shaker* potassium channels (Pongs et al., 1988). These models orient the six proposed transmembrane segments S1-S6 in a pseudo-symmetric fashion across the membrane such that the amino and carboxyl termini are located on the cytoplasmic side and that the

amino acid sequences joining segments S1 and S2, S3 and S4, and S5 and S6 are located on the extracellular side. Only sequences in the core region that join the proposed membrane-spanning segments S1 / S2 and S3 / S4 at the extracellular side of the membrane have not been conserved. To obtain the epitope specific antibody, fifteen or ten amino acids sequence of rat among the RCK family RCK potassium channels extracellular portion, between S1 and S2 region (Fig 10; ELKD-DKDFTGTIHRI for RCK1, SSFSTLGGSF for RCK2 and ENEDMHGGGVTFHTY for RCK5), were selected. The peptides corresponding to this region was synthesized by MilliGen solid phase Fmoc-polyamide method. Synthetic peptide was purified by C_{18} reverse phase HPLC chromatography. Structure of the synthetic peptides were confirmed by sequencing (Applied Biosystems, 477A Protein Sequencer). These synthetic peptides were conjugated to BSA, purified by Sephadex G-50 gel filtration chromatography and used as antigens. Rabbits were immunized 3 times by injecting corresponding peptide conjugates. The titer against peptide was measured using ELISA system (Fig 11). Antiserum against RCK2 peptide was obtained from Research Genetic (USA).

Rat brain membrane was solubilized with SDS and fractionated by 15 % SDS-PAGE, then blotted onto nitrocellulose filter. After blocking, the filter was incubated with 1:50 dilution of each antiserum followed by incubation in peroxidase-conjugated goat anti-rabbit IgG (1:50). The immunoreactive products were visualized by developing in 4-chloro-1-naphtol. The band of 70 K-*Mr* was recognized by antiserum for

RCK1, RCK2 and RCK5 (Fig 12) and was specifically competed by an excess amount of corresponding peptide (data not shown). Although their predicted molecular size from cDNA is about 60 K-*Mr*, they possess a possible N-linked glycosylation sites; such a modification could contribute to the increased molecular size of the band on the immunoblot. RCK1 and RCK5 with the same molecular size were also detected in DTxI-Sepharose eluate and DE-5PW eluate (Fig 13). Therefore, at least RCK1 and RCK5 were contained in the purified fraction, and these channels may have contributed to the potassium current which was blocked by a low concentration of MCD.

Immunohistochemical Localization of MCD Binding Sites in Rat Brain and in Hippocampal Primary Culture

Adult rats were perfused with 4 % paraformaldehyde and brains were post-fixed using the same solution. Serial sagittal paraffin sections (6 μm thick) were prepared. After deparaffinization and blocking, they were incubated with 1:50 dilution of each antiserum followed by incubation in 1:200 dilution of FITC-conjugated anti-rabbit IgG. Their immunoreactivity were examined using confocal laser-scanning microscope.

Specific immunoreactivity of RCK proteins (RCK1, RCK2 and RCK5) were found in several regions of the brain, including the cerebellum, hippocampus, thalamus, olfactory tubercle, striatum, and cerebral cortex, in a good agreement with their mRNA distribution (Beckh and Pongs, 1990). The characteristic immunostaining

pattern was specific to the immune serum and was absent when preimmune serum was used.

The pattern of RCK protein immunoreactivity in the CA1 region of hippocampus indicated that these potassium channel proteins are differentially localized (Fig 14). The highest level of RCK1 immunoreactivity was found in pyramidal cell layer of CA1 region. RCK1 was present in pyramidal cell bodies and the stem of dendrites (Fig 14 RCK1). This staining pattern for RCK1 antiserum was close to that of N-biotinyl-MCD binding. RCK2 and RCK5 had less immunoreactivity in the CA1 region of hippocampus (Fig 14 RCK2, RCK5).

In the cerebellum, RCK1 immunoreactivity were found in the granule cell layer, fiber of white matter and faint reactivity in Purkinje cell bodies (Fig 15 RCK1). Immunoreactivity of RCK2 in granule cell layer was stronger than that of RCK1 and no reactivity in the white matter and Purkinje cells (Fig 15 RCK2). Highest density of immunoreactivity in Purkinje cell layer was found for RCK5. RCK5-immunoreactivity could clearly be observed in the somata of Purkinje cells and their dendrites that across the molecular layer (Fig 15 RCK5). These results show that the strong immunoreactivity for RCK1 protein found in the pyramidal cell layer in hippocampus is not caused by the difference in the titer of antibodies used.

Primary cultures of dissociated hippocampal cells were prepared from the brains of 18-day fetal mouse as described in Materials and Methods. After 1 day culture in serum containing medium, it was changed to a serum-free chemically defined medium

and cultured for two weeks before use. Cells were fixed with ice cold ethanol containing 5 % acetic acid, incubated with anti-RCK peptide antibodies (1:100) and reacted with FITC-conjugated goat anti-rabbit IgG (1:200). Visualization was carried out by fluorescence microscopy.

Immunoreactivity specific for each proteins (RCK1, RCK2 and RCK5) were found in several types of cells (Fig 16). The characteristic immunostaining pattern was specific to the immune serum and was absent when its preimmune serum was used. Strong immunoreactivity for RCK1 was found in triangular cells, which resembles pyramidal neuron cells morphologically. Comparing with the immunoreactivity for MAP2 in the hippocampal cells (Fig 17), RCK1 was less abundant in axon than MAP2. This localization was very close to that of N-biotinyl-MCD binding sites. Immunoreactivity of RCK2 was similar to GFAP staining (Fig 17), which indicates that RCK2 channel is present in glial cell in culture. Immunoreactivity for RCK5 was found in both type of cells, pyramidal cells and glial cells, however it was very weak (Fig 16). These results strongly indicate that RCK1 channel is responsible for MCD binding in pyramidal cells in CA1 region of hippocampus.

Functional and pharmacological properties of the channels formed by the various RCK proteins have been intensively explored *in vitro* using *Xenopus* oocytes expression system (Stuhmer et al., 1988). Current produced by RCK1 channel has been shown to be rapidly activating and non-inactivating current. However, we found that MCD reduced the peak amplitude of fast-inactivating (A-type) current and showed no

effect on non-inactivating (K-type) current in hippocampal slice preparation. Whole-cell recording of hippocampal neurons, which was a collaboration with Dr. Hiroshi Kastuki, revealed that approximately 80 % of A-type current was reduced by 1 μM MCD.

Recent experiments have shown that members of the same subfamily of potassium channel proteins can oligomerize to form mixed subunit channels in *Xenopus* oocytes (Christie et al., 1990; Isacoff et al., 1990; McCormack et al., 1990; Ruppersberg et al., 1990; Covarrubias et al., 1991). If a single gene product of RCK family was inject into *Xenopus* oocyte, only RCK4 channel can mediate transient A-type potassium current. Therefore, I hypothesized that the A-current suppressed by MCD in the pyramidal cell is mediated by a heteromultimer composed of RCK1 and RCK4 protein. It was reported that RCK4 could form mixed subunit channels with RCK1 in *Xenopus* oocytes to mediate fast inactivating current (Ruppersberg et al., 1990). Since RCK1 is MCD-sensitive, this hybrid channel may also be MCD-sensitive. Thus, I studied localization of RCK4 protein in the hippocampus.

Localization of RCK4 protein

Fifteen amino acids sequence corresponding to the extracellular portion of rat RCK4 potassium channel, GGHSRLLNDTSAPHL (Fig 10), were selected to make an antibody. The peptide was synthesized by solid phase Fmoc-polyamide method. Synthetic peptide was purified and checked as described in Materials and Methods.

This synthetic peptide was conjugated with BSA and injected into rabbit as an antigen. Antisera was obtained and used for immunohistochemical analysis of mouse hippocampal primary culture and paraffin section of rat brain. In hippocampal primary cultured cells, the antisera against RCK4 stained the triangular cells, which were identified morphologically as pyramidal neurons, and smaller circular cells (Fig 16 RCK4). The triangular cells were also stained with anti-RCK1 and N-biotinyl-MCD. Immunoreactivity of RCK4 in hippocampus was localized in the pyramidal cell layer (Fig 14 RCK4). In cerebellum, RCK4 immunoreactivity was found in fibers of white matter and granule cell layer (Fig 15 RCK4). Co-localization of RCK4 and RCK1 immunoreactivity in hippocampal CA1 region strongly suggested RCK4 oligomerized with RCK1.

Characterization of Signal Transduced after G Protein Activation by MCD

During the course of studies on reconstituting potassium channels in a lipid bilayer, another channel with a large conductance (about 3 times larger than the previous current) was found even in the presence of 50 nM MCD. This channel closed after further addition of 100 nM MCD to give final concentration of 150 nM (Fig 18). This concentration of MCD that was required for suppressing a large conductance potassium current was quite similar to the concentration of MCD needed for G protein activation. Thus, the presence of G protein in this fraction was examined.

By collaboration with Dr. Toshiaki Katada, we performed immunoblot analysis for

G protein subunits and ADP-ribosylation by pertussis toxin, IAP. Positive immunoreactivity was found in the purified fraction using antibodies raised against rat brain αo and αi (data not shown). The presence of βγ subunits of G protein in the same fraction was also shown by the immunoblot analysis (data not shown). Thus, the affinity purified fraction from rat brain contained both Go and Gi. Furthermore, IAP could ADP-ribosylate proteins in the purified fraction, whose mobility on SDS-PAGE was identical to those of Gi-1, Gi-2 and Go (data not shown). These results indicate that the affinity purified fraction contains Gi and Go in an active form.

Therefore, I investigated the effect of G protein activator on the current produced by the purified sample in the lipid bilayer system. When 10 μM GTP-γ-S, a G protein activator, was added to *trans* chamber, there was no change in this current. However, addition of GTP-γ-S to *cis* chamber produced significant change in the current. It blocked the current within 10 min (Fig 19). Therefore, this potassium channel showing a large conductance is suppressed directly by the activated G protein, however, it was not characterized enough.

DISCUSSION

MCD is a highly basic peptide isolated from bee venom, with 22 amino acid residues. Brief application of MCD to hippocampal slices induces LTP of the synaptic response evoked by Scaffer collateral stimulation in the CA1 region. LTP is the persistent enhancement in the strength of excitatory synaptic transmission at certain brain synapses. LTP occurs in several areas of the nervous systems, but is most widely studied in the hippocampus. The presence of an endogenous MCD-like substance in the brain has been suggested by the binding assay and radioimmunoassay. Since this substance inhibits binding of ^{125}I-MCD to its high affinity binding sites, which have been shown to be a voltage sensitive potassium channel, it is reasonable to consider that this substance is involved in regulations of neuronal activity, and thus contributing to produce neuronal plasticity. Therefore, to determine the mechanisms that underlie the LTP induction by MCD would provide us with valuable information on molecular mechanisms underlying synaptic plasticity. Previous studies in our laboratory have shown that it is important for MCD to bind to the high affinity binding site and also to activate G protein to induce LTP. The concentration of MCD required for binding to the high affinity binding site is less than 1 nM, whereas more than 100 nM is required for activation of G protein, and this concentration (100 nM) is the lower limit for LTP induction by MCD. Since high and low affinity binding sites were shown to be present in rat brain (Rehm and Lazdunski, 1988), G protein may be the low affinity binding sites for MCD, however, we have not measured binding constant

of MCD to G proteins. The aims of this study were (1) Purification and characterization of the high affinity binding sites for MCD in rat brain. (2) Identification of the signaling pathway following G protein activation by MCD.

Characterization of the High Affinity Binding Sites for MCD

A major conclusion that is obtained through this study is that the high affinity binding sites for MCD in hippocampal CA1 region is a heteromultimer channel composed of RCK1 and RCK4 proteins. This conclusion is drawn from the following evidences. First, this type of channel has been shown to mediate transient A-type potassium current in *Xenopus* oocyte system (Ruppersberg et al., 1990), and may be blocked by MCD. Second, RCK1 and RCK5 were present in highly purified fraction of high affinity binding sites for MCD (Fig 13). Third, through the immunohistochemical investigation on the localization of several RCK channels, RCK1 proteins was found in pyramidal cell layer of CA1 region at the highest level (Fig 14), and in hippocampal primary cultured cells which morphologically resembled pyramidal cells (Fig 16). This localization of RCK1 was quite similar to that of N-biotinyl-MCD. Therefore, RCK1 is one of the component of the high affinity binding sites for MCD. RCK1 channel has been shown to be a rapidly activating and non-inactivating current when it is expressed in *Xenopus* oocyte (Stuhmer, et al., 1989). However, we found that MCD reduces the peak amplitude of fast-inactivating (A-type) current and shows no effects on non-inactivating (K-type) current though a whole-cell recording study of

hippocampal neurons, (collaboration with Dr. Hiroshi Kastuki). Therefore, RCK1 should form heteromultimer with other RCK channels which mediate fast-inactivating current. Among RCK subfamily only RCK4 channel can mediate transient A-type potassium current, and RCK4 localized in pyramidal cells in hippocampal CA1 region (Fig 14, 16). Therefore, it is strongly suggested that RCK1 is oligomerizing with RCK4 and mediating MCD-sensitive transient A-current in hippocampal CA1 region *in vivo*. A-current has the function of inducing fast rate of repolarization of the action potential and would therefore result in an action potential of shorter duration. 4-aminopyridine, which is also a blocker of A-current in hippocampal pyramidal cells, has been reported to mediate increase in LTP (Haas and Greene, 1985; Lee et al., 1986). Therefore, blocking this A-current will induce the excitation of cells, which is a favorable condition for inducing LTP.

