A-53930A and B, Novel N-Type Ca^{2+} Channel Blockers

MARIE HISAMOTO, YOSHINORI INAOKA, YOSHIHARU SAKAIDA[††], TAKESHI KAGAZAKI[††], RYUZO ENOKIDA[†††], TAKAO OKAZAKI[†††], HIDEYUKI HARUYAMA[†], TAKESHI KINOSHITA[†] and KEIICHI MATSUDA

Neuroscience Research Laboratories,
[†]Analytical and Metabolic Research Laboratories, Sankyo Co., Ltd.,
1-2-58 Hiromachi, Shinagawa-ku, Tokyo 140-8710, Japan
[††]Biomedical Research Laboratories, Sankyo Co., Ltd.,
389-4 Shimokawa, Izumi-machi, Iwaki-shi, Fukushima 971-8183, Japan
[†††]Tsukuba Research Laboratories, Sankyo Co., Ltd.,
Miyukigaoka 33, Tsukuba-shi, Ibaraki 305-0841, Japan

(Received for publication January 8, 1998)

A-53930A, B and C, which inhibit N-type Ca^{2+} channels, were isolated from the culture broth of *Streptomyces vinaceusdrappus* SANK 62394. A-53930A and B were new compounds which contained a carbamoyl group on the 6-hydroxyl group of the D-gulosamine part of streptothricin. A-53930C was identical to streptothricin B. A-53930A, B and C inhibited [^{125}I]ω-conotoxin MVIIA binding to N-type Ca^{2+} channels (IC_{50}= 0.17, 0.091 and 0.071 μM), but did not inhibit [^{3}H]PN200-110 binding to L-type Ca^{2+} channels ($IC_{50} > 50\ \mu$M). These compounds also inhibited [^{3}H]norepinephrine release from chick cerebral cortex synaptosomes (IC_{50}=91.0, 20.6 and 39.5 μM), indicating these compounds selectively block N-type Ca^{2+} channels which are important for neurotransmitter release. It was also revealed that although A-53930C had antimicrobial activity against Gram-negative and -positive bacteria and fungi, A-53930A and B showed weak activity only against Gram-negative bacteria.

Following brain ischemia, there is an excessive release of excitatory neurotransmitters such as glutamate from depolarized nerve terminals[1]. The increased concentration of extracellular neurotransmitters stimulates influx of calcium into neurons, which initiates a cascade that results in cell death. A key step in secretion of neurotransmitters is the influx of calcium through voltage-gated calcium channels which are located at presynaptic nerve terminals[2]. Thus calcium channel blockers, especially those that block excitatory transmitter release, might be expected to be neuroprotective against neuronal damage following brain ischemia.

Voltage-gated calcium channels have been classified according to pharmacological and biophysical characteristics into several types (T, L, N, P/Q and R). N-Type calcium channels are mainly identified in neuronal cells[3] and are distinguished from other types of voltage-gated calcium channels by their sensitivity to the peptide toxins ω-conotoxin MVIIA (ω-CgTx MVIIA) and GVIA, which are 25 and 27 amino acids in length[4,5]. Through the use of these peptide toxins, it is now clear that N-type calcium channels support depolarization-evoked release of neurotransmitters from presynaptic nerve terminals[6~8]. Recent studies have also shown ω-CgTx MVIIA to be neuroprotective in animal models of brain ischemia[9,10]. These results suggest the potential use of N-type calcium channel blockers in the prevention of neuronal damage resulting from ischemia.

To elucidate the function of N-type calcium channels and also for therapeutic applications, small organic N-type calcium channel antagonists are required, but to date, ω-CgTx MVIIA and GVIA are the only known specific N-type calcium channel blockers. In the course of screening for N-type calcium channel blockers, we have isolated A-53930A, B and C from the culture broth of *Streptomyces vinaceusdrappus* SANK 62394. In this paper, we report on the isolation, physico-chemical properties, biological properties and antimicrobial properties of novel compounds A-53930A and B.

Materials and Methods

Taxonomic Studies

The producing organism, strain SANK 62394, was isolated from a soil sample collected in Amakusa, Kumamoto Prefecture, Japan. Morphological, cultural and physiological characterization of strain SANK 62394 was carried out by the methods described by the International Streptomyces Project (ISP)[11] and WAKSMAN[12]. Several other media were also used for morphological studies. The color names were assigned according to "Guide to Color Standard" (a manual published by Nippon Shikisai Kenkyusho, Tokyo, Japan). The cultures were observed after incubation at 28°C for 14 days unless otherwise stated. Diaminopimelic acid in the whole-cell was analyzed by the method of HASEGAWA *et al.*[13].

Fermentation

All cultivations were carried out using PY medium consisting of (%): glucose (2), starch (1), pressed yeast (0.9), beef extract (0.5), polypepton (0.5), $CaCO_3$ (0.3), NaCl (0.5), CB442 (0.02), at a pH of 7.4. A loopful of spores from a slant culture of strain SANK 62394 was inoculated into a 2-liter baffled flask which contained 700 ml of PY medium. The inoculated flasks were incubated on a rotary shaker (210 rpm) at 28°C for 4 days. Then 2.1 liters of this seed culture was transferred into a 200-liter jar fermentor containing 100 liters of PY medium. Cultivation was carried out at 28°C for 115 hours with aeration (1.0 v/v/m) and agitation (110~145 rpm).

