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The Effects of Strychnine on the Regulation of Voltage-Dependent Calcium Channels by Dihydropyridines in Brain and Heart

S. K. O'NEILL* AND G. T. BOLGER†[1]

**Memorial University of Newfoundland, St. John's, Newfoundland, Canada A1B 3V6*
†BioMega Inc., Laval, Quebec, Canada H7S 2G5

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O'NEILL, S. K. AND G. T. BOLGER. *The effects of strychnine on the regulation of voltage-dependent calcium channels by dihydropyridines in brain and heart.* PHARMACOL BIOCHEM BEHAV **35**(4) 833–840, 1990.—The effects of strychnine (STR) were investigated on K^+-stimulated $^{45}Ca^{2+}$-uptake into mouse brain neurons, the contractile activity of spontaneously beating rat atria and on [^{3}H]nitrendipine and [^{3}H]BAY K 8644 binding to dihydropyridine calcium channel antagonist and agonist binding sites on brain and cardiac membranes. STR (10^{-6}–10^{-4} M) had no effect on neuronal $^{45}Ca^{2+}$-uptake. When combined at equimolar concentrations (10^{-5} M), STR and nifedipine produced a potent (nM) inhibition (40%) of neuronal $^{45}Ca^{2+}$-uptake. In the spontaneously beating rat atria, STR produced a dose-dependent (10^{-7}–3×10^{-4} M) decrease in chronotropy but did not affect inotropy. STR (10^{-4} M) completely inhibited the positive chronotropic, but did not affect the positive inotropic effects of (−)-S-BAY K 8644. [^{3}H]Nitrendipine and [^{3}H]BAY K 8644 binding to brain and cardiac membranes was enhanced by STR in a concentration-dependent manner (EC_{50} 8×10^{-6} M). Scatchard analysis revealed that STR increased the affinity (decreased the K_d) of [^{3}H]BAY K 8644 to a greater degree than that of [^{3}H]nitrendipine for dihydropyridine binding sites. STR decreased the K_d of [^{3}H]nitrendipine binding by increasing and decreasing the microassociation and microdissociation constants respectively. STR enhanced [^{3}H]nitrendipine binding to the same extent in the cerebral cortex, striatum, hippocampus, cerebellum, brainstem and spinal cord. The enhancement of [^{3}H]nitrendipine binding in brain was completely inhibited by Ca^{2-} and partially inhibited by Na^+ in a concentration-dependent manner. Glycine (10^{-2} M) did not affect the STR enhancement of [^{3}H]nitrendipine binding. Extensive rinsing of brain membranes increased (~two-fold) the affinity of STR for enhancement of [^{3}H]nitrendipine binding. The effects of STR on [^{3}H]nitrendipine binding both resembled and were unique to those of phencyclidine. These findings suggest that STR interacts specifically with calcium channels in brain and cardiac tissues at a site similar, but not identical to that of phencyclidine and which is distinct from the dihydropyridine calcium channel regulatory site.

Strychnine Calcium channels Dihydropyridine binding sites

STRYCHNINE (STR) is a potent convulsant drug which exerts its actions by antagonism of the inhibitory neurotransmitter glycine primarily in the spinal cord, but also in higher CNS centers (7, 12, 25, 28). Specific nanomolar affinity STR binding sites that interact with glycine and mediate the neurophysiological effects of STR have been well characterized and exist in their highest density in the spinal cord, but also are present in significant quantities in the midbrain, hypothalamus, thalamus, substantia nigra and medulla (6, 10, 12, 14, 28).

In addition to its antagonism of glycine receptors in the CNS, STR has been reported to interact with a number of ion channels, although at much higher concentrations. In the frog node of Ranvier, STR (60–300 μM) blocked potassium and sodium channels in the same manner and by a mechanism similar to channel block mediated by procaine (20,21). In rat cerebral cortex, it has been proposed that the STR spiking activity recorded during EEG measurements is due to an increased calcium conductance through calcium channels (16). STR also produced an increased amplitude and duration of spontaneous electrical and mechanical activity in rat portal vein, an effect mediated by a reduction in potassium conductance and/or an increase in smooth muscle cell calcium conductance.

While the actions of STR on sodium and potassium channels are well understood, there is a paucity of information on the mechanism of STR at calcium channels. Preliminary studies indicated that STR increased the affinity of [^{3}H]nitrendipine for

[1]Requests for reprints should be addressed to Dr. Gordon T. Bolger, BioMega, Inc., 2100 rue Cunard, Laval, Quebec H7S 2G5 Canada.