Identification of signaling pathway following G protein activation

I identified that one of the targets located down stream of the G protein activated by MCD is also a potassium channel. I fortuitously found this potassium channel in the purified fraction of high affinity binding sites of MCD. The purified fraction also contained G proteins (Go and Gi) in their active form. The channel which was reconstituted in the lipid bilayer in the presence of 50 nM MCD, was blocked by addition of higher concentration of MCD, which was sufficient to activate G protein. This channel was also blocked by a G protein activator, GTP-γ-S. Therefore, this potassium

current was suppressed direct by the action of the G protein.

Many G protein-coupled potassium channels have been found in the brain, however, most of them are inwardly rectifying potassium channel, which has been cloned recently (Kubo et al., 1993). The mechanisms by which muscarinic acetylcholine receptors modulate potassium channels, both suppression and activation, may be divided into two pathways based on G protein activity (Brown and Birnbaumer, 1990). M1 muscarinic acetylcholine receptors suppress a time- and voltage-dependent potassium channel within sympathetic and hippocampal neuron, termed the M-current; suppression of M-current is responsible for slow excitatory postsynaptic potentiation of these cells. Normally this outward potassium current increases as the hippocampal pyramidal neuron is depolarized, with a resulting return of membrane potential toward resting levels. Therefore, blocking of this M-current by G protein activation may increase the excitability of the cells and induce LTP. This M-current seemed to be consist with MCD-sensitive large conductance current observed, however, M-current is not suppressed directly by activated G protein. Diffusible cytoplasmic second messenger is involved in suppression of M-current. The m1 muscarinic acetylcholine receptors utilizes a signal transduction pathway involving PLC-generated signals and cellular tyrosine kinase activity to suppress potently a delayed rectifier currents (Huang et al. 1993). Therefore, the channel I found was novel and a future challenge will be to characterize and determine the functionally significance of this MCD-sensitive currents. Recently, another potassium current that was suppressed via

G protein was found in cardiac muscle by Grand (1993). This current was a time- and voltage-dependent transient outward current, which was suppressed by addition of GTP-γ-S. Relation between the MCD-sensitive A-current and this current should also be investigated.

Functional Correlation between Potassium Channels and LTP

Suppression of potassium channels has a close relationship with the induction of LTP. LTP can be induced by a specific potassium current blocker, tetraethylammonium (TEA) (Aniksztejі and Ben-Ari, 1991). The blockade of the potassium channels by TEA produced a powerful activation of quisqualate receptors as a result of the large increase in glutamate release. This leads to depolarization at the dendritic level, which will facilitate the activation of voltage-dependent calcium channels and induce LTP. Suppression of MCD-sensitive potassium channel may also results in induction of LTP via the same process.

Furthermore, some of the potassium channel genes which are expressed in hippocampus are down-regulated, at least at the mRNA level, following seizure activity of neurons (Tsaur et al., 1992). Since regulation of gene expression is required for maintenance of LTP, this suggests that regulation of potassium channel gene expression is important for LTP. In *Hermissenda* it is known that some potassium channels are blocked during the process of learning and memory and that these potassium channels are suppressed by G protein activation (Nelson et al., 1990). Therefore,

suppression of potassium channels seems to be greatly involved not only in LTP induction, but also in the process of learning and memory.

In the present studies, it became clear that a large conductance potassium current is suppressed by G protein activated by MCD. It is important to characterize these MCD-sensitive channels in more detail and to explore some other signaling pathway following the activation of G protein by MCD.

Aniksztejn, L. and Ben-Ari, Y. (1991)
Novel form of long-term potentiation produced by a K^+ channel blocker in the hippo campus, *Nature*, 349, 67-69

Anderson, A. J. and Harvey, A. L. (1988)
Effects of the potassium channel blocking dendrotoxins on acetylcholine release and motor nerve terminal activity, *Br. J. Phamacol.* 93, 215-221 (1988)

Artola, A. and Singer, W.(1987)
Long-term potentiation and NMDA receptors in rat visual cortex, *Nature* 330,649

Banks, B. E. C., Graman A. J. and Habermann, E. (1978)
Structure-activity studies on apamin and mast cell degranulating peptide (MCDP)-401, *J. Physil., Lond.* 284, 160-161

Banks, B. E. C., Brown, C., Burgess, G. M., Burnstock G., Claret, M., Cocks, T. M. and Jenkinson, D. H. (1979)
Apamin blocks certain neurotransmitter-induced increase in potassium permeability, *Nature* 282, 415-417

Baranyi, A. and Szente, M.B.(1987)
Long-Lasting potentiation of synaptic transmission requires postsynaptic modifications in the neocortex, *Brain Research* 423,378

Beckh, S. and Pongs, O. (1990)
Members of the RCK family are differentially expressed in the rat nervous system, *The EMBO Journal* 9, 777-782

Bennett, V., Baines, A. J. and Davis, J. (1986)
Purification of brain analogs of red blood cell membrane skeletal proteins:ankyrin,protein 4.1 (synapsin), spectrin, and spectrin subunits, *Methods Enzymol.* 134, 55-69

Benoit, E. and Dubois, J. M. (1986)
Toxin from the snake *Dendroaspis polylepis polylepis*: a highly specific blocker of one type of potassium channel in myelinated nerve fiber,*Brain Research* 377, 374-377

Bidard, J. N., Mourre, C., Gandolfo, G., Schweitz, H., Widmann, C., Gottesmann, C. and Lazdunski, M. (1989)
Analogies and difference in the mode of action and properties of binding sites (localization and mutual interactions) of two K^+channel toxins, MCD peptide and dendrotoxin I, *Brain Research* 495, 45-57

Black, A. R., Donegan, C. M., Denny, B. J. and Dolly, J. O. (1988)
Solubilization and physical characterization of acceptors for Dendrotoxin and β-Bungarotoxin from synaptic membranes of rat brain, *Biochemistry* 27, 6814-6820

Bruns,R.F.,Lawson-Wendling,K.and Pugsley,T.A. (1983)
A rapid filtration assay for soluble receptors using polyethyleneimine-treated filters, *Anal. Biochem.* 32, 74-81

Christie, M. J., North, R. A., Osborne, P. B., Douglass, J. and Adelman, J. P. (1990)
Heteropolymeric potassium channels expressed in *Xenopus* oocytes from cloned subunits, *Neuron* 4, 405-411

Cherubini, E., Ben Ari, Y., Gho, M., Bidard, J. N. and Lazdunski, M. (1987)
Long-term potentiation of synaptic transmission in the hippocampus induced by a bee venom peptide, *Nature* 328, 70-73

Collingridge, G.L., Kehl, S.J. and McLennan, H. (1983)
Excitatory amino acids in synaptic transmission in the Schaffer collateral commissural pathway of the rat hippocampus. *J. Physiol.* 334, 33-46.

Collingridge, G.L., and Bliss, T.V.P.(1987)
NMDA receptors-their role in long-term potentiation, *Trends Neurosci.* 10,288

Covarrubias,M.,Wei,A.and Salkoff,L. (1991)
Shaker,shal,shab,and shaw express independent K^+ current systems, *Neuron* 7, 763-773

Creighton, T. E. (1977)
Energetics of folding and unfolding of pancreatic trypsin inhibitor, *J. Mol. Biol.* 113, 295-312

Dolly, J. O., Halliwell, J. V., Black, J. D., Williams, R. S., Pelchen-Matthews, A., Breeze, A. L., Mehraban, F., Othman, I. B. and Black, A. R. (1984)
Botulinum neurotoxin and dendrotoxin as probes for studies on transmitter release, *J. Physiol.(Paris)* 79, 280-303

Dolly, J. O., Black, J. D., Black, A. R., Pelchen-Matthews, A. and Halliwell, J. V. (1986)
Novel roles of neural acceptors for inhibitory and facilitatory toxins, *Animal, Plant and Microbial Toxins*, 237-264

Dreyer,F. and Penner,R. (1987)
The actions of presynaptic snake toxins on membrane currents of mouse motor nerve terminals,*J. Physiol.* 386, 455-463

Feltz A. and Stansfeld,C. (1988)
Dendrotoxin-sensitive small conductance K^+ channels in isolated rat sensory neurones, *J. Physiol.* 406, 208P

Fujimoto, I., Ikenaka, K., Kondo, T., Aimoto, S., Kuno, M. and Mikoshiba, K. (1991)
Mast cell degranulating (MCD) peptide and its optical isomer active GTP binding protein in rat mast cells, *FEBS Lett.* 287 15-18

Goh, J. W. and Pennefather, P. S. (1989)
A pertussis toxin-sensitive G protein in hippocampal long-term potentiation, *Science* 244, 980-983

Grand, B. L. (1993)
Regulation of transient outward K^+ current in human atria involves G proteins, *Biochem. Biophys. Res. Commun.* 193, 1143-1147

Haas, H. L. and Greene, R. W. (1985)
Long-term potentiation and 4-aminopyridine, *Cellular and Molecular Neurobiology* 5, 297-301

Habermann, E. (1977)
Neurotoxicity of apamin and MCD peptide upon central application. *Naunyn-Schmiedeberg's Arch. Pharmac.* 300, 189-191

Harvey, A. L. and Anderson, A. J. (1986)
Dendrotoxin: snake toxins that block potassium channels and facilitate neurotransmitter release, *Pharmac. Ther.* 31, 33-35

Halliwell, J. V., Othman, I. B., Pelchen-Matthews, A. and Dolly, J. o. (1986)
Central action of dendrotoxin: selective reduction of a transient K conductance in hippocampus and binding to localized receptors, *Proc. Natl. Acad. Sci. U. S. A.* 83, 493-497

Hollecker, M. and Creighton, T. E. (1983)
Evolutionary conservation and variation of protein folding pathways, *J. Mol. Biol.* 168, 409-437

Hughes, M., Romey, G., Duval, D., Vincent, J. P. and Lazdunski, M. (1982)
Apamin as a selective blocker of the calcium-dependent potassium channel in neuroblastoma cells: voltage clamp and biochemical characterization of the toxin receptor, *Proc. Natl. Acad. Sci. U. S. A.* 79, 1308-1312

Ide, T., Taguchi, T., Morita, T., Sato, M., Ikenaka, K., Aimoto, S., Kondo, T., Hojo, H., Kasai, M. and Mikoshiba, K. (1989)
Mast cell degranulating peptide forms voltage gated and cation-selective channels in lipid bilayers, *Biochem. Biophys. Res. Commun.* 163, 155-160

Isacoff, E. Y., Jan, Y. N. and Jan, L. Y. (1990)
Evidence for the formation of heteromultimeric potassium channels in *Xenopus* oocytes, *Nature* 345, 530-534

Kato, H., Ikenaka, K., Kondo, T., Fujimoto, I., Ito, K. and Mikoshiba, K. (1993)
The induction mechanism of long term potentiation by synthetic mast cell degranulating peptide (MCD) differs from that evoke by tetanic stimulation in hippocampal CA1 neurons studied in vitro, *Yamagata Med, J.* 11, 1-16

Kirsch, G. E., Drewe, J. A., Verma, S., Brown, A. M. and Joho, R. H. (1991)
Electrophysiological characterization of a new member of the RCK family of rat brain K+ channels, *FEBS Lett.* 278, 55-60

Kobayashi, Y., Sato, A., Takashima, H., Tamaoki, H., Nishimura, S., Kyogoku, Y., Ikenaka, K., Kondo, T., Mikoshiba, K., Hojo, H., Aimoto, S. and Moroder, L. (1991)
A new helical motif in membrane active peptides, *Neurochemistry International* 18, 525-534

Komatsu, Y., Fujii,K.,Maeda,J.,Sakaguchi,H. and Toyama,K. (1988)
Long-term potentiation of synaptic transmission in kitten visual cortex, *J.Neurophysiol.* 59,124 (1988)