Isolation of A-53930A, B and C

The cultured broth (220 liters) was filtered with the aid of Celite 545. The filtrate (200 liters) was absorbed on a Diaion HP20 column (20L) and after washing with 40 liters of 10% MeOH, the active material was eluted with 100 liters of 10% MeOH, 0.5% CH_3COOH. The eluate was concentrated to 20 liters under reduced pressure and then absorbed on an Amberlite IRC-50 (NH_4) column (10L). After washing with 40 liters of water, the column was eluted with 40 liters of 1 N NH_4OH and the eluate was concentrated and lyophilized to obtain 38.18 g of the crude powder. For separation of A-53930A, B and C, preparative reverse phase HPLC was employed. A 19 g aliquot of this crude powder was applied and eluted repeatedly on a YMC ODS (15/30) column (100 × 500 mm, YMC Co. Ltd.) with acetonitrile - water - TFA (5 : 95 : 0.2) at 200 ml/minute, and the compounds were detected by UV absorption at 215 nm. The crude fraction of A-53930A, B and C were eluted at 48, 79 and 91 minutes after injection, respectively. Each fraction was collected and concentrated to 5.2 liters and absorbed on a carbon column (600 ml). After washing with 3 liters of water, the column was eluted with 3 liters of 40% MeOH, 0.5% CH_3COOH. After each fraction was concentrated and lyophilized to powder, it was again applied on the same HPLC. A-53930A (5.12 g) was dissolved and developed with MeOH - water - TFA (5 : 95 : 0.2). A-53930B (2.04 g) and A-53930C (1.20 g) were each dissolved and developed with MeOH - water - TFA (7 : 93 : 0.2). The main peaks were collected, and after concentration and lyophilization, 2.6, 1.4 and 0.6 g of A-53930A, B and C were obtained, respectively. In order to remove TFA, each powder was dissolved in 242 ml of water and passed through a Dowex-1-X2 (Cl^-) column (290 ml), and the column was eluted with 200 ml of water. The collected fractions were concentrated and lyophilized to obtain A-53930A (1.55 g), B (0.70 g) and C (0.20 g) in powder form. In the course of purification, activity was monitored by inhibition of [^{125}I]ω-CgTx GVIA binding to guinea-pig brain membrane.

Measurement of [^{125}I]ω-CgTx MVIIA Binding to Rat Brain Membrane

Preparation of rat brain membrane—Rat cerebrums were homogenized in 10 volumes of ice cold 50 mM HEPES-NaOH buffer, pH 7.4, in a Teflon-glass homogenizer. The homogenate was centrifuged at 48,000 × *g* for 15 minutes and the pellet was suspended in 50 mM HEPES-NaOH buffer, pH 7.4. After centrifugation at 48,000 × *g* for 15 minutes, the pellet was resuspended in the same buffer and stored at −80°C.

Binding assay—The assay was carried out according to ABE *et al.*[14]. Membrane (1 μg protein/tube) was incubated with [^{125}I]ω-CgTx MVIIA (20 pM final concentration, 2200 Ci/mmol) for 30 minutes at 37°C in 20 mM Tris-HCl buffer, pH 7.2, containing 0.1% BSA, 1 mM EDTA, in a total assay volume of 0.2 ml. The incubation was terminated by rapid filtration through a Whatman GF/C glass fiber filter presoaked in 0.3% polyethylenimine. After washing with ice-cold 20 mM Tris-HCl buffer, pH 7.2, the filter was dried and the radioactivity trapped was determined by γ-counter. Non-specific binding was measured in the presence of 10 μM unlabeled ω-CgTx MVIIA.

Preparation of guinea-pig brain membrane was done by the same method as preparation of rat brain membrane except the buffer used was 20 mM Tris-HCl buffer,

pH 7.2, containing 0.1 mM PMSF. Measurement of [^{125}I]ω-CgTx GVIA binding to guinea-pig brain membrane was performed by the same method as [^{125}I]ω-CgTx MVIIA binding assay.

Saturation binding analysis of [^{125}I]ω-CgTx MVIIA to rat brain membrane was performed using 1.21~43.3 pM ligand. Tissue aliquots were incubated in the absence or presence of A-53930B (100 nM) at 37°C for 30 minutes. Reactions were terminated by vacume filtration and washing, followed by determination of bound [^{125}I]ω-CgTx MVIIA.

Measurement of Association and Dissociation Rate of [^{125}I]ω-CgTx MVIIA Binding to Rat Brain Membrane

For kinetic analyses, samples were incubated at room temperature (22°C). The association of binding of [^{125}I]ω-CgTx MVIIA was started by adding membrane to [^{125}I]ω-CgTx MVIIA (20 pM) in the absence or presence of 100 nM A-53930B. After Incubation for 1, 5, 10, 20, 30 or 60 minutes at 22°C, reactions were terminated by vacume filtration and washing, followed by determination of bound [^{125}I]ω-CgTx MVIIA.

To measure the dissociation of binding of [^{125}I]ω-CgTx MVIIA, membrane was incubated for 60 minutes with 20 pM [^{125}I]ω-CgTx MVIIA. Measurement of dissociation was initiated by adding 10 μM unlabelled ω-CgTx MVIIA $\pm$ A-53930B (100 nM or 1 μM) at zero time. Reactions were terminated (at 1, 5, 10, 20, 30, 60 or 90 minutes) by vacume filtration and washing, followed by determination of bound [^{125}I]ω-CgTx MVIIA.

Measurement of [^{3}H]PN 200-110 Binding to Rabbit Skeletal Muscle T-Tubule Membrane

The preparation of membrane fraction and the binding assay were carried out according to GLOSSMAN *et al.*[15].