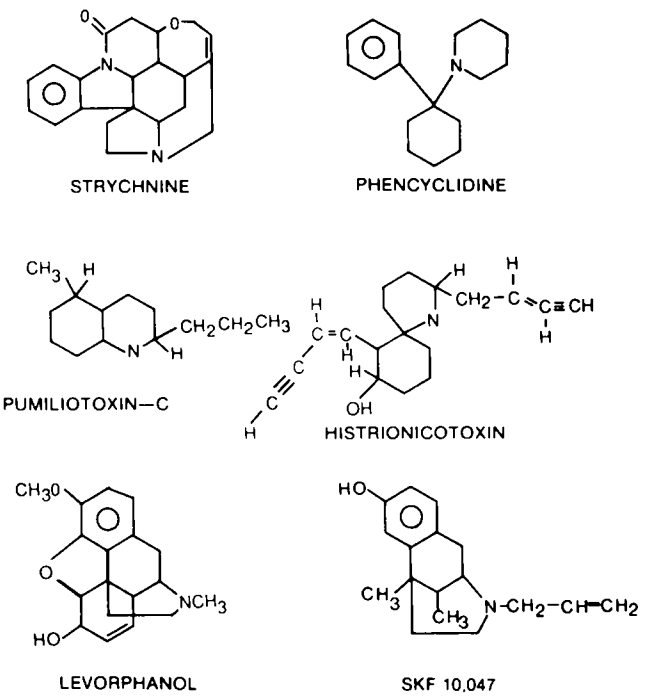

FIG. 1. Structures of drugs that increase the affinity of dihydropyridine binding sites.

voltage-dependent calcium channel (VDCC)-linked dihydropyridine (DHP) regulatory sites in mouse brain, an effect shared by phencyclidine (PCP) and structurally related compounds, levorphanol, SKF 10,047 and a number of ion channel toxins (Fig. 1) (2–4). In this report, we have characterized the effects of STR on K^+-stimulated $^{45}Ca^{2+}$-uptake into mouse brain neurons and on the contractile activity of the spontaneously beating rat atria in the presence and absence of DHP ligands. In addition, the effects of STR were characterized on the binding of [^{3}H]nitrendipine and [^{3}H]BAY K 8644 to VDCC agonist and antagonist binding sites on mouse brain membranes. Owing to the similar effect of STR and PCP on DHP binding sites, the potential of a similar site and mechanism of interaction for these two ligands was investigated. We now report that STR produced changes in the interaction of nifedipine and (−)-S-BAY K 8644 with neuronal calcium uptake and with spontaneous contraction in rat atrium. These effects were accompanied by specific changes in the kinetics of [^{3}H]nitrendipine and [^{3}H]BAY K 8644 binding to brain and cardiac membranes. A comparison of the effects of STR and PCP on contraction in the atrium and on DHP binding sites revealed a similar but biochemically different site of interaction.

METHOD

Materials

[^{3}H]Nitrendipine (specific activity 81 Ci/mmole), [^{3}H]BAY K 8644 (specific activity 89 Ci/mmole) and $^{45}CaCl_2$ were obtained from New England Nuclear-Dupont, Boston, MA. Strychnine (HCl) and phencyclidine were obtained from the Sigma Chemical Co., St. Louis, MO and the Bureau of Dangerous Drugs, Health and Welfare, Ottawa, Canada. Nifedipine was obtained from Pfizer, Groton, CT. (−)-S-BAY K 8644 was a gift from Dr. A. Scriabine, Miles Laboratories, West Haven, CT. The phencyclidine acylating derivative FOURPHIT [4-isocyanato-1-(phencylcyclohexyl) piperidine] was synthesized by Dr. Ralph A. Lessor and kindly supplied by Dr. Kenner C. Rice, Laboratory of Chemistry, NIADDK/NIH. Nitex 210 and 310 were purchased from Tetko, Elmsford, NY. All other reagents and chemicals were obtained from standard commercial sources and were of the highest purity possible.

Procedure

$^{45}Ca^{2+}$-uptake. K^+-stimulated $^{45}Ca^{2+}$-uptake was conducted in mouse brain neurons according to the procedure of Skattebol and Triggle (19). Briefly, male mice (18–22 g, Charles River, St. Constant, Quebec, Canada) were killed by decapitation and their brains rapidly placed into an ice-cold physiologic salt solution (PSS) of the following composition (mM): NaCl (138); KCl (5.4); $NaHPO_4$ (0.17); KH_2PO_4 (0.22); glucose, (5.5); and sucrose (46.9) pH adjusted to 7.35 with 1 N NaOH and containing 0.1 mM phenylmethylsulfonyl fluoride. The cerebellum and underlying brain stem were removed by blunt dissection. The remaining brain was sieved through a 210 μM mesh screen (Nitex 210) into 75 vol. of ice-cold PSS and twice more through a 130 μM screen (Nitex 130). The subsequent steps for the preparation of mouse brain neurons are as described for the preparation of rat brain neurons (19). Light microscope examination of the preparation of mouse brain neurons revealed vesicular-like structures with much larger irregularly shaped structures dispersed throughout. The average viability for all the structures identified using the trypan blue exclusion test ranged from 80–85%.