Kubo, Y., Reuveny, E., Slesinger, P. A., Jan, Y. N. and Jan, L. Y. (1993)
Primary structure and functional expression of a rat G-protein-coupled muscarinic potassium channel, *Nature* 364, 802-806

Kondo, T., Ikenaka, K., Fujimoto, I., Aimoto, S., Kato, H., Ito, K., Taguchi, T., Morita, T., Kasai, M. and Mikoshiba, K. (1992)
K+ channel involvement in induction of synaptic enhancement by mast cell degranulating (MCD) peptide, *Neuroscience Research* 13, 207-216

Laemmli, U. K. (1970)
Cleavage of structure proteins during the head of bacteriophage T4. *Nature* 227, 680-685

Lee, W. L., Anwyl, R. and Rowan, M. (1986)
4-Aminopyridine-mediated increase in long-term potentiation in CA1 of the rat hippocampus *Neuroscience Letters* 70, 106-109

McCormack, K., Lin, J. W., Iverson, L. E. and Rudy, B. (1990)
Shaker K+ channel subunits form heteromultimeric channels with novel functional properties, *Biochem. Biophys. Res. Commun.* 171, 1361-1371

Mehraban, F., Breeze, A. L. and Dolley, J. O. (1984)
Identification by cross-linking of a neuronal acceptor protein for dendrotoxin,a convulsant polypeptide,*FEBS Lett.* 173, 116-122

Muniz,Z.M.,Diniz,C.,R.and Dolly,O.(1990)
Characterization of binding sites for δ-Dendrotoxin in Guinea-Pig synaptosomes: Relationship to acceptors for the K^+-channel probe a-Dendrotoxin,*Journal of Neurochemistry* 54,343-346

Nelson, T. J., Collin, C. and Alkon, D. L. (1990)
Isolation of a G protein that is modified by learning and reduces potassium currents in *Hermissenda, Science*, 247, 1479-1483

Nicoll, R.A., Kauer, J.A. and Malenka, R.C.(1988)
The current excitement in long-term potentiation, *Neuron* 1,97

Noda,M., Simizu,S., Tanabe,T., Takai,T., Kayano,T., Ikeda,T., Takahasi,H., Nakayama,H., Kanaoka,Y., Minamino,N., Kangawa,K., Matsuo,H., Raftery,M.A., Hirose,T., Inayama,S., Hayasida,H., Miyata,T. and Numa,S. (1984)
Primary structure of electrophorus electricus sodium channel deduced from cDNA sequence, *Nature*, 312, 121-127

Paxinos, G. and Watson, C. (1982)
The rat brain in stereotaxic coordinates, *Academic, New York*, 150 pp

Parcej, D. N. and Dolly, J. O. (1989)
Dendrotoxin acceptor from bovine synaptic plasma membranes, *Biochem. J.* 257, 899-903

Penner, R., Peterson, M., Pierau, F. K. and Dreyer, F. (1986)
Dendrotoxin, a selective blocker of a non-inactivating potassium current in guinea pig dorsal root ganglion neurones, *Pflugers Arch.* 407, 365-369

Perkins, A.T. and Teyler, T.J. (1988)
A critical period for long-term potentiation in the developing rat visual cortex, *Brain Research* 439,222-229

Pongs, O., Kecskemethy, N., Muller, R., Krah-Jentgens, I., Baumann, A., Kiltz, H. H., Canal, I., Llamazares, S. and Ferrus,A. (1988)
Shaker encodes a family of putative potassium channel proteins in the nervous system of *Drosophila, The EMBO Journal* 7, 1087-1096

Rehm, H. and Lazdunski, M. (1988)
Existence of different population of the dendrotoxin I binding protein associated with neuronal K^+channels, *Biochem. Biophys. Res. Commun.* 153, 231-240

Rehm, H., Bidard, J.-N., Schweits, H. and Lazdunski, M. (1988)
The receptor site for the bee venom mast cell degranulating peptide. Affinity labeling and evidence for a common molecular target for mast cell degranulating peptide and dendrotoxin I,a snake toxin active on K^+-channels, *Biochemistry* 27, 1827-1832

Ruppersberg, J. P., Schroter, K. H., Sakmann, B., Stocker, M., Sewing, S. and Pongs, O. (1990)
Heteromultimeric channels formed by rat brain potassium-channel proteins. *Nature* 345, 535-537

Stansfeld, C. E. and Feltz, A.(1988)
Dendrotoxin-sensitive K+-channels in dorsal root ganglion cells, *Neurosci. Lett.* 93, 49-55

Stansfeld, C. E., Marsh, S. J., Parcej, D. N., Dolly, J. O. and Brown, D. A. (1987)
Mast cell degranulating peptide and dendrotoxin selectively inhibit a fast-acting potassium current and bind to common neuronal proteins, *Neuroscience* 23, 893-902

Stuhmer, W., Ruppersberg, J. P., Schroter, K. H., Sakmann, B., Stocker, M., Giesse, K. P., Perschke, A., Baumann, A. and Pongs, O. (1989)
Molecular basis of functional diversity of voltage-gated potassium channels in mammalian brain,*The ENBO Journal* 8, 3235-3244

Stuhmer,W.,Stocker,M.,Sakmann,B.,Seeburg,P.,Baumann,A.,Grupe,A. and Pongs,O. (1988)
Potassium channels expressed from rat brain cDNA have delayed rectifier properties,*FEBS Lett.* 242, 199-206

Taylor, J. W., Bidard, J. N. and Lazdunski, M. (1984)
The characterization of high affinity binding sites in rat brain for the mast cell degranulating peptide from bee venom using the purified monoiodinated peptide. *J. Bio. Chem.* 259, 13957-13967

Tomita, U., Takahashi, K., Ikenaka, K., Kondo, T., Fujimoto, I., Aimoto, S., Mikoshiba, K., Ui, M. and Katada,T. (1991)
Direct activation of GTP-binding proteins by venom peptides that contain cationic clusters within their alpha-helical structures, *Biochem. Biophys. Res. Commun.* 178, 400-406

Trimmer, J. S. (1991)
Immunological identification and characterization of a delayed rectifier K^+ channel polypeptide in rat brain, *Proc. Natl. Acad. Sci. U. S. A.* 88, 10764-10768

Tsaur, M. L., Sheng, M., Lowenstein, D. H., Jan, Y. N. and Jan, L. Y. (1992)
Differential expression of K^+ channel mRNAs in the rat brain and down-regulation in the hippocampus following seizures, *Neuron*, 8, 1055-1067

Weller, U., Bernhardt, U., Siemen, D., Dreyer, F., Vogel, W. and Habermann, E. (1985)
Electrophysiological and neurochemical evidence for the blockade of a potassium channel by dendrotoxin, *Naunyn-Schmiedeberg's Arch. Pharmacol.* 330, 77-83

	LTP	Binding activity (K^+-channel)	Channel formation	G-protein activation
MCD	+	+	+	+
I-MCD	+	+	+	+
D-MCD	-	-	-	+
Apamin	-	-	+	-
DTX-I	+	+	-	+
Mastoparan	-	-	NT	+

NT: not tested

Table 1

LTP inducibility and other function of MCD derivatives and its analogues. Previous results (Fujimoto et al., 1991; Kondo et al., 1992, Fig 4) are summarized in this table. Binding activity (K^+ channel) shows binding activity of the MCD binding site to it's high affinity binding sites in brain membranes, channel formation shows channel forming activity by itself in planar lipid bilayers, and G protein activation shows ability to increase intracellular Ca^{2+} concentration via Ptx-sensitive pathway in mast cells. NT: not tested.

VOLTAGE DEPENDENT K^+CHANNEL GENES

Shaker type	rat	mouse	human
Kv1.1	RCK1	MBK1	HK1
	RBK1	MK1	
	RK1		
Kv1.2	RCK5	MK2	HK4
	RBK2		
	RK2		
Kv1.3	RCK3	MK3	HPCN3
	RGK5		
	KV3		
Kv1.4	RCK4		HPCN2
	RHK1		HK2
	RK3		
Kv1.5	KV1		HPCN1
Kv1.6	RCK2		HBK2
	KV2		
Kv1.7	RK6	MK6	HaK6

Table 2

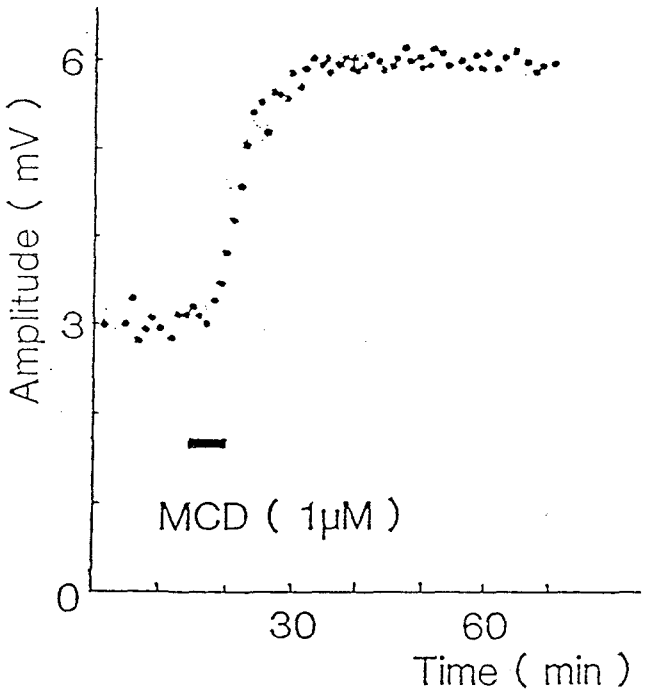
A simplified nomenclature for a family of vertebrate voltage-dependent potassium channel genes. This table is described by Candy, 1991 Nature, 352, 26.

Fig 1

Induction of LTP by MCD application in CA1 region of hippocampus.

Left: Amplitude of field population spike was increased by addition of MCD. MCD dissolved in the standard perfusion medium was perfused for 5 min (black bar), and then returned to the standard medium again.

Right: Structure of MCD. MCD is a highly basic peptide isolated from bee venom, with two disulphide bonds.



Structure of MCD Peptide

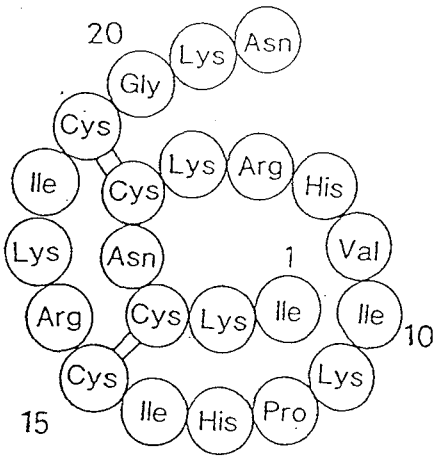


Fig 2

Effect of compound 48/80 on LTP inducibility of MCD. Varying doses of MCD were applied to the hippocampal slice. Mean percentage increase in population spike amplitude is plotted against varying concentration of MCD. Compound 48/80: G protein activator, histamine releaser, condensation product of N-methyl-p-methoxy-phenethylamine with formaldehyde.

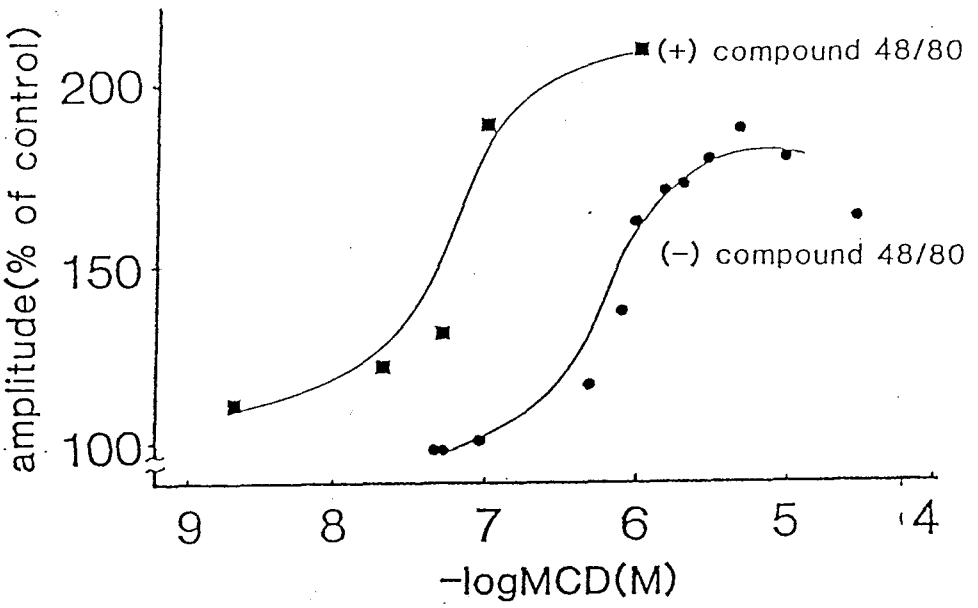

amplitude(% of control)
200
150
100
9
8
7
6
5
4
-logMCD(M)
(+) compound 48/80
(−) compound 48/80

Fig 3

Distribution of N-biotinyl-MCD binding sites in a hippocampal slice. N-biotinyl-MCD (1 μM) was applied to the hippocampal slice as described in Materials and Methods. N-biotinyl-MCD binding sites were still present in cell bodies and stems of dendrites of pyramidal cells in the CA1 region of the hippocampal slice.