Preparation of rabbit skeletal muscle T-tubule membrane—Rabbit skeletal muscle from back and hind limb was finely minced with scissors in ice-cold solution containing 0.1 mM PMSF and 20 mM $NaHCO_3$, and homogenized with a Polytron. The homogenate was centrifuged at 1,500 $\times g$ for 15 minutes. Then the supernatant was filtered through gauze and the filtrate was centrifuged at 48,000 $\times g$ for 15 minutes. The pellet was resuspended with 50 mM Tris-HCl buffer, pH 7.4, containing 0.25 M sucrose and 0.1 mM PMSF, and layered on a discontinuous sucrose gradient (30 and 25% (w/w) sucrose in 50 mM Tris-HCl buffer, pH 7.4, containing 0.1 mM PMSF). Gradients were centrifuged at 100,000 $\times g$ for 17 hours. The membrane fraction at the 25% sucrose layer/overlay interface was collected, diluted in 50 mM Tris-HCl buffer, pH 7.4, containing 0.1 mM PMSF, and centrifuged at 100,000 $\times g$ for 30 minutes. The pellet was resuspended in 50 mM Tris-HCl buffer, pH 7.4, containing 0.25 M sucrose and 0.1 mM PMSF, and stored at −80°C.

Binding assay—Membrane fraction (5 μg protein/tube) was incubated with [^{3}H]PN 200-110 (5 nM final concentration, 87.0 Ci/mmol) for 60 minutes at 4°C in 50 mM Tris-HCl buffer, pH 7.4, containing 2 mM $CaCl_2$. Total assay volume was 0.2 ml. Incubation was terminated by rapid filtration through a Whatman GF/C glass fiber filter. Each filter was incubated with 300 μl of 10% Triton-X over-night and the radioactivity released was determined by liquid scintillation spectrometry. Non-specific binding was measured in the presence of 10 μM unlabeled PN 200-110.

Measurement of [^{3}H]Norepinephrine Release from Chick Brain Synaptosomes

Preparation of synaptosomes and assay of [^{3}H]norepinephrine release were done by the method according to ODA *et al.*[16].

Preparation of synaptosomes—All steps were performed at 4°C. Chick cerebrums were homogenized in 9 volumes of ice-cold 0.32 M sucrose containing 1 mM EDTA and 0.25 mM dithiothreitol, pH adjusted to 7.4 with dilute NaOH, in a Teflon-glass homogenizer, and the homogenate was centrifuged at 1,000 $\times g$ for 10 minutes. The supernatant was layered onto four-step discontinuous Percoll gradients (23, 15, 10 and 3% (vol./vol.) Percoll in 0.32 M sucrose containing 1 mM EDTA and 0.25 mM dithiothreitol, pH adjusted to 7.4 with dilute NaOH) and centrifuged at 32,400 $\times g$ for 9 minutes. The membrane fraction at the 15/23% interface was collected, washed twice by centrifugation at 15,000 $\times g$ for 15 minutes with HPS buffer (composition in mM: NaCl 132, KCl 4.8, $MgCl_2$ 2.4, KH_2PO_4 0.5, glucose 10, HEPES (pH 7.4) 20, and ascorbate 0.5, gassed with 95% O_2/5% CO_2), containing 0.1% (wt/vol.) BSA and 50 μM pargyline.

Potassium-induced [^{3}H]norepinephrine release—The synaptosomes were incubated with [^{3}H]norepinephrine (95 nM final concentration, 41 Ci/mmol) for 15 minutes at 30°C to preload the radioactive transmitter. Once loaded, synaptosomes were washed four times with HPS buffer containing 50 μM pargyline, by centrifugation at 15,000 $\times g$ for 2 minutes. Synaptosomes were resuspended in HPS buffer containing 50 μM pargyline and 0.1 μM desipramine at a concentration of 500 μg protein/ml.

Aliquots (50 μl) of synaptosomal suspensions were incubated at 30°C for 3.5 minutes with 4 volumes of depolarizing HPS buffer containing 50 μM pargyline, 0.1 μM desipramine and 1.5 mM $CaCl_2$. The composition of depolarizing HPS buffer was the same as that of HPS buffer except that NaCl was isosmotically replaced by KCl to provide a final KCl concentration of 15 mM. The incubation was terminated by adding 2 ml of ice-cold HPS buffer containing 1.2 mM EGTA, followed by centrifugation at 15,000 $\times g$ for 5 minutes. The supernatant was collected and the radioactivity released was determined by liquid scintillation spectrometry.

Results

Taxonomy of Strain SANK 62394

The substrate mycelium of the strain SANK 62394 is observed generally on various agar media. The strain forms spiral sporophores, branching monopodially on aerial hyphae, with a smooth surface (Fig. 1). Special structures such as sporangia, zoo-spores, ball-like bodies, whorls, or sclerotia were not observed on the media employed.

The color of the substrate mycelium is pale brown to dull red. With age, the color of the substrate mycelium became brownish. The strain forms a brownish-gray to pale reddish-purple aerial mycelium. Neither melanoid pigment nor soluble pigment was produced. The strain grows within the temperature range of 11 to 46°C. Hydrolysis of starch was negative. Gelatin liquefaction was weakly positive. All of the carbohydrates tested were utilized. Other cultural and physiological properties are summarized in Table 1, 2 and 3, respectively.

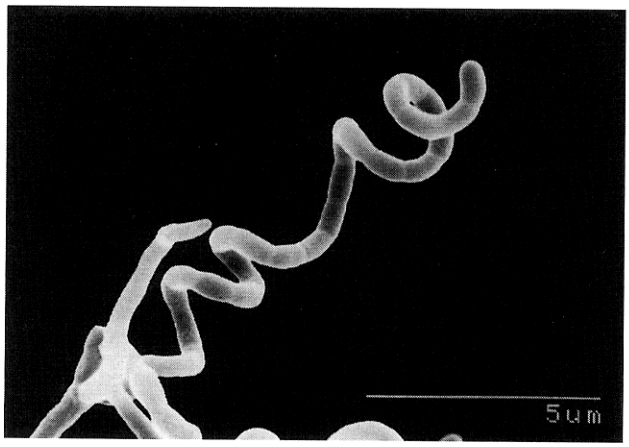
Based on the taxonomic properties described above, the strain SANK 62394 is considered a member of the genus *Streptomyces*. By comparison of the description of strain SANK 62394 with those of the *Streptomyces* species and by direct comparison with *S. vinaceusdrappus* ATCC 25511 as the most related strain, the strain was identified as *S. vinaceusdrappus* and designated *S. vinaceusdrappus* SANK 62394.