Spontaneously beating rat atria. Sprague-Dawley rats (175–200 g, Charles River, St. Constant, Quebec) were killed by cervical dislocation. The hearts were rapidly removed and placed into a physiologic salt solution (PS) at 37°C which had previously been equilibrated with 95% O_2/5% CO_2. The PS had the following composition (mM): NaCl (137); KCl (2.7); $CaCl_2$ (1.8); $MgCl_2$ (0.49); NaH_2PO_4 (0.36); dextrose (5.6); and $NaHCO_3$ (11.9). The right and left atria were dissected free from the surrounding tissue and mounted with 0.5 g tension to a high compliance strain gauge transducer (Grass Model FT03C 4, Grass Instruments Co., Quincy, MA) in a 10 ml organ bath containing PS at 37°C equilibrated with 95% O_2/5% CO_2. Atrial tissues were equilibrated for 60 min at 37°C (with one bath change every 15 min) before any drug addition(s). Contractile activity was recorded on a Grass Polygraph (Model 7D, Grass Instruments, Quincy, MA).

[^{3}H]Nitrendipine and [^{3}H]BAY K 8644 binding. For preparation of brain membranes, male CD-1 mice (Charles River, St. Constant, Quebec) were killed by decapitation and the brain rapidly removed and placed into ice-cold 5 mM tris-HCl buffer, pH 7.4 at 22°C. The cerebellum and brain stem were removed by blunt dissection and the remaining brain homogenized in 80 vol. of buffer using a 10-sec burst from a polytron (Brinkman, speed setting, 6). The homogenate was centrifuged at 24,000 × g for 15 min, the supernatant discarded and the pellet resuspended in 80 vol. of buffer. Unless otherwise specified, this membrane preparation was used for radioligand binding. For well washed membranes, the resuspended membranes were centrifuged at 24,000 × g for 15 min and resuspended by polytron (5-sec burst) four more times prior to resuspension in 80 vol. of buffer. For studies involving isolated rat brain regions, male rats (Sprague-Dawley, 150–175 g, Charles River, St. Constant, Quebec) were killed by decapitation. The brain was rapidly removed and placed into ice-cold buffer. The spinal cord was removed by pressure injection of air into the lower thoracic spinal cord column and placed into ice-cold buffer. The cerebral cortex, striatum, hippocampus, cerebellum and brain stem were isolated by blunt dissection. All rat CNS tissues were disrupted and membrane fractions prepared as described for mouse brain.

For preparation of atrial membranes, rats were killed by decapitation and the heart rapidly removed and placed into ice-cold buffer. Atrial muscle was dissected free from ventricular muscle and minced into small pieces. The tissue pieces were homogenized in 50 vol. of buffer using two 10-sec bursts from a polytron. The homogenate was centrifuged at $1,000 \times g$ for 10 min. The pellet was discarded and the supernatant recentrifuged at $25,000 \times g$ for 30 min. The membrane pellet was resuspended in 30 vol. of buffer.

[^{3}H]Nitrendipine and [^{3}H]BAY K 8644 binding were performed in a total assay volume of 2.0 ml consisting of 0.5 ml of protein (0.2–0.3 mg protein for cardiac membranes; 0.4–0.6 mg protein for brain membranes), 0.1 ml radioligand and 1.4 ml of buffer. Nonspecific binding was determined in the presence of 1 μM nifedipine. Radioligand binding was initiated by the addition of membrane protein. The assay tubes were incubated at 25°C under subdued lighting for 1 hr. Binding was terminated by rapid filtration through Whatman GF/B filters, followed by 2×5 ml washes with ice-cold buffer. The filters were placed in 8 ml of a scintillation cocktail (Beckman Ready Solv-MP) and the radioactivity determined in a Beckman LS-5000 scintillation counter (counting efficiency 0.45; Beckman Instruments, Fullerton, CA). Specific binding represents the difference between total and nonspecific binding. In the case where [^{3}H]nitrendipine binding was conducted in mouse brain neurons, the total volume of the binding assay was 1.0 ml and consisted of 0.85 ml of HEPES/physiologic salt containing buffer in the presence of KCl for either resting (5.3 mM) or stimulating (53.0 mM) conditions [see (19)], 0.05 ml of mouse brain neurons [130–170 μg protein; see (19)] and 0.1 ml of radioligand. The assay tubes were incubated for 30 min at 37°. The binding assay was terminated as described above. Protein was determined by the method of Miller (15).