O: striatum oriens, P: striatum pyramidale, R: striatum radiatus.

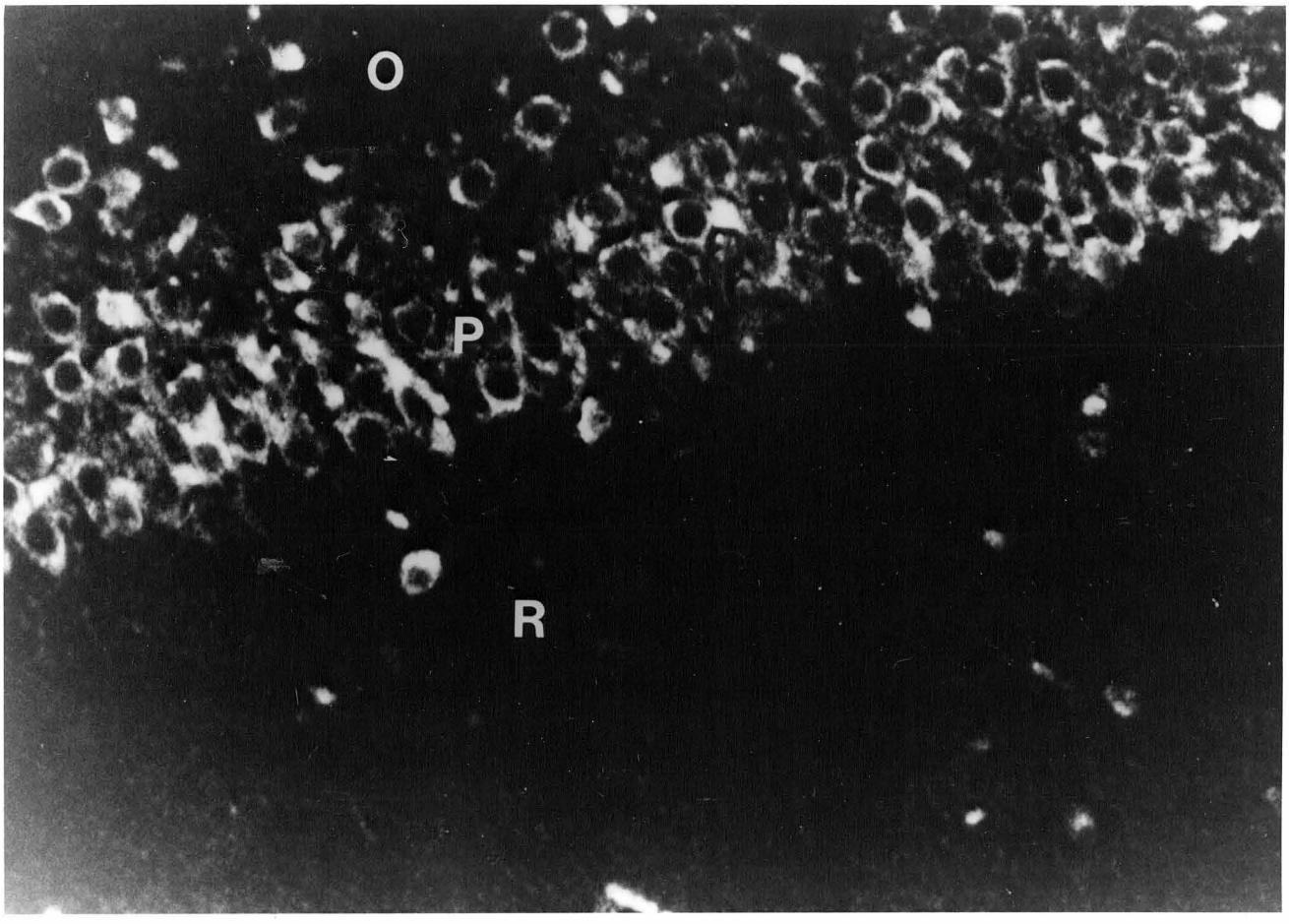

O
P
R

Fig 4

Distribution of N-biotinyl-MCD binding sites in primary cultured hippocampal cells. Primary cultured hippocampal cells were fixed with 4 % paraformaldehyde on ice for 40 min. Cells were incubated with N-biotinyl-MCD (1 μM). N-biotinyl-MCD was present in cell bodies and stems of dendrites of pyramidal cells.

Fig 5

Inhibition of ^{125}I-MCD binding by various derivatives and analogues of MCD. Brain membranes (0.2 mg protein) were incubated with 40-50 pM ^{125}I-MCD in the presence of various concentrations of unlabeled MCD (●), DTxI (▲), apamin (○), mastoparan (△), or D-MCD (□). The amount of ^{125}I-MCD bound was determined by the centrifugation method. All binding data represent the mean ± SD from triplicate determinations.

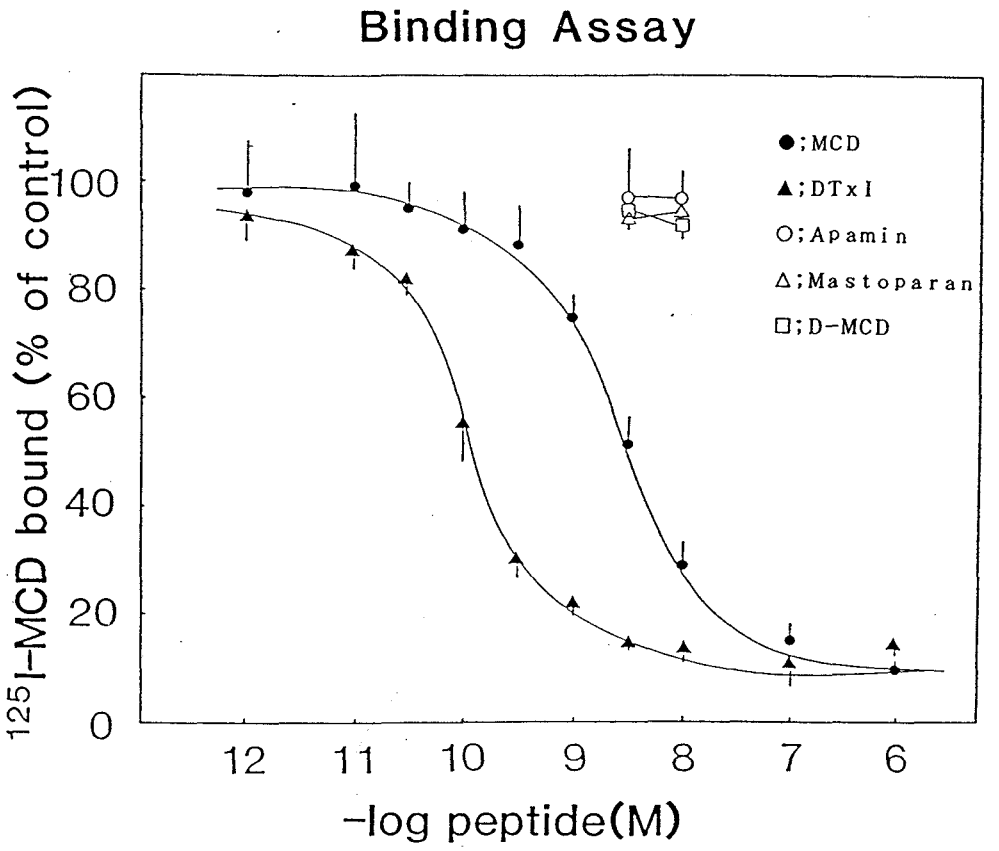

Binding Assay
^{125}I-MCD bound (% of control)
100
80
60
40
20
0
12
11
10
9
8
7
6
-log peptide(M)
●;MCD
▲;DTxI
○;Apamin
△;Mastoparan
□;D-MCD

Fig 6

SDS-PAGE pattern of the purified fraction after each purification step. After each purification step protein profiles were analyzed with SDS-PAGE. The lines are (1) low molecular weight marker; (2) synaptic plasma membranes solubilized with 4 % (w/v) Lubrol PX; (3) flow through of DTxI affinity column; (4) DTxI binding protein eluted from DTxI affinity column; (5-8) eluate fraction from DE-5PW ion exchange HPLC. Size of molecular weight marker was indicated at left of the panel.

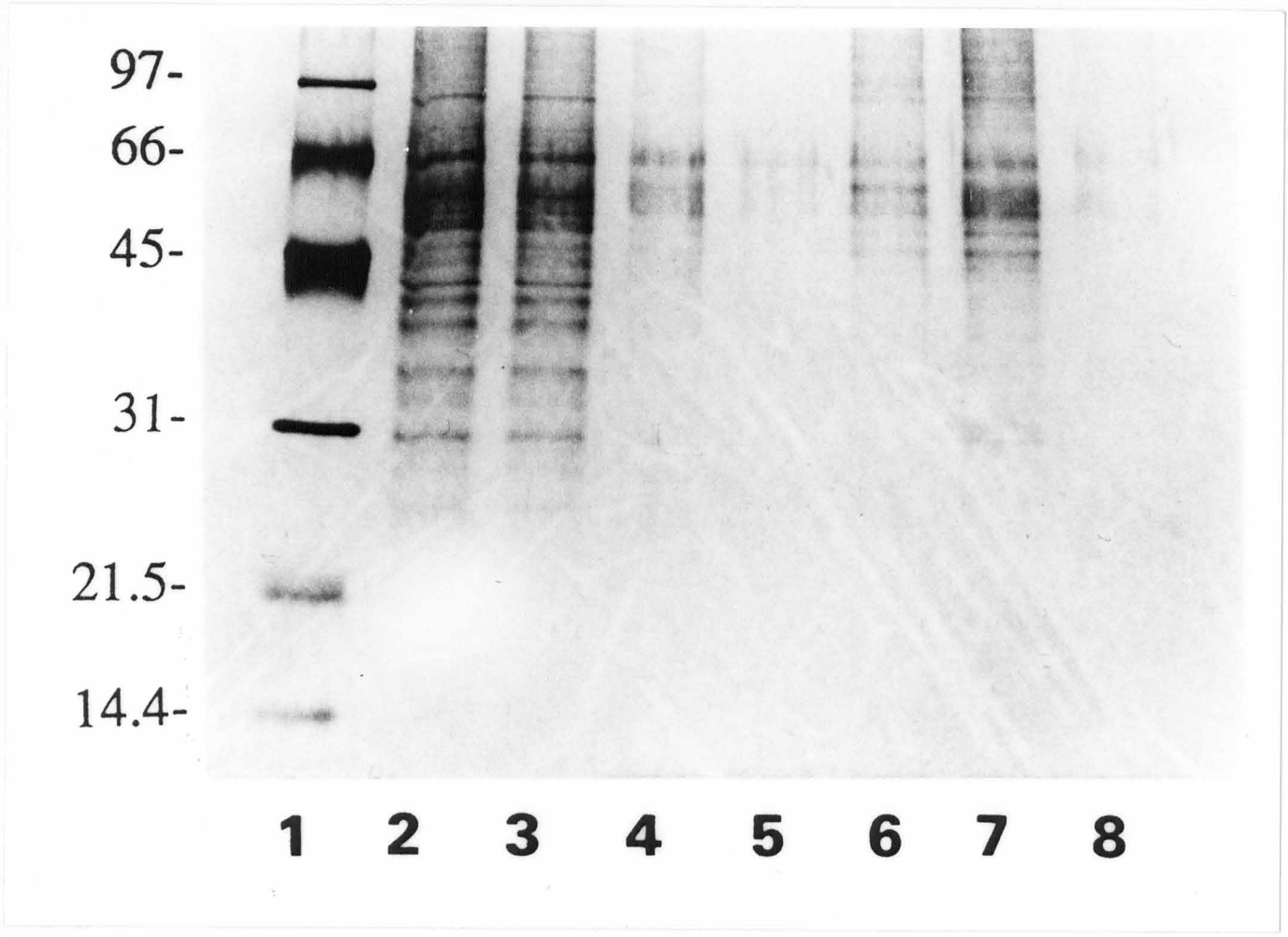

97-
66-
45-
31-
21.5-
14.4-
1
2
3
4
5
6
7
8

Fig 7

Binding assay of crude extract and purified DTxI binding protein. Crude extract and purified DTxI binding protein were incubated with 40-50 pM ^{125}I-DTxI in the presence of various concentrations of unlabeled DTxI. The amount of ^{125}I-DTxI bound was determined by the filtration method using GF/C filter discs soaked in 0.3 % (w/v) polyethyleneimine.