The progeny of the strain SANK 62394 has been deposited in the National Institute of Bioscience and Human Technology, Agency of Industrial Science and Technology, Ibaraki Prefecture, Japan, with the accession number of FERM BP-4811.

Structure Elucidation

The structures of A-53930-A, B and C were deduced as shown in Fig. 2. Determination of the chemical structures in detail will be reported elsewhere.

Fig. 1. Scanning electron micrograph of strain SANK 62394 on potato extract-carrot extract agar at 28°C for 7 days.



Physico-chemical Properties

The physico-chemical properties of A-53930-A, B and C are summarized in Table 4. The compounds are basic white powders, and soluble in water and methanol. Their molecular weight and molecular formulae (in parenthesis) were determined to be 887 ($C_{37}H_{70}N_{14}O_{11}$), 1015 ($C_{43}H_{82}N_{16}O_{12}$) and 1015 ($C_{43}H_{82}N_{16}O_{12}$) by mass spectrometry and elemental analysis, respectively. A-53930A contained 4β-lysine molecules and A-53930B and C contained 5β-lysine molecules. The differences of their molecular weights were exactly accounted for by this difference of the constituents.

Biological Properties

Biological properties of A-53930A, B and C are summarized in Table 5. A-53930A, B and C blocked the binding of [^{125}I]ω-CgTx MVIIA to rat brain membrane at IC_{50} values of 0.17, 0.091 and 0.071 μM, respectively. The IC_{50} value of streptothricin F which contains one β-lysine was 50 μM.

To evaluate the selectivity of A-53930A, B and C between neuronal N-type calcium channels and skeletal muscle L-type calcium channels, the binding of [^{3}H]-PN200-110 to rabbit skeletal muscle T-tubule membrane was determined. A-53930A, B and C did not inhibit the [^{3}H]PN200-110 binding up to 50 μM.

Calcium entry through N-type calcium channels mediates a neurotransmitter release from presynaptic nerve terminals. We investigated the effects of A-53930A, B and C on the neurotransmitter release from chick brain

Table 1. Cultural characteristics of strains SANK 62394 and *Streptomyces vinaceusdrappus* ATCC 25511.

		SANK 62394	ATCC 25511
Yeast extract-malt extract agar (ISP 2)	G :	Abundant, flat, reddish brown	Abundant, flat, pale yellowish brown
	AM :	Good, velvety, grayish red to light brownish gray	Abundant, velvety, light brownish gray
	R :	Brown	Pale yellowish brown
	SP :	None	None
Oatmeal agar (ISP 3)	G :	Abundant, flat, pale brown	Good, flat, pale brown
	AM :	Abundant, velvety, light brownish gray	Abundant, velvety, light brownish gray
	R :	Pale brown to pale red	Pale yellowish brown
	SP :	None	None
Inorganic salts-starch agar (ISP)	G :	Abundant, flat, light brwonish gray	Abundant, flat, pale brown
	AM :	Abundant, velvety, light brownish gray	Abundant, velvety, light brownish gray
	R :	Pale red	Pale yellowish brown
	SP :	None	None
Glycerol-asparagine agar (ISP 5)	G :	Abundant, flat, pale yellowish orange to dull red	Abundant, flat, pale brown
	AM :	Abundant, velvety, light brownish gray to pink white	Good, velvety, white to light brownish gray
	R :	Light brownish gray to brownish purple	Pale yellow
	SP :	None	None
Peptone-yeast extract-iron agar (ISP 6)	G :	Abundant, flat, olive gray	Abundant, flat, light olive gray
	AM :	Poor, white	Poor, white
	R :	Pale yellowish brown	Light olive gray
	SP :	None	None
Tyrosine agar (ISP 7)	G :	Abundant, flat, pale brown to dark reddish brown	Abundant, flat, pale yellowish brown
	AM :	Abundant, velvety, light brownish gray to pale red purple	Good, velvety, brownish white to light brownish gray
	R :	Brown purple	Yellowish brown
	SP :	None	None
Sucrose-nitrate agar	G :	Abundant, flat, pale yellowish brown	Abundant, flat, pale yellowish orange
	AM :	Good, velvety, light brownish gray	Abundant, velvety, light brownish gray
	R :	Pale yellowish brown	Pale yellowish orange
	SP :	None	None
Glucose-asparagine agar	G :	Good, flat, pale red	Moderate, flat, brownish white
	AM :	Poor, white to pale pink	None
	R :	Pale red	Pale yellowish orange
	SP :	None	None
Nutrient agar (Difco)	G :	Abundant, flat, pale yellowish brown	Good, flat, pale brown
	AM :	Moderate, velvety, white	Good, velvety, white to light brownish gray
	R :	Pale yellowish brown	Light olive gray
	SP :	None	None
Potato extract-carrot extract agar	G :	Poor, flat, pale yellowish orange	Poor, flat, brownish white
	AM :	Moderate, velvety, light brownish gray	Moderate, velvety, light brownish gray
	R :	Light brownish gray	Light brownish gray
	SP :	None	None
Water agar	G :	Poor, flat, brownish white	Poor, flat, brownish white
	AM :	Moderate, velvety, light brownish gray	Poor, light brownish gray
	R :	Light brownish gray	Light brownish gray
	SP :	None	None

G:Growth, AM:Aerial mycelium, R:Reverse, SP:Soluble pigment

Table 2. Physiological properties of strains SANK 62394 and *Streptomyces vinaceusdrappus* ATCC 25511.