RESULTS

$^{45}Ca^{2+}$-Uptake

Net K^+-stimulated $^{45}Ca^{2+}$-uptake into mouse brain neurons had a $t_{1/2}$ of 6 ± 1 sec and reached maximal uptake (5.5 ± 0.6 nmoles/mg protein) by 10–12 sec (Fig. 2). These results are consistent with those obtained by Skattebol and Triggle (19) for rat brain neurons in which a preparation containing 80–85% neuron viability was obtained. STR (10^{-6}–10^{-4} M), nifedipine (10^{-5} M) and (−)-S-BAY K 8644 (10^{-5} M) did not affect $^{45}Ca^{2+}$-uptake (Table 1). However, in combination with STR (10^{-5} M), nifedipine produced a concentration-dependent partial inhibition (maximum inhibition of 40%) of $^{45}Ca^{2+}$-uptake (Fig. 3). Nifedipine inhibited $^{45}Ca^{2+}$-uptake in the presence of STR with an IC_{50} of $1.0 \pm 0.4 \times 10^{-7}$ M. (−)-S-BAY K 8644 in the same concentration range as nifedipine produced a markedly different inhibition of $^{45}Ca^{2+}$-uptake than nifedipine. In contrast to nifedipine, a maximal inhibition of $^{45}Ca^{2+}$-uptake was not attained for (−)-S-BAY K 8644 when employed at concentrations up to 10^{-5} M. Significant inhibition of $^{45}Ca^{2+}$-uptake in the presence of STR was only observed for concentrations of (−)-S-BAY K 8644 greater than 10^{-6} M (Table 1).

Spontaneously Beating Rat Atrium

STR (10^{-6}–3.0×10^{-4} M) produced a concentration-dependent, rapidly reversible reduction in the rate of spontaneous contractions, which was neither accompanied by a change in basal tension nor developed tension (Table 2). The EC_{50} for the STR-mediated reduction of contractile rate in the atrium was $4.2 \pm 0.5 \times 10^{-6}$ M (Table 2). Similar findings were made for PCP. PCP (10^{-5} M) reduced the rate of contraction in the atrium from a control value of 182 ± 15 beats per min to 97 ± 16 beats per min (values are the mean ± S.E.M. of four tissues). (−)-S-BAY K 8644 produced a significant increase in the rate of contractions in the atrium (Table 2) and did not affect the resting tension. STR inhibited the increases in the rate of contractions in the atrium but did not affect developed tension (Table 2).

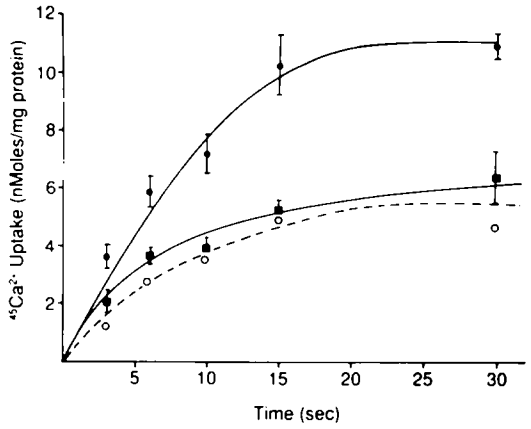

FIG. 2. Time dependence of $^{45}Ca^{2+}$-uptake into mouse brain neurons. Stimulated (●), resting (■) and net (○) $^{45}Ca^{2+}$-uptake in response to 53 mM K^+ are illustrated. The half time for net (depolarization-dependent) $^{45}Ca^{2+}$-uptake was 6 ± 1 sec with a maximal uptake of 5.5 ± 1.2 nmol/mg protein obtained between 8 and 12 sec. Each data point is the mean ± S.E.M. of three experiments.

Radioligand Binding

STR produced concentration-dependent effects on [^{3}H]nitrendipine binding to mouse brain membranes. In the concentration range 10^{-7}–10^{-4} M, STR increased specific [^{3}H]nitrendipine binding, while between 10^{-4} M and 10^{-3} M the increases in [^{3}H]nitrendipine binding were reduced (Fig. 4a). The EC_{50} for enhancement of [^{3}H]nitrendipine binding by STR was $8.2 \pm 2.6 \times 10^{-6}$ M. Extensive washing (five times) of mouse brain membranes increased the potency of STR for enhancement of [^{3}H]nitrendipine binding by ~two-fold to $4.1 \pm 1.0 \times 10^{-6}$ M (Fig. 4a). PCP also produced a concentration-dependent biphasic enhancement (EC_{50} $2.5 \pm 0.3 \times 10^{-6}$ M) of [^{3}H]nitrendipine binding to mouse brain membranes (Fig. 4b) consistent with previous observations in rat brain (2,3). However, in contrast to STR, extensive washing of membranes, while not affecting the potency, reduced the maximum enhancement of [^{3}H]nitrendipine binding obtainable with PCP; 10^{-4} M PCP producing a significant inhibition of binding.