BINDING ASSAY

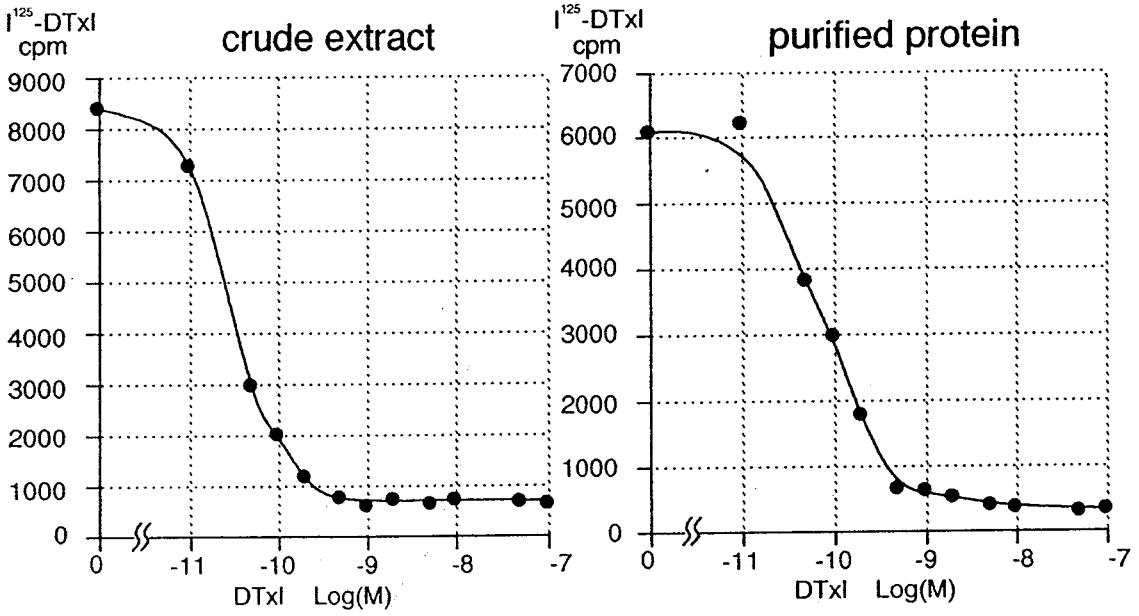


Fig 8

Inactivation of reconstituted channels by 50 nM MCD. The purified DTxI binding protein was reconstituted into planar lipid bilayer membrane. Traces (Control, 1, 2 and 3) show enlarged portions of record on the top. Planar lipid bilayer was formed from phosphatidylcholine. The chamber, designated *cis*, contained 300 mM KCl and 10 mM Tris-Hepes, pH 7.2. The other chamber, designated *trans*, contained 100 mM KCl and 10 mM Tris-Hepes, pH 7.2. Baths were voltage clamped at -100 mV. Signal was filtered with a low-pass filter with cutoff frequency of 500 Hz.

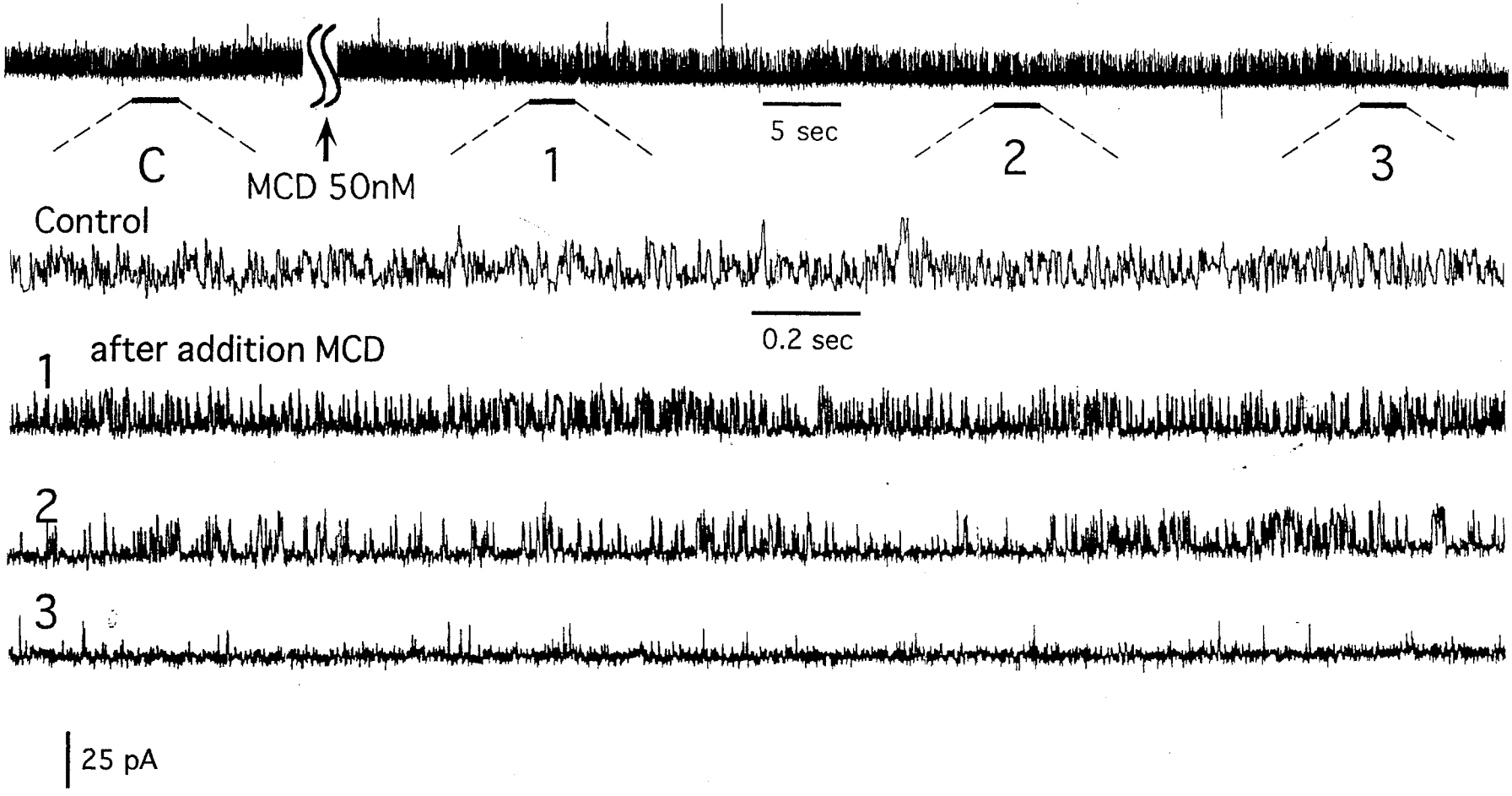

C
MCD 50nM
1
5 sec
2
3
Control
0.2 sec
after addition MCD
1
2
3
25 pA

Fig 9

Current-voltage (I-V) relationship of MCD-inactivated channel. The single channel conductance of 16.2 pS was calculated from the slope of the linear regression curve between -100 and -40 mV. Reversal potential was estimated to be -11 mV.

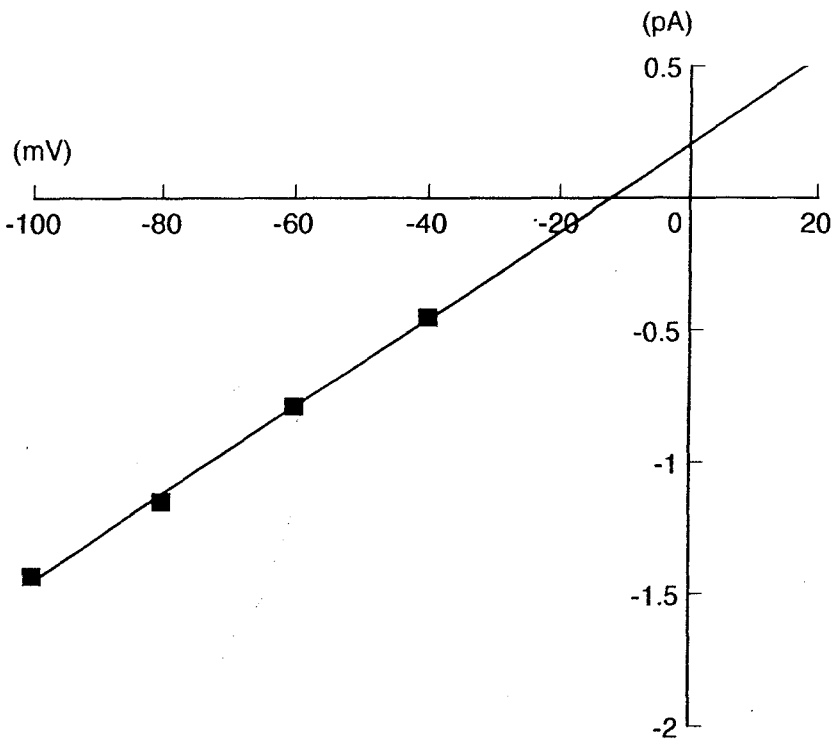
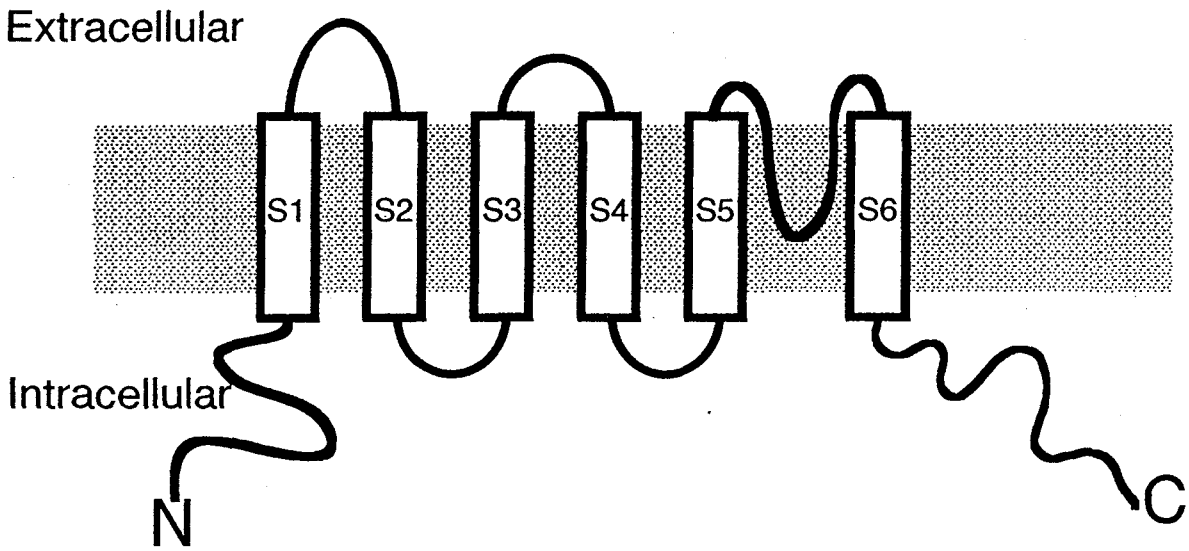