	SANK 62394	ATCC 25511
Hydrolysis of starch	Negative	Negative
Liquefaction of gelatin	Weakly positive	Weakly positive
Reduction of nitrate	Positive	Positive
Coagulation of milk	Positive	Negative
Peptonization of milk	Positive	Positive
Production of melanoid pigment	Negative	Negative
Decomposition of:		
Casein	Negative	Positive
Tyrosine	Positive	Positive
Xanthine	Negative	Negative
Temperature range for growth (Medium 1)	11 - 46℃	12 - 46℃
Optimum temperature for growth (Medium 1)	28 - 42℃	26 - 42℃
Sodium chloride resistance (Medium 1)	10%	7%

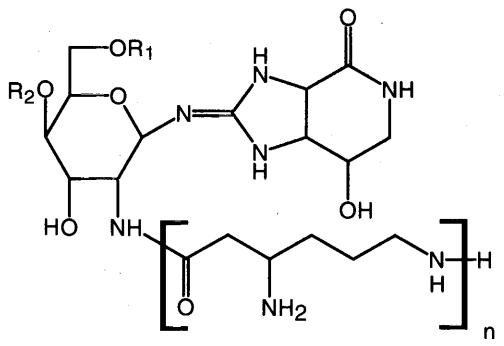
Medium 1: Yeast extract-malt extract agar(ISP 2).

Table 3. Carbohydrate utilization of strains SANK 62394 and *Streptomyces vinaceusdrappus* ATCC 25511.

	SANK 62394	ATCC 25511
D-Glucose	+	+
L-Arabinose	+	+
D-Xylose	+	+
Inositol	+	+
D-Mannitol	+	+
D-Fructose	+	+
L-Rhamnose	+	+
Sucrose	+	±
Raffinose	+	±
Control	−	−

+: Positive utilization, ±: doubtful utilization, −: negative utilization.

Fig. 2. Structures of A-53930A, B and C.



	R_1	R_2	n
A-53930A	$CONH_2$	H	4
A-53930B	$CONH_2$	H	5
A-53930C	H	$CONH_2$	5

synaptosomes. The release of [^{3}H]norepinephrine from chick brain synaptosomes by high potassium (15 mM) stimulation was blocked by ω-CgTx GVIA (IC_{50} = 0.47 μM, data not shown), indicating this release was mediated by the calcium influx through N-type calcium channels. A-53930A, B and C also effectively blocked the [^{3}H]norepinephrine release with IC_{50} values of 91.0, 20.6 and 39.5 μM, respectively. These results suggest A-53930A, B and C inhibited the function of N-type calcium channels by affecting N-type calcium channels.

Figure 3 shows a representative Scatchard analysis of the binding of [^{125}I]ω-CgTx MVIIA in control and in the presence of A-53930B (100 nM). The equilibrium dissociation constant for [^{125}I]ω-CgTx MVIIA (K_D) in control was 2.96 pM and a maximal binding capacity (B_{max}) of 0.81 pmol/mg protein. A-53930B increased the K_D for [^{125}I]ω-CgTx MVIIA to 12.8 pM without

Table 4. Physico-chemical properties of A-53930A, B and C.

	A-53930A	A-53930B	A-53930C
Nature	White basic powder		
Solubility	Soluble in Water, MeOH Insoluble in $CHCl_3$, Acetone, Ethyl acetate, DMSO, Hexane		
Ninhydrin reaction	Positive		
MP (°C)	55~57	61~63	61~65
$[\alpha]_D^{25}$ (C=0.5, H_2O)	-15.09°	-4.4°	-12.8°
Molecular formula	$C_{37}H_{70}N_{14}O_{11}$	$C_{43}H_{82}N_{16}O_{12}$	$C_{43}H_{82}N_{16}O_{12}$
Elemental analysis (%)			
Calcd :	C 38.81, H 6.99, N 17.13, Cl 18.88 as $C_{37}H_{70}N_{14}O_{11}$ · 6HCl· $2H_2O$	C 37.83, H 7.04, N 16.42, Cl 20.17 as $C_{43}H_{82}N_{16}O_{12}$ · 8HCl· $3H_2O$	C 39.42, H 7.10, N 17.11, Cl 18.99 as $C_{43}H_{82}N_{16}O_{12}$ · 7HCl· $2H_2O$
Found :	C 38.05, H 7.43, N 16.74, Cl 18.89	C 37.75, H 7.10, N 16.19, Cl 20.17	C 36.59, H 7.03, N 15.78, Cl 18.99
FAB-MS (m/z) $(M+H)^+$	887.5433	1015	1015
IR υ_{max}^{KBr} (cm^{-1})	468,521,591,671,818, 850,914,949,1023, 1078,1132,1164,1190, 1257,1299,1349,1386, 1419,1451,1473,1488, 1650,2037,2349,2936, 3069,3267,3387	470,517,592,672,818, 850,914,949,1077, 1130,1163,1190,1251, 1300,1349,1387,1450, 1473,1487,1558,1651, 2029,2350,2929,3034, 3260,3360	408,467,568,590,820, 850,929,950,980, 1030,1070,1128,1163, 1190,1256,1307,1389, 1473,1558,1650,1714, 1886,2029,2350,2936, 3062,3260,3379
UV (H_2O)	End adsorption		

Table 5. Inhibitory activities of A-53930 A, B, C and streptothricin F.