Scatchard analysis of the effects of 10^{-5} M STR on [^{3}H]nitrendipine binding revealed a decrease (34%) in the macromolecular dissociation constant (K_d) (increased affinity), with no change in the maximum binding site capacity (B_{max}) (Fig. 5a). STR also enhanced [^{3}H]BAY K 8644 binding to mouse brain membranes. Scatchard analysis of the effects of 10^{-5} M STR on [^{3}H]BAY K 8644 binding revealed a 67% decrease in the K_d with no change in the B_{max} (Fig. 5b). STR produced similar effects on [^{3}H]nitrendipine and [^{3}H]BAY K 8644 binding to rat atrial membranes. The K_d and B_{max} values obtained for [^{3}H]nitrendipine binding to atrial membranes were 239.2 ± 28.4 pM, 508.2 ± 5.1 fmol/mg protein in the absence and 153.9 ± 19.2* pM, 509.3 ± 6.2 fmol/mg protein in the presence of 10^{-5} M STR. For [^{3}H]BAY K 8644 binding the values were 4.2 ± 0.9 nM, 329.1 ± 4.2 fmol/mg

TABLE 1

THE EFFECTS OF STR ON NET $^{45}Ca^{2+}$-UPTAKE IN THE PRESENCE AND ABSENCE OF NIFEDIPINE AND (−)-S-BAY K 8644

Drug	Net $^{45}Ca^{2+}$-Uptake (nmoles/mg protein)	(N)
None (control)	2.64 ± 0.13	6
STR 10^{-6} M	3.27 ± 0.59	6
STR 10^{-5} M	2.61 ± 0.30	6
STR 10^{-4} M	2.70 ± 0.25	6
Nifedipine 10^{-5} M	2.24 ± 0.28	12
(−)-S-BAY K 8644 10^{-5} M	2.04 ± 0.22	11
STR 10^{-5} M plus		
nifedipine 10^{-7} M	2.06 ± 0.30	4
nifedipine 5×10^{-7} M	1.60 ± 0.10*	4
nifedipine 10^{-6} M	1.59 ± 0.14*	4
nifedipine 10^{-5} M	1.58 ± 0.14*	12
STR 10^{-5} M plus		
(−)-S-BAY K 8644 5×10^{-7} M	2.56 ± 0.52	4
(−)-S-BAY K 8644 10^{-6} M	2.01 ± 0.30	4
(−)-S-BAY K 8644 10^{-5} M	0.99 ± 0.13*	12

The results are presented as the mean ± S.E.M. of (N) determinations. Drug or drug combinations were incubated with mouse brain neurons for 10 min prior to depolarization with 53 mM KCl for 5 sec in the presence of the same drug(s) [see (19)]. Net calcium uptake represents the difference between total and resting calcium uptake. Ethanol (the vehicle used to dissolve nifedipine), STR or a combination of the two did not affect resting (nonvoltage-dependent calcium channel) mediated $^{45}Ca^{2+}$-uptake at all the concentrations of STR employed.

*Significantly different $p<0.05$ from control, unpaired Student's t-test.

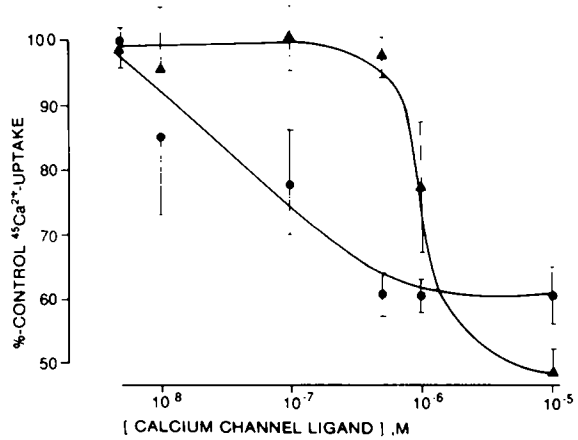

FIG. 3. The effects of nifedipine and (−)-S-BAY K 8644 on $^{45}Ca^{2+}$-uptake in the presence of STR. The inhibition of K^+-stimulated net $^{45}Ca^{2+}$-uptake by nifedipine (●) and (−)-S-BAY K 8644 (▲) in the presence of STR 10^{-5} M is shown. STR, nifedipine and (−)-S-BAY K 8644 were incubated with mouse brain neurons 10 min prior to measurement of $^{45}Ca^{2+}$-uptake in an assay medium containing a combination of STR and the appropriate DHP. The IC_{50} of nifedipine for maximal inhibition of $^{45}Ca^{2+}$-uptake is $1.0\pm0.4\times10^{-7}$ M. This result and each data point are presented as the mean ± S.E.M. of four experiments. For further details of the experimental conditions, please refer to the legend of Table 1.