(pA)
0.5
(mV)
-100
-80
-60
-40
-20
0
20
-0.5
-1
-1.5
-2

Fig 10

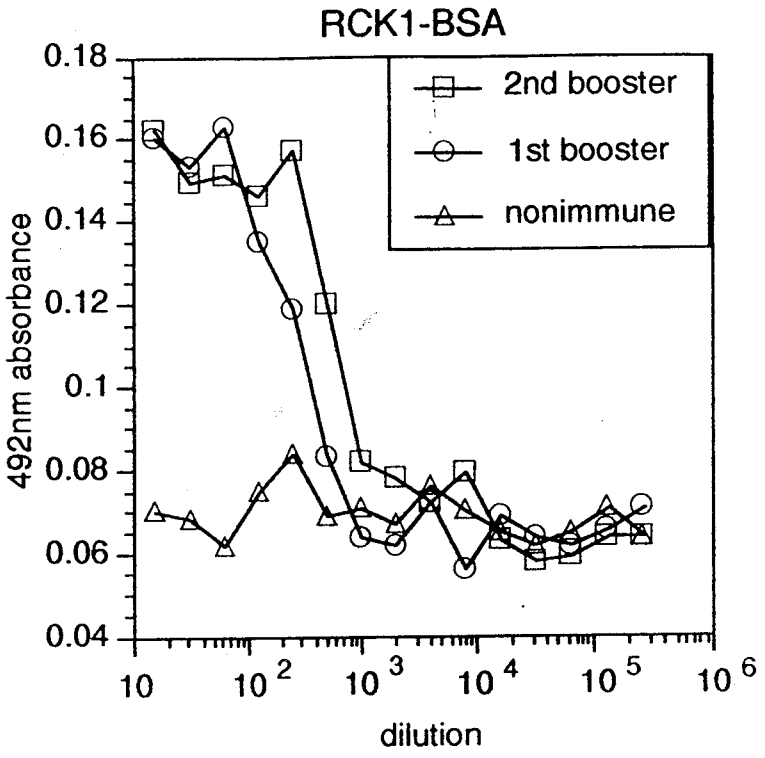
Schematic representation of voltage-dependent potassium channel and amino acid sequences used for production of specific antibodies. To obtain the epitope specific antibody, 15 or 10 amino acids sequences (box) corresponding to extracellular domain, between S1 and S2, of rat RCK channels were selected.




```
RCK1 PARVIAIVSVMVILISIVIFCLETLP----------ELK--------------D-DKD---FTGTIHRIDN-T--TVIY-T---SNIFTDPFFIVETLCIIWFSFELVVRFFA
RCK2 PARGIAIVSVLVILISIVIFCLETLPQFRADGRGGSNEGSGTRMSPASRGSHEEEDEDEDSYAFPGSIPSGGLGTGGTSSFSTLGGSF-FTDPFFLVETLCIVWFTFELLVRFSA
RCK4 PARGIAIVSVLVILISIVIFCLETLPEFRDD-RDLI-------M-ALSAGGHSRLLNDT-S-A-PHLENSGH--TI----FN---------DPFFIVETVCIVWFSFEFVVRCFA
RCK5 PARIIAIVSVMVILISIVSFCLETLPIFR-DE----NED----M-------H----------G---GGVTFHTYSNSTIGYQQSTSFTDPFFIVETLCIIWFSFELVRFFA
          S1                                                                                                  S2
```

Fig 11

Enzyme-linked immunosorbent assay (ELISA) of RCK1 and RCK5 antisera. Various concentrations of each antiserum were added into a 96-well microtitre plate coated with the purified synthesized peptides (2 μg/well). After treatment with biotinylated anti-(rabbit IgG) antibody followed by avidin-conjugated peroxidase, peroxidase activity was measured.


RCK1-BSA
2nd booster
1st booster
nonimmune
492nm absorbance
0.18
0.16
0.14
0.12
0.1
0.08
0.06
0.04
10
10 2
10 3
10 4
10 5
10 6
dilution

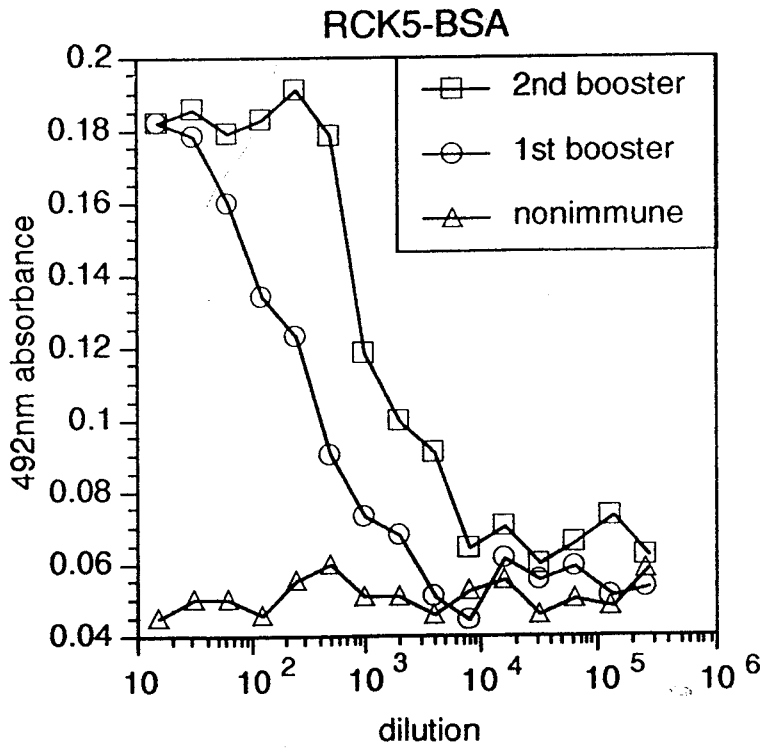

RCK5-BSA
2nd booster
1st booster
nonimmune
492nm absorbance
0.2
0.18
0.16
0.14
0.12
0.1
0.08
0.06
0.04
10
10 2
10 3
10 4
10 5
10 6
dilution

Fig 12

Western blotting analysis of brain extracts with three different antibodies. P_2 membrane fraction of rat brain was fractionated by 15 % SDS-PAGE and blotted onto nitrocellulose filter. The lanes are (1) low molecular weight marker; (2) amide black staining of proteins; (3) reaction with anti-RCK1 antibody; (4) reaction with anti-RCK2 antibody; (5) reaction with anti-RCK5 antibody.

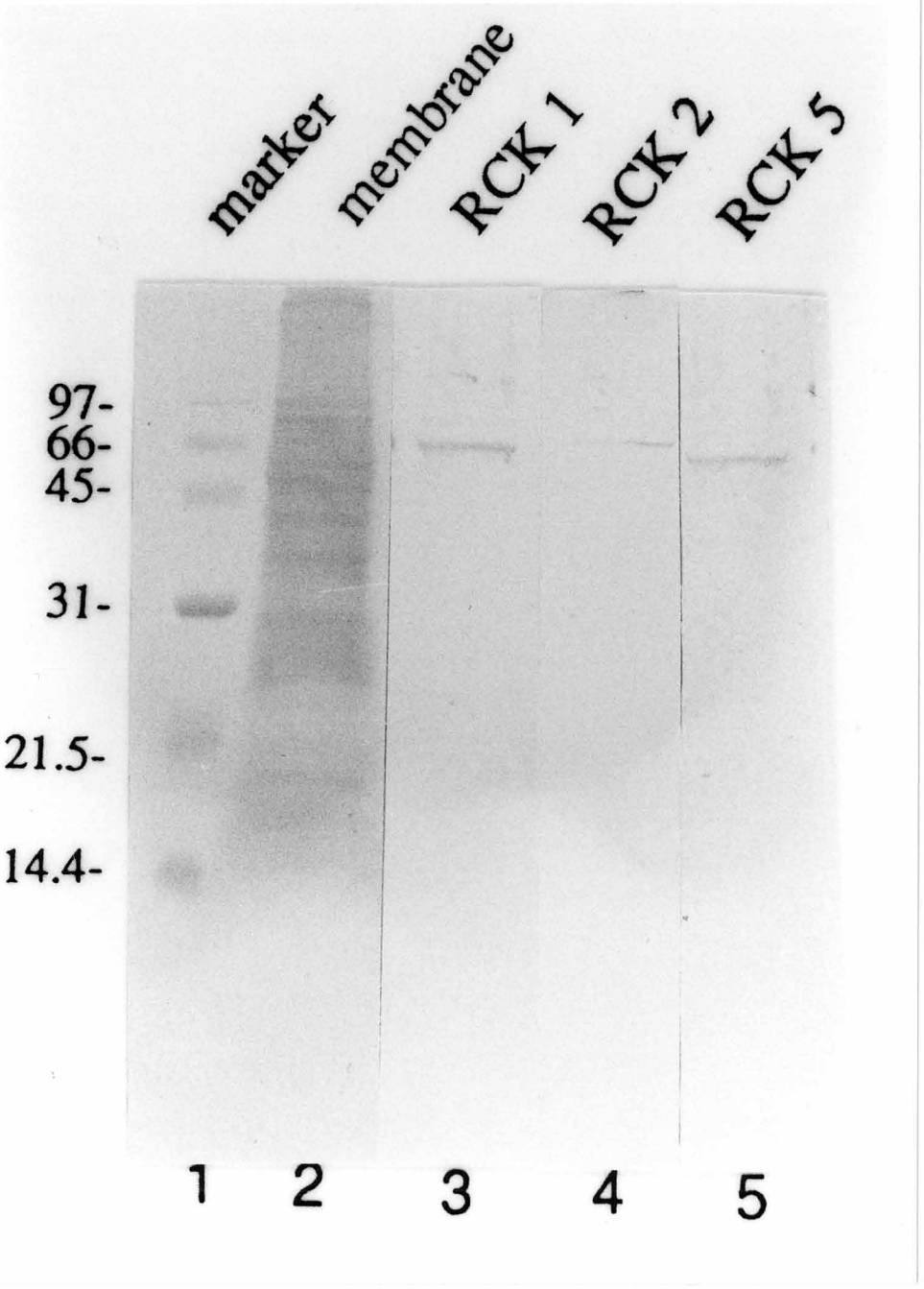

marker
membrane
RCK 1
RCK 2
RCK 5
97-
66-
45-
31-
21.5-
14.4-
1
2
3
4
5

Fig 13

Western blotting analysis of purified proteins with anti-RCK1 and anti-RCK5 antibodies. The eluates of DTxI-Sepharose and DE-5PW were fractionated by 15 % SDS-PAGE and blotted onto nitrocellulose filter. The lanes are (1) low molecular weight marker; (2,4) eluate of DTxI-Sepharose; (3,5) eluate of DE-5PW; (2,3) reaction with anti-RCK1 antibody; (4,5) reaction with anti-RCK5 antibody.

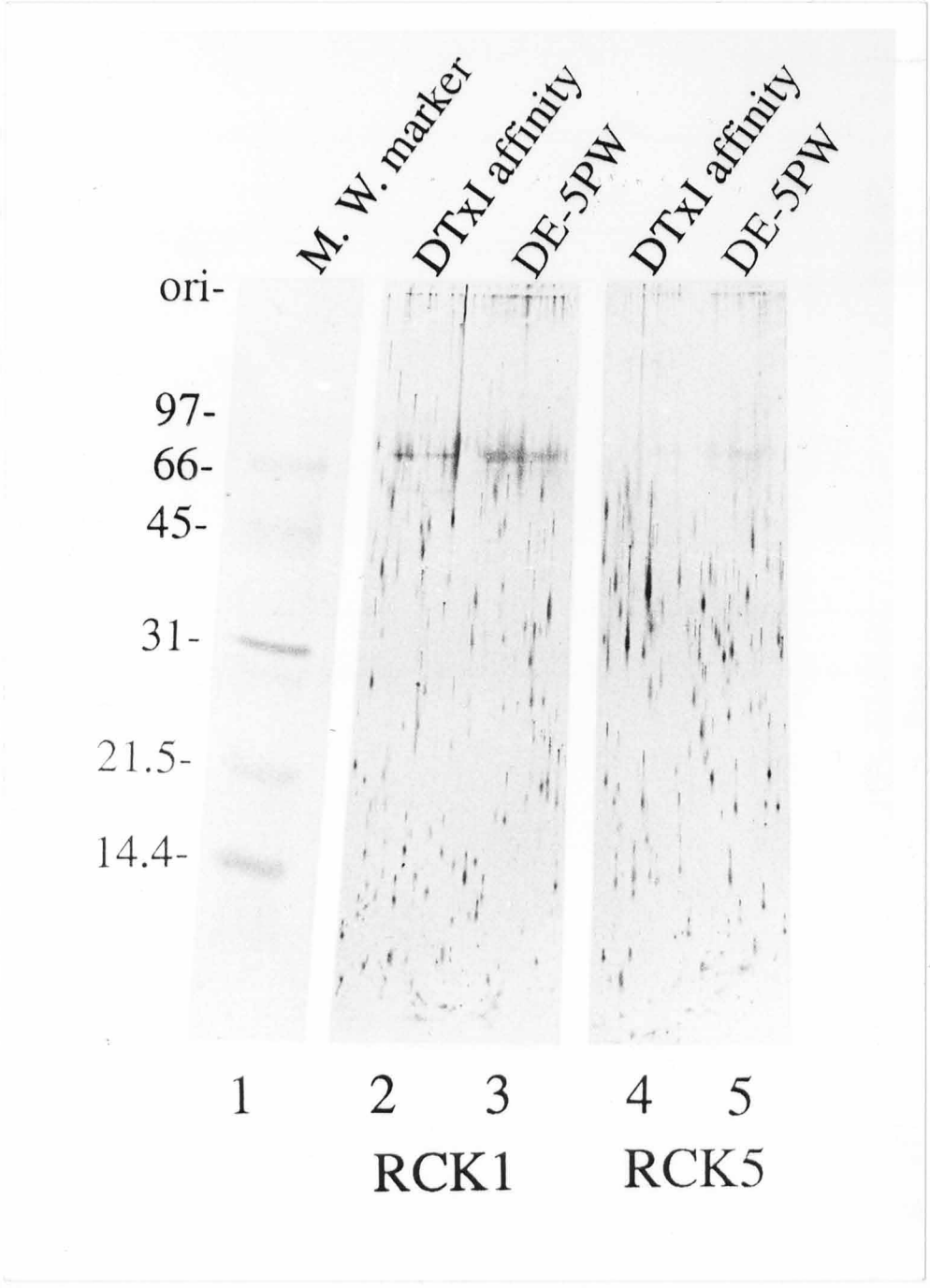

M. W. marker
DTxI affinity
DE-5PW
DTxI affinity
DE-5PW
ori-
97-
66-
45-
31-
21.5-
14.4-
1
2
3
4
5
RCK1
RCK5

Fig 14

Immunohistochemical reaction with RCK1, RCK2, RCK4 and RCK5 on sections of the rat hippocampus. Adult rat brain was perfused with 4 % paraformaldehyde. Serial sagittal paraffin sections (6 μm thick) were incubated overnight at 4 °C in 1:50 dilution of each antiserum. Then they were incubated in 1:200 dilution of FITC-conjugated anti-rabbit IgG and their immunoreactivity were examined by laser-scanning confocal microscope.