		IC_{50} (μM)		
	numbers of β-lysines	[^{125}I]ω-CgTx MVIIA binding	[^{3}H]PN200-110 binding	[^{3}H]norepinephrine release
A-53930 A	n=4	0.17	>50	91.0
A-53930 B	n=5	0.091	>50	20.6
A-53930 C	n=5	0.071	>50	39.5
streptothricin F	n=1	50	>50	-

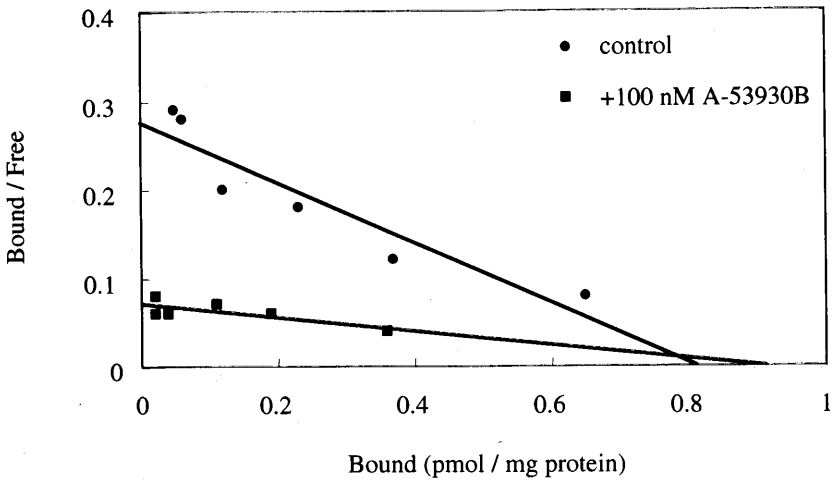
−: Not tested.

significantly affecting B_{max} (0.91 pmol/mg protein).

Effects of A-53930B on the association and the dissociation rate of [^{125}I]ω-CgTx MVIIA binding to N-type calcium channels are shown in Figure 4.

A-53930B 100 nM decreased the association rate but did not markedly affect the dissociation rate of [^{125}I]ω-CgTx MVIIA binding. Higher concentration of A-53930B (1 μM) slightly increased the dissociation rate constant of [^{125}I]ω-CgTx MVIIA binding.

Fig. 3. Scatchard analysis of [^{125}I]ω-CgTx MVIIA binding to rat brain membrane.



Membrane was incubated with various concentrations of [^{125}I]ω-CgTx MVIIA in the absence or presence of 100 nM A-53930B.

Antimicrobial Activities

Antimicrobial activities of A-53930A, B, C, and streptothricin F against Gram-positive and -negative bacteria and fungi were determined by conventional agar dilution method with Muller-Hinton broth as shown in Table 6. Although A-53930C (streptothricin B) and streptothricin F had strong antimicrobial activities against Gram-positive and -negative bacteria and fungi, A-53930A and B were moderately active only against Gram-negative organisms.

Acute toxicity was found in mice. When given intravenously, no toxicity was observed for A-53930A, B and C, up to 12.5, 6.25 and 3.13 mg/kg, respectively. Delayed death was seen by administration of 100 mg/kg streptothricin F.

Discussion

A-53930A and B are novel N-type calcium channel blockers isolated from microbial fermentation broth, which selectively inhibit N-type calcium channel bindings. They also inhibited N-type calcium channel dependent neurotransmitter release.

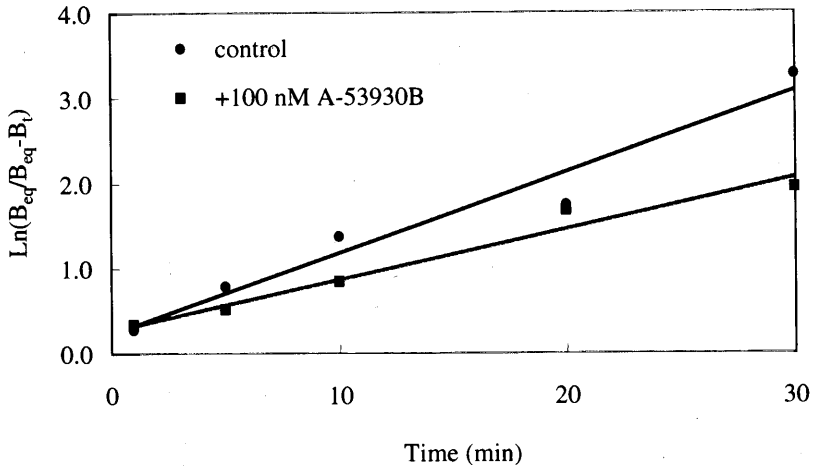
A-53930B, which is the most potent compound between two novel N-type calcium channel blockers, was used to characterize the inhibition of [^{125}I]ω-CgTx MVIIA binding to rat brain membrane. Scatchard analysis revealed A-53930B decrease the affinity of binding site for [^{125}I]ω-CgTx MVIIA binding without changing the number of binding site. Kinetic analysis showed that A-53930B decreased the rate of association of [^{125}I]ω-CgTx MVIIA binding but had no effect on the rate of dissociation. These data indicate that A-53930B interacts with [^{125}I]ω-CgTx MVIIA binding site in a competitively manner to inhibit the binding of [^{125}I]ω-CgTx MVIIA. A-53930B at 1 μM (about 10 times its IC_{50} value) slightly increased the dissociation rate of [^{125}I]ω-CgTx MVIIA binding. This may indicate the possibility of allosteric binding of A-53930B at higher concentration.