TABLE 2

THE EFFECT OF STR ON THE SPONTANEOUSLY BEATING RAT ATRIA AND ITS RESPONSE TO (−)-S-BAY K 8644

Drug	Rate of Atrial Contractions (beats/min)
None (control)	218 ± 15
STR 10^{-6} M	210 ± 8
10^{-5} M	184 ± 7*
6×10^{-5} M	149 ± 14*
10^{-4} M	130 ± 14*
3×10^{-4} M	120 ± 12*
(−)-S BAY K 8644 2×10^{-6} M	301 ± 18*
(−)-S-BAY K 8644[a] 2×10^{-6} M plus STR 10^{-4} M	120 ± 12*†

The results are presented as the mean ± S.E.M. of five experiments. At the concentrations indicated, STR affected neither the inotropy nor the positive inotropic response to (−)-S-BAY K 8644 in rat atria. The IC_{50} value of STR for inhibition of the inotropy of rat atria is $4.2\pm0.5\times10^{-6}$ M.

[a]STR was present with the tissues for 5 min prior to the administration of (−)-S-BAY K 8644.

*Significantly different from control $p<0.05$; †significantly different from (−)-S-BAY K 8644 $p<0.05$, unpaired Student's t-test.

protein in the absence and 1.8 ± 0.2* nM, 314.6 ± 10.8 fmol/mg protein in the presence of 10^{-5} M STR. The results are the mean ± S.E.M. of four experiments (*significantly different, $p<0.05$, unpaired Student's t-test). The kinetic values obtained for [^{3}H]BAY K 8644 binding in brain and cardiac membranes are consistent with previously reported values (9). STR did not affect the nonspecific binding of [^{3}H]BAY K 8644 and [^{3}H]nitrendipine binding to brain and cardiac membranes.

Nonequilibrium binding kinetic analyses employing [^{3}H]nitrendipine revealed that 10^{-5} M STR produced a decrease in the microdissociation constant (k−1) and a substantial increase in the microassociation constant (k1) (Table 3). The calculated K_d values derived from the nonequilibrium binding constants are in excellent agreement with those obtained from equilibrium binding studies. The effects of STR on the competition for [^{3}H]nitrendipine and [^{3}H]BAY K 8644 binding by nifedipine and (−)-S-BAY K 8644 are shown in Table 4. STR (10^{-5} M) produced a 1.5-fold increase in the affinity of nifedipine and a 2.6-fold increase in the affinity of (−)-S-BAY K 8644 for DHP calcium antagonist and agonist binding sites without significantly affecting the Hill coefficient (nH) of binding (Table 4). The effects of Na^+, Ca^{2+} and glycine were investigated on the enhancement of [^{3}H]nitrendipine binding to mouse brain membranes by STR (10 μM). Na^+ (10^{-2}–10^{-1} M) partially inhibited the enhancement of [^{3}H]nitrendipine binding by STR (Fig. 6a). In contrast, Ca^{2+} (10^{-6}–10^{-3} M) produced a complete inhibition of the enhancement of [^{3}H]nitrendipine binding by STR with an IC_{50} of $5.0\pm2.0\times10^{-5}$ M (Fig. 6b). Glycine at concentrations up to 1.0×10^{-2} M did not affect the enhancement of [^{3}H]nitrendipine binding by STR either in minimally or extensively washed membranes.

The brain region dependence of STR's enhancement of [^{3}H]nitrendipine binding was studied in rat brain. STR (10^{-5} M) significantly enhanced [^{3}H]nitrendipine binding to the cerebral cortex, striatum, hippocampus, cerebellum, brain stem and spinal cord (Fig. 7). The magnitude of the increase expressed as a