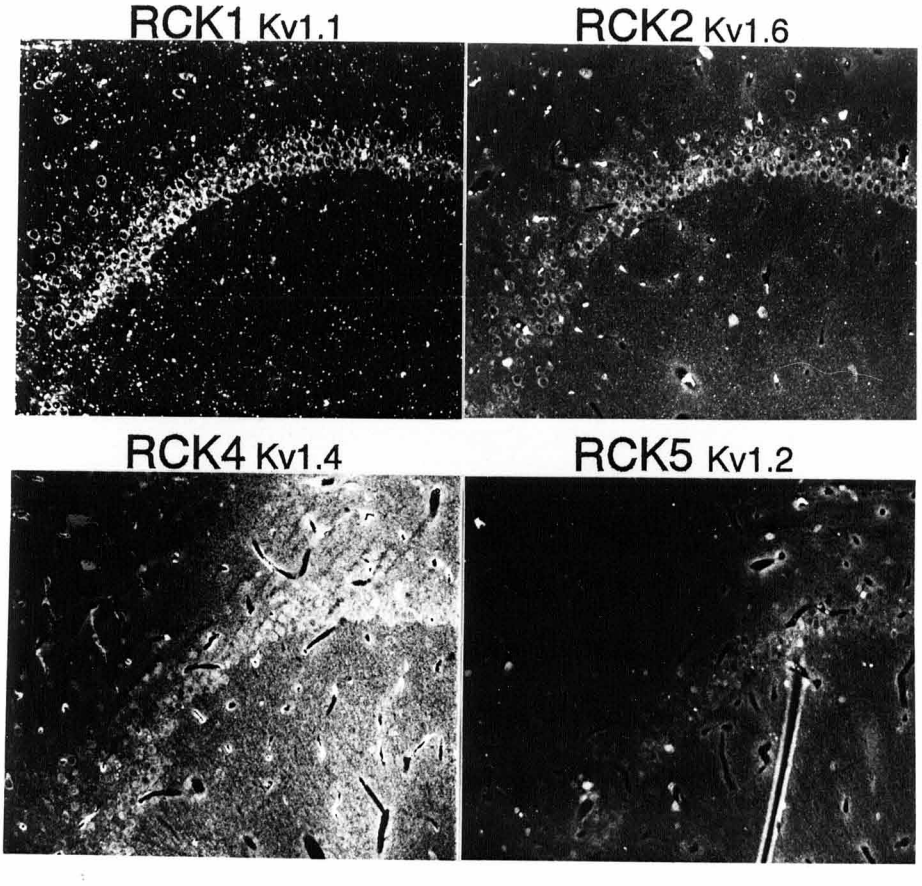
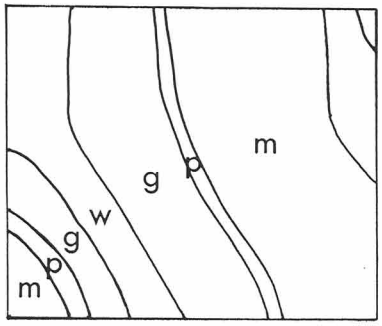
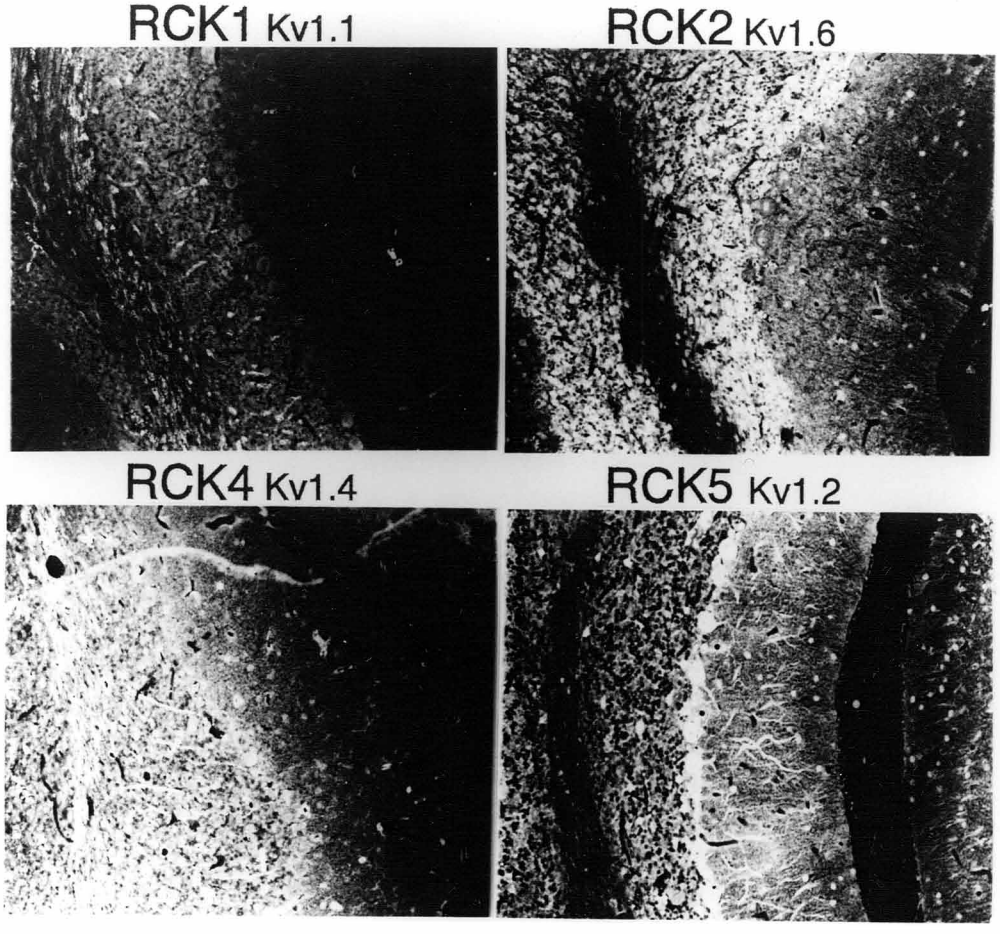

RCK1 Kv1.1
RCK2 Kv1.6
RCK4 Kv1.4
RCK5 Kv1.2

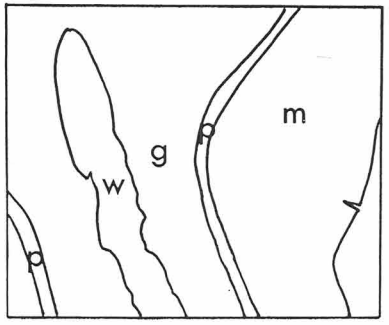
Fig 15

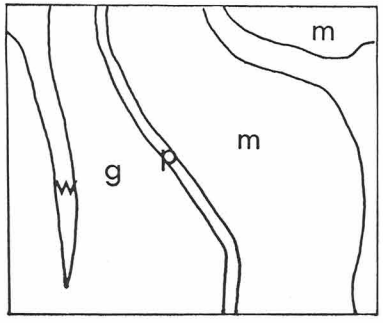
Immunohistochemical reaction with RCK1, RCK2, RCK4 and RCK5 on sections of the rat cerebellum. Adult rat brain was perfused with 4 % paraformaldehyde. Serial sagittal paraffin sections (6 μm thick) were incubated overnight at 4 °C in 1:50 dilution of each antiserum. Then they were incubated in 1:200 dilution of FITC-conjugated anti-rabbit IgG and their immunoreactivity were examined by laser-scanning confocal microscope.

m: molecular layer, P: Purkinji cell, g: granule cell layer, w: white matter.


m
g
p
w
g
p
m


RCK1 Kv1.1
RCK2 Kv1.6
RCK4 Kv1.4
RCK5 Kv1.2


m
g
p
w
p


m
m
g
p
w

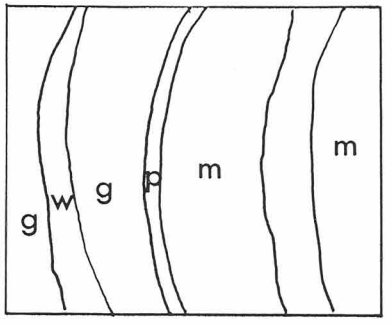
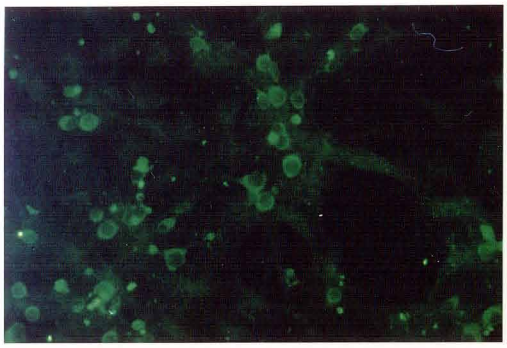

m
m
g
w
g
p

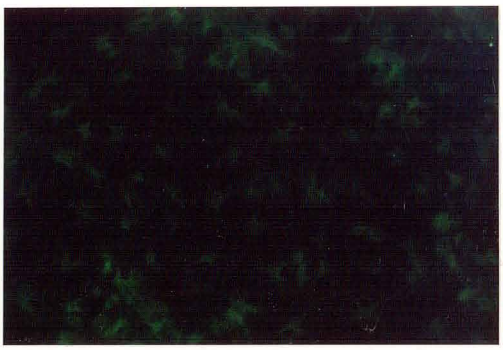
Fig 16

Immunohistochemistry of RCK1, RCK2, RCK4 and RCK5 in primary cultured hippocampal cells. Primary cultured hippocampal cells were fixed with ice cold ethanol containing 5 % acetic acid. Cells were incubated with anti-RCK peptide antibodies (1:100) and reacted with FITC-conjugated goat anti-rabbit IgG (1:200). Samples were observed using fluorescence microscopy.

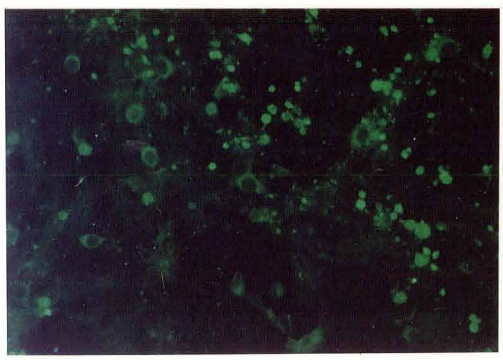
RCK1 Kv1.1



RCK2 Kv1.6



RCK4 Kv1.4



RCK5 Kv1.2

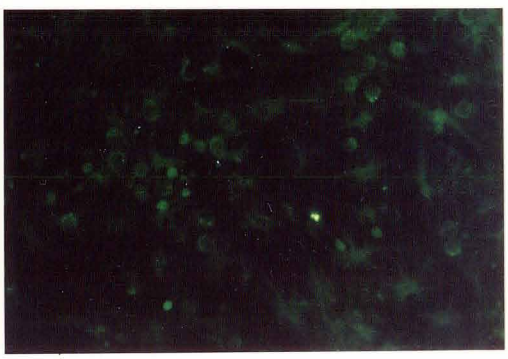
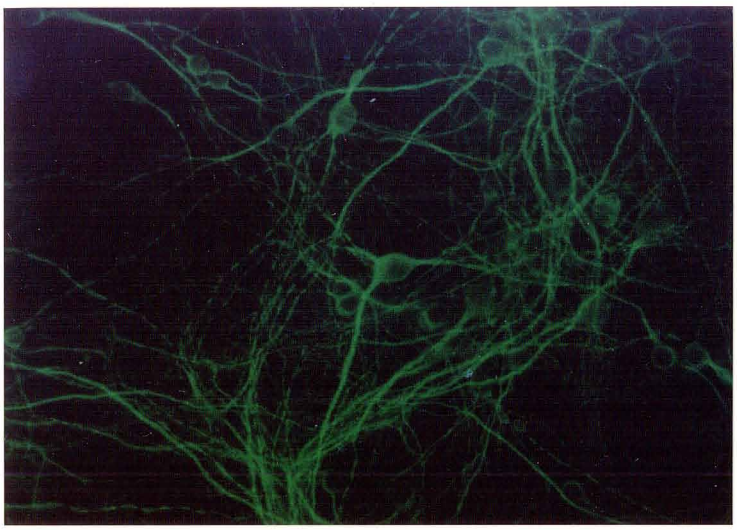


Fig 17

Immunohistochemistry of MAP2 and GFAP in primary cultured hippocampal cells. Primary cultured hippocampal cells were fixed with ice cold ethanol containing 5 % acetic acid. Cells were incubated with rabbit anti-MAP2 antibodies (1:2000) or mouse anti-GFAP monoclonal-antibody (1:200) and reacted with FITC-conjugated goat anti-rabbit IgG (1:200) or FITC-conjugated goat anti-mouse IgG (1:200). Samples were observed using fluorescence microscopy.