In our [^{125}I]ω-CgTx MVIIA binding assay, IC_{50}s are well correlated with the numbers of basic β-lysines (streptothricin F < A-53930A < A-53930B = C). It was shown that ionic interaction between the basic Lys re-sidue of ω-CgTx MVIIA and the extracellular surface of the calcium channels are important for the ω-CgTx GVIA binding[17,18], and the basicity of β-lysine moiety is also supposed to contribute to the binding of A-53930A, B and C to N-type calcium channels. We also investigated the inhibition of N-type calcium channel labeling by lysine peptides which contain 2 to 5 α-lysines, but it was far less effective (data not shown). It is possible that the position of the amino group in the β-lysine moiety is also important for the inhibitory effects of A-53930A, B and C to the N-type calcium channel binding.

There is a discrepancy between the IC_{50} values of [^{125}I]ω-CgTx MVIIA binding and [^{3}H]norepinephrine release. The reasons for this discrepancy may be multiple and could be due in part to experimental condition used in each experiment. It has been shown that [^{125}I]ω-CgTx

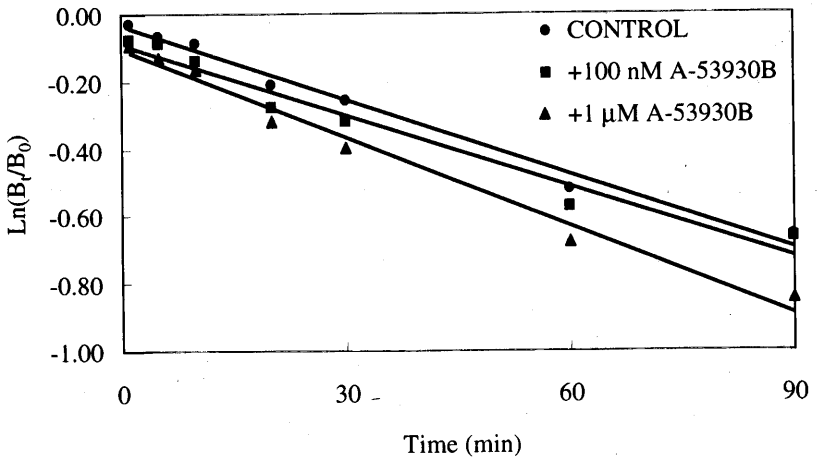
Fig. 4.

(A) Effect of A-53930B on the association rate of [^{125}I]ω-CgTx MVIIA binding to rat brain membrane.



Membrane was incubated with [^{125}I]ω-CgTx MVIIA in the absence or presence of 100 nM A-53930B. B_{eq} = specific binding of [^{125}I]ω-CgTX MVIIA at equilibrium time (60 minutes), B_t = specific binding of [^{125}I]ω-CgTx MVIIA at time t.

(B) Effect of A-53930B on the dissociation rate of [^{125}I]ω-CgTx MVIIA binding from rat brain membrane.



Membrane was incubated for 60 minutes with 20 pM [^{125}I]ω-CgTx MVIIA, after which dissociation was initiated by addition of 10 μM unlabelled ω-CgTx MVIIA in the absence or presence of 100 nM or 1 μM A-53930B. B_t = specific binding of [^{125}I]ω-CgTx MVIIA at time t, B_0 = specific binding of [^{125}I]ω-CgTx MVIIA at time zero.

MVIIA and GVIA binding is very sensitive to the presence of monovalent and divalent cations[14,19]. Functional assays using physiologic buffering systems often require 3~4 orders of magnitude increase in ω-CgTx concentrations to show efficacy, as compared to the binding studies which were done with low ionic strength buffer[19,20]. A-53930B is supposed to affect the [^{125}I]ω-CgTx MVIIA binding in competitive mechanism of inhibition and the interaction of cations with this binding site can be the reason for the large IC_{50} values of [^{3}H]norepinephrine release inhibition.

A-53930A and B are the first compounds which contain a carbamoyl group on the 6-hydroxyl group of the D-gulosamine part of streptothricin in place of the 4-hydroxyl group. As we have mentioned above, this difference in the D-gulosamine part had little effect on N-type calcium channel bindings because A-53930A and C inhibited N-type calcium channel binding in the same order. But this difference had some effect on antimicrobial activities. It is well known that streptothricins have

Table 6. Antimicrobial activities of A-53930A, B, C and streptothricin F.

Organism	MIC (μg/ml) A-53930A	A-53930B	A-53930C	streptothricin F
S. aureus 209P	0.4	0.4	<=0.01	1.5
S. aureus 56R	50	50	1.5	6.2
S. aureus 535 (MRSA)	200	200	50	>200
B. subtilis ATCC 6633	1.5	1.5	0.05	0.4
E. faecalis 681	>200	>200	200	200
E.coli NIHJ	200	200	25	12.5
E.coli 609	200	200	12.5	6.2
S. enteritidis	200	100	6.2	3.1
S. flexneri IID 642	200	200	12.5	6.2
K. pneumoniae 806	200	>200	12.5	6.2
K. pne 846 (R)	50	50	1.5	0.8
E. cloacae 963	200	>200	12.5	12.5
S. marcescens 1184	200	>200	25	12.5
P. vulgaris 1420	200	>200	25	12.5
M. morganii 1510	200	200	100	3.1
P. aeruginosa 1001	>200	>200	3.1	12.5
P. aeruginosa NO7	>200	>200	6.2	25
P. aeruginosa 3719	>200	>200	6.2	50
C. albicans	>100	>100	12.5	100
T. mentagrophytes	>100	>100	>100	25
T. interdigitale	>100	>100	100	25
T. rubrum	>100	>100	>100	50

strong antimicrobial activities with a broad spectrum of antibacterial effects, and the β-amino group of the β-lysine residue in this antibiotic is an important site for antimicrobial activity[21,22]. In our findings, A-53930A and B showed only weak antimicrobial activities compared to A-53930C (streptothricin B) and streptothricin F, although they contained β-lysine residues. This result suggests the position of the carbamoyl group in the D-gulosamine part is also important for the antimicrobial activities of streptothricins.