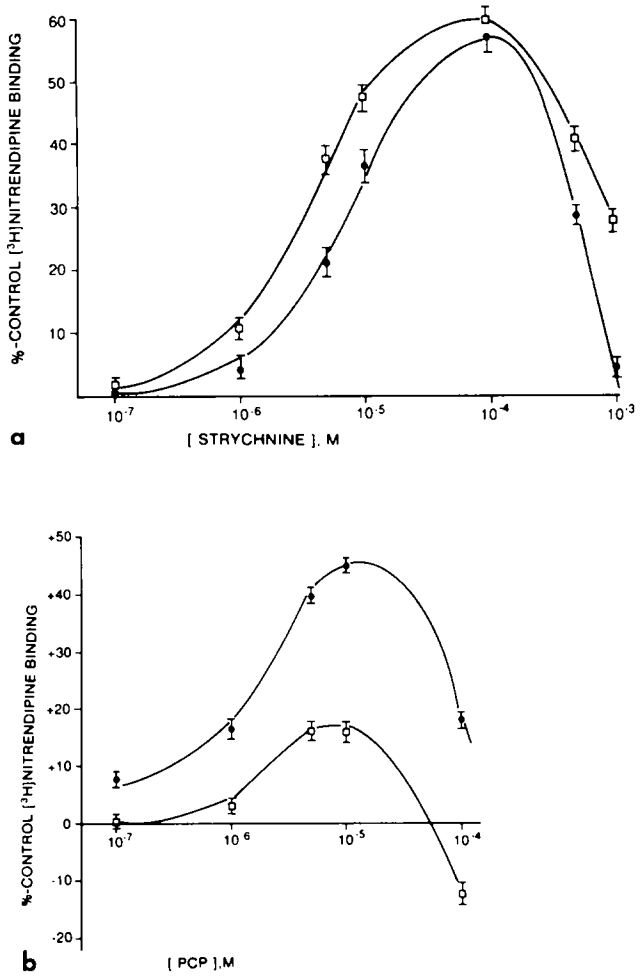

FIG. 4. The concentration dependence for enhancement of [^{3}H]nitrendipine binding by STR and PCP in minimally and extensively washed membranes. The concentration dependence for enhancement of [^{3}H]nitrendipine binding by (a) STR and (b) PCP in extensively washed (five times) (□) and minimally washed (one time) (●) mouse brain membrane is shown. In (a) the EC_{50} of STR is $8.2 \pm 2.1 \times 10^{-6}$ M in minimally washed and $4.1 \pm 1.0 \times 10^{-6}$ M* in extensively washed membranes. In (b) the EC_{50} of PCP is $2.5 \pm 0.3 \times 10^{-6}$ M in minimally washed and $1.8 \pm 0.4 \times 10^{-6}$ M in extensively washed membranes. The results are the mean ± S.E.M. of four experiments. The concentration of [^{3}H]nitrendipine in the binding assay was 120 pM. *Significantly different from the effects of STR in minimally washed membranes, $p<0.05$, unpaired Student's t-test.