MAP2



GFAP

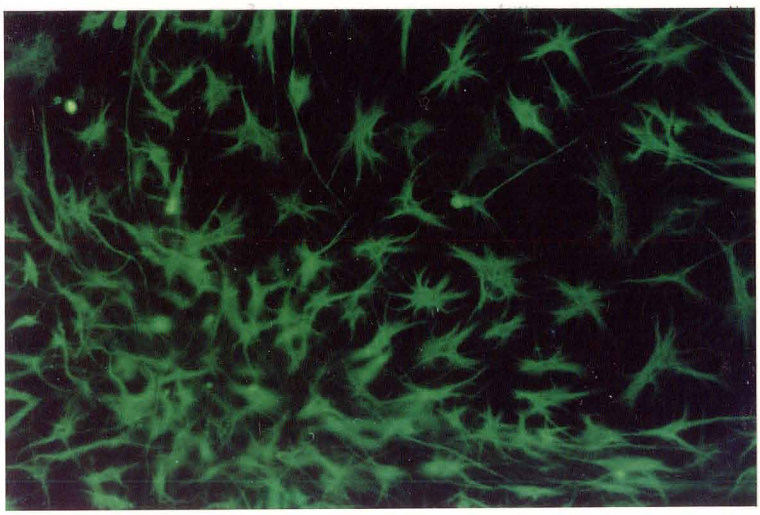


Fig 18

Inactivation of reconstituted channels by 150 nM MCD. The purified DTxI binding protein was reconstituted into the planar bilayer membrane. More than three channels were reconstituted in this experiment. MCD was applied at the point indicated by a large arrow. Almost all currents were suppressed by 150 nM MCD within 25 sec. Planar lipid bilayer was formed from phosphatidylcholine. The chamber, designated *cis*, contained 300 mM KCl and 10 mM Tris-Hepes, pH 7.2. The other chamber, designated *trans*, contained 100 mM KCl and 10 mM Tris-Hepes, pH 7.2. Baths were voltage clamped at 100 mV. Signal was filtered with a low-pass filter with cutoff frequency of 500 Hz. Base line was indicated by small arrows.

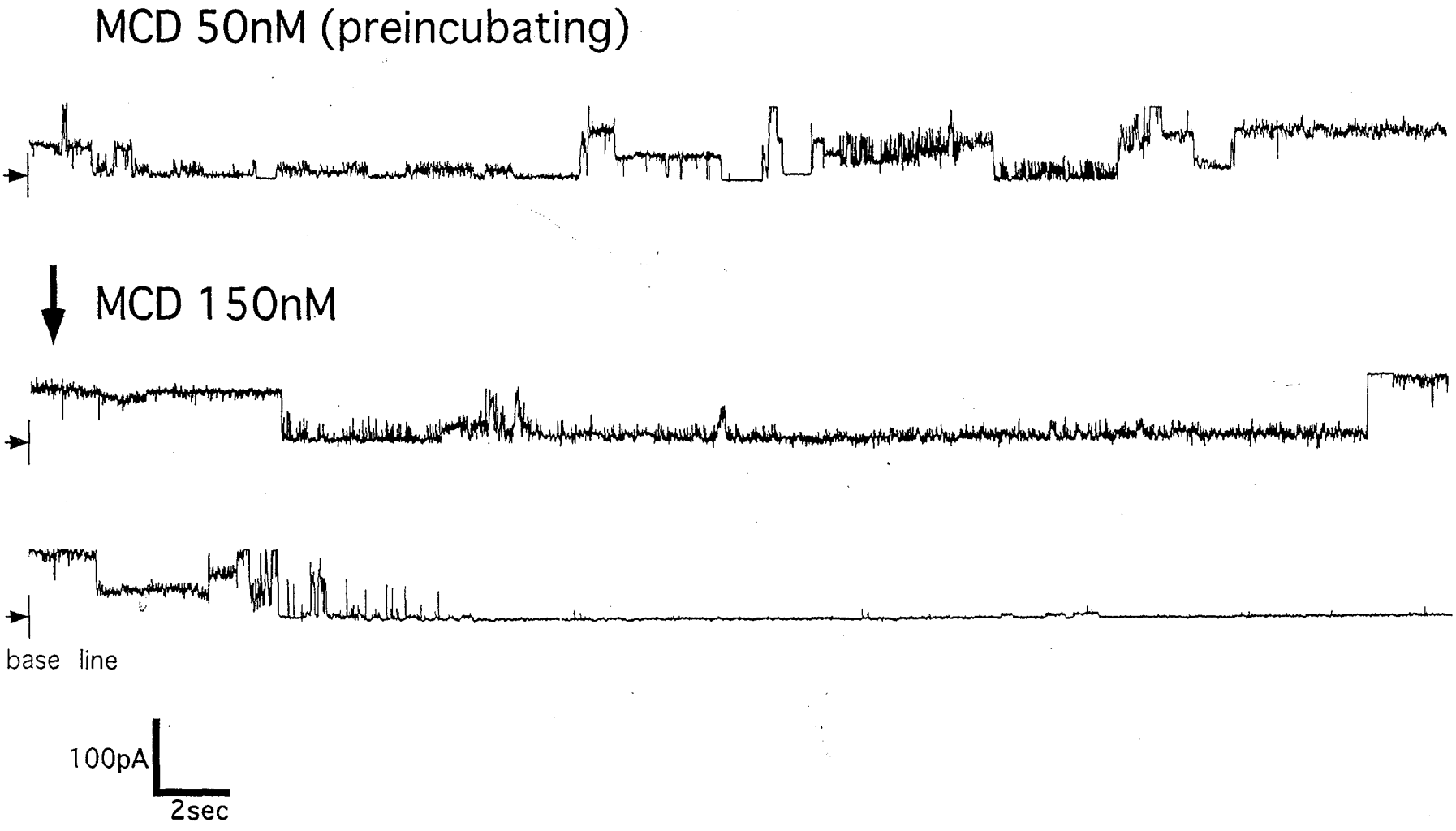
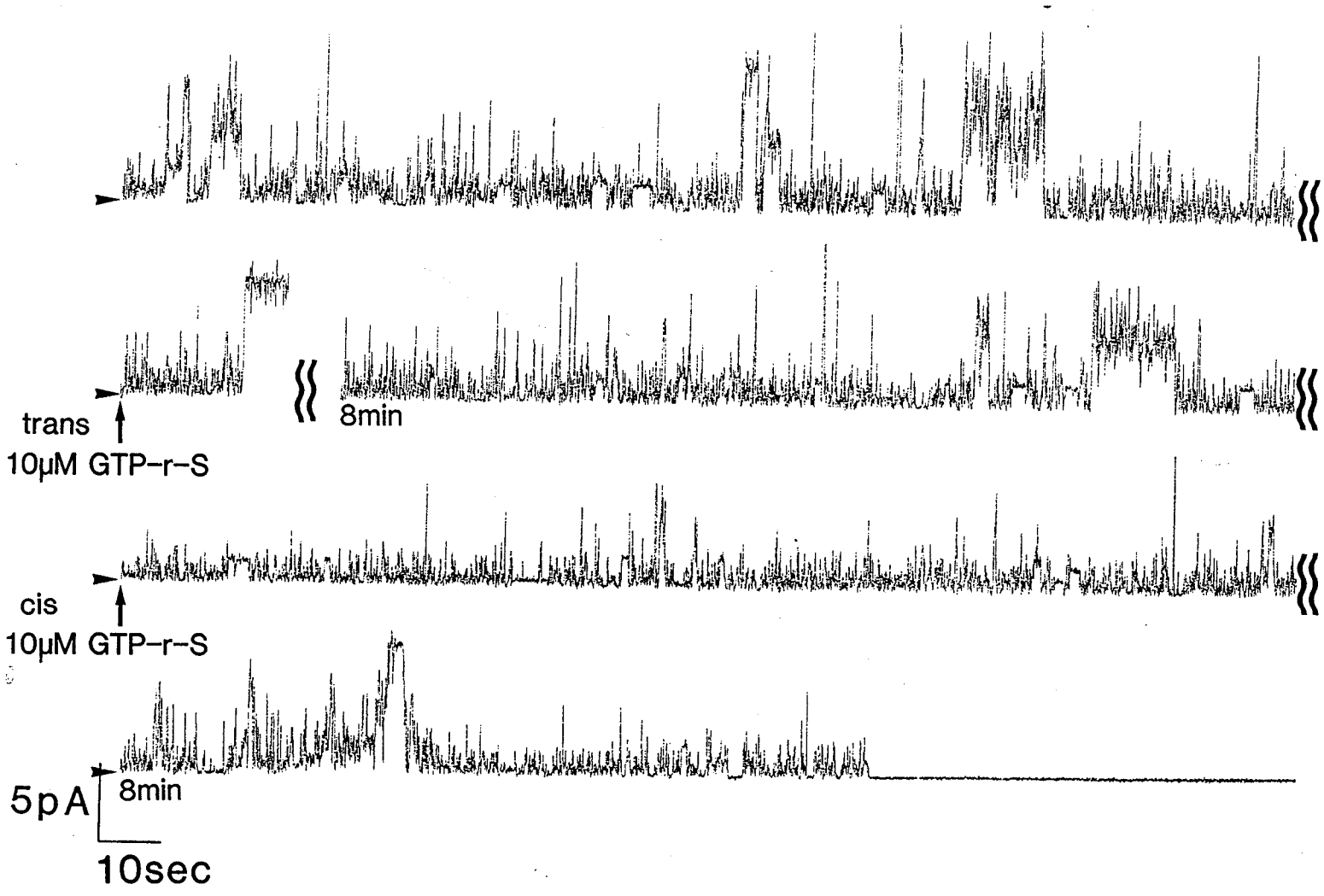

MCD 50nM (preincubating)
MCD 150nM
base line
100pA
2sec

Fig 19

Inactivation of reconstituted channels by guanosine 5'-γ-thio triphosphate (GTP-γ-S). The purified DTxI binding protein was reconstituted into planar bilayer membrane. The chamber, designated *cis*, contained 300 mM KCl and 10 mM Tris-Hepes, pH 7.2. The other chamber, designated *trans*, contained 100 mM KCl and 10 mM Tris-Hepes, pH 7.2. No effect was observed by addition of 10 μM GTP-γ-S to the *trans* bath. Addition of 10 μM GTP-γ-S to the *cis* bath suppressed the current within 10 min.


trans
10μM GTP-r-S
8min
cis
10μM GTP-r-S
8min
5pA
10sec

ACKNOWLEDGMENTS

I wish to express gratitude to Prof. Kazuhiro Ikenaka and Prof. Katsuhiko Mikoshiba. I wish to thank Prof. Toshiaki Katada for immunoblotting analysis of G protein, Dr. Hiroshi Katsuki for patch-clamp analysis of hippocampal neurons, Prof. Hiroshi Kato for LTP measurement in hippocampal slice, Prof. Saburo Aimoto for synthesis of MCD peptide, Drs. Shigetoshi Oiki, Takashi Yamamoto, Takahisa Taguchi and Takashi Kawasaki for their helpful suggestions in the lipid bilayer system and Dr. Tetsuro Kondo for helpful discussion. I am also grateful to all colleagues in the Laboratory of Neural Information, Department of Information Physiology, National Institute for Physiological Sciences, Okazaki National Research Institutes, especially Drs. Kensuke Nakahira, Tetsushi Kagawa for valuable discussions.