References

1) BENVENISTE, H.; J. DREJER, A. SCHOUSBOE & N. H. DIEMER: Elevation of the extracellular concentrations of glutamate and aspartate in rat hippocampus during transient cerebral ischemia monitored by intracerebral microdialysis. J. Neurochem. 43: 1369~1374, 1984
2) SIESJÖ, B. K. & F. BENGTSSON: Calcium fluxes, calcium antagonists, and calcium-related pathology in brain ischemia, hypoglycemia, and spreading depression: A unifying hypothesis. J. Cereb. Blood. Flow. Metab. 9: 127~140, 1989
3) MCCLESKY, E. W.; A. P. FOX, D. H. FELDMAN, L. J. CRUZ, B. M. OLIVERA, R. W. TSIEN & D. YOSHIKAMI: ω-Conotoxin: Direct and persistent blockade of specific types of calcium channels in neurons but not muscle. Pros. Natl. Acad. Sci. 84: 4327~4331 1987
4) OLIVERA, B. M.; G. P. MILJANICH, J. RAMACHANDRAN & M. E. ADAMS: Calcium channel diversity and neurotransmitter release: The ω-conotoxins and ω-agatoxins. Ann. Rev. Biochem. 63: 823~867, 1994
5) UCHITEL, O. D.: Toxins affecting calcium channels in neurons. Toxicon 35: 1161~1191, 1997
6) LUEBKE, J. I.; K. DUNLAP & T. J. TURNER: Multiple calcium channel types control glutamatergic synaptic transmission in the hippocampus. Neuron 11: 895~902, 1993
7) WHEELER, D. B.; A. RANDALL & R. W. TSIEN: Roles of N-Type and Q-Type Ca^{2+} Channels in Supporting Hippocampal Synaptic Transmission. Science 264: 107~111, 1994
8) TAKAHASHI, T. & A. MOMIYAMA: Different types of calcium channels mediate central synaptic transmission. Nature 366: 156~158, 1993
9) VALENTINO, K.; R. NEWCOMB, T. GADBOIS, T. SINGH, S. BOWERSOX, S. BITNER, A. JUSTICE, D. YAMASHIRO, B. B. HOFFMAN, R. CIARANELLO, G. MILJANICH & J.

RAMACHANDRAN: A selective N-type calcium channel antagonist protects against neuronal loss after global cerebral ischemia. Proc. Natl. Acad. Sci. 90: 7894～7897, 1993

10) TAKIZAWA, S.; K. MATSUSHIMA, H. FUJITA, K. NANRI, S. OGAWA & Y. SHINOHARA: A selective N-type calcium channel antagonist reduces extracellular glutamate release and infarct volume in focal cerebral ischemia. J. Cereb. Blood. Flow. Metab. 15: 611～618, 1995

11) SHIRLING, E. B. & D. GOTTLIEB: Methods for characterization of *Streptomyces* species. Int. J. Syst. Bacteriol. 16: 313～340, 1966

12) WAKSMAN, S. A. (*Ed.*): The Actinomycetes Vol. 2. Classification, Identification and Description of Genera and Species. Williams & Wilkins Co., Baltimore, 1961

13) HASEGAWA, T.; M. TAKIZAWA & S. TANIDA: A rapid analysis for chemical grouping of aerobic actinomycetes. J. Gen. Appl. Microbiol. 29: 319～322, 1983

14) ABE, T.; K. KOYANO, H. SAISU, Y. NISHIUCHI & S. SAKAKIBARA: Binding of ω-conotoxin to receptor sites associated with the voltage-sensitive calcium channel. Neurosci. Lett. 71: 203～208, 1986

15) GLOSSMANN, H. & D. R. FERRY: Assay for calcium channels. Methods in Enzymology 109: 513～550, 1985

16) ODA, T.; M. S. SHEARMAN & Y. NISHIZUKA: Synaptosomal protein kinase C subspecies: B. Down regulation promoted by phorbol ester and its effect on evoked norepinephrine release. J. Neurochem. 56: 1263～1269, 1991

17) KIM, J.-I.; M. TAKAHASHI, A. OHTAKE, A. WAKAMIYA & K. SATO: Tyr^{13} is essential for the activity of ω-conotoxin MVIIA and GVIA, specific N-type calcium channel blockers. Biochem. Biophys. Res. Commun. 206: 449～454, 1995

18) KOHNO, T.; J.-I. KIM, K. KOBAYASHI, Y. KODERA, T. MAEDA & K. SATO: Three-dimentional structure in solution of the calcium channel blocker ω-conotoxin MVIIA. Biochemistry 34: 10256～10265, 1995

19) STOEHR, S. J. & D. J. DOOLEY: Characteristics of [^{125}I]ω-conotoxin MVIIA binding to rat neocortical membranes. Neuroscience Letters 161: 113～116, 1993

20) YAMADA, K.; T. TERAOKA, S. MORITA, T. HASEGAWA & T. NABESHIMA: Neuropharmacological characterization of voltage-sensitive calcium channels: Possible existence of neomycin-sensitive, ω-conotoxin GVIA-and dihydropyridines-resistant calcium channels in the rat brain. Japan. J. Pharmacol. 63: 423～432, 1993

21) SAWADA, Y.; H. SAKAMOTO & H. TANIYAMA: Studies on chemical modification of streptothricin-group antibiotics. III. Partial *N*-acetylation of racemomycins and their biological activity. Yakugaku Zasshi 94: 176～180, 1974

22) SAWADA, Y. & H. TANIYAMA: Studies on chemical modification of streptothricin-group antibiotics. IV. Preparation of β-*N*-acetyl-racemomycin-A delivative and its antimicrobial activity. Yakugaku Zasshi 94: 264～266, 1974