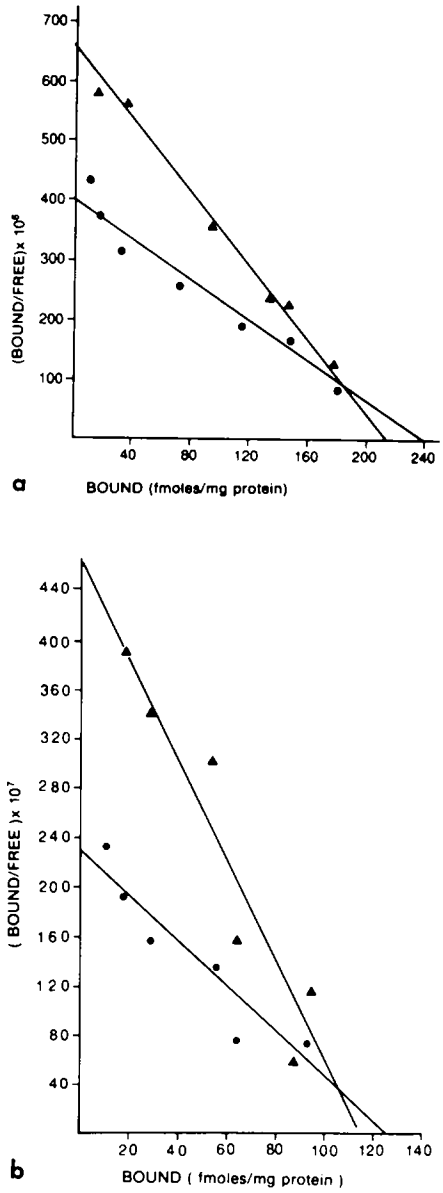

FIG. 5. The effects of STR on the equilibrium binding of [^{3}H]nitrendipine and [^{3}H]BAY K 8644 to mouse brain membranes. (a) Representative Scatchard plots of [^{3}H]nitrendipine binding to brain membranes in the presence (▲) and absence (●) of 10^{-5} M STR. The K_d and B_{max} values for [^{3}H]nitrendipine binding are (●) 420.9 ± 23.9 pM, 191 ± 6.5 fmol/mg protein and (▲) 276.2 ± 36.8 pM,* 204.1 ± 7.9 fmol/mg protein. (b) Representative Scatchard plot of [^{3}H]BAY K 8644 binding to brain membranes in the presence (▲) and absence (●) of 10^{-5} M STR. The K_d and B_{max} values for [^{3}H]BAY K 8644 binding are (●) 5.5 ± 0.6 nM, 112 ± 5.1 fmol/mg protein, and (▲) 1.8 ± 0.2 nM,* 105.5 ± 7.9 fmol/mg protein. The results are the mean ± S.E.M. of four experiments. [^{3}H]BAY K 8644 binding was performed at concentrations of radioligand ranging from 0.2–10 nM. [^{3}H]Nitrendipine binding was performed at concentrations of radioligand ranging from 0.2–1,200 pM. *Significantly different from binding in the absence of STR, $p<0.05$, unpaired Student's t-test. The correlation coefficients of the Scatchard plots were >.96.

fraction of [^{3}H]nitrendipine binding in the absense of STR was the same for each brain region (~50%).

In order to address the possibility that STR was enhancing [^{3}H]nitrendipine binding by acting at a site similar to PCP (2–4), FOURPHIT was employed. FOURPHIT produced an irreversible enhancement of [^{3}H]nitrendipine binding to mouse brain membranes (Table 5), consistent with previous observations (3). Following FOURPHIT treatment, STR (10^{-5} M) no longer produced a significant increase in [^{3}H]nitrendipine binding.

[^{3}H]Nitrendipine binding and the effects of STR were also measured in the same preparation of mouse brain neurons as that used to measure $^{45}Ca^{2+}$-uptake. The K_d and B_{max} values obtained were for control, 677.2 ± 110.1 pM, 551.5 ± 36.5 fmol/mg protein and in the presence of STR (10^{-4} M) 419.2 ± 81.0* pM, 500.3 ± 45.5 fmol/mg protein. The results are the mean ± S.E.M. of four experiments (*significantly different from control, unpaired Student's t-test).

TABLE 3

KINETIC ANALYSIS OF THE EFFECTS OF STR ON [^{3}H]NITRENDIPINE BINDING TO MOUSE BRAIN MEMBRANES

Drug	Kinetic Constant	Value
None (control)	k − 1	$3.9 \pm 0.1 \times 10^{-2}$ min^{-1}
	k1	$8.3 \pm 0.9 \times 10^{5}$ min^{-1}pM^{-1}
	K_d calc.	470.6 pM
	K_d equilibrium	420.9 pM
plus STR 10^{-6} M	k − 1	$2.6 \pm 0.1 \times 10^{-2}$ min^{-1}
	k1	$1.3 \pm 0.1 \times 10^{-4}$ min^{-1}pM^{-1}
	K_d calc.	191.0 pM
	K_d equilibrium	276.2 pM

The results are presented as the mean (± the range) of two experimental determinations. The microdissociation constant, k − 1, was determined by addition of 10^{-6} M nifedipine (final concentration) to membranes which had been previously equilibrated with 120 pM [^{3}H]nitrendipine for 60 min. Dissociation of bound [^{3}H]nitrendipine was studied for 60 min. Plots of ln[Bo/Bt] versus t (Bo bound at time o; Bt bound at time t) were linear (correlation coefficient, r = .97–.99). The microassociation constant k1 was determined by addition of [^{3}H]nitrendipine (1000 pM) to membranes at t = 0 and studied for up to 30 min. The K_d calc. refers to the K_d value obtained from the ratio k − 1/k1; K_d equilibrium refers to the K_d value obtained under equilibrium binding conditions.

DISCUSSION

Through the use of specific calcium channel ligands and functional measurements of tissue calcium translocation, STR has now been shown to affect VDCC. In brain, the ability of STR to alter the function of VDCC was observed indirectly through its interaction with DHPs to inhibit neuronal calcium uptake. DHP calcium antagonists have been shown either to be ineffective or potent (nM) partial inhibitors of calcium channels in neurons and synaptosomes (5, 17, 19, 23, 27). Thus, the potent concentration-dependent partial inhibition of $^{45}Ca^{2+}$-uptake into mouse brain neurons by nifedipine in the presence of STR, while consistent with its inhibition of the VDCC (8), may be due to STR-dependent changes in the biochemical environment of VDCC, possibly the 'L'-subtype. This is certainly suggested by the interaction of STR

TABLE 4

THE EFFECTS OF STR ON DISPLACEMENT OF [^{3}H]NITRENDIPINE AND [^{3}H]BAY K 8644 BINDING FROM MOUSE BRAIN MEMBRANES

Drug Displacement	IC_{50} (M)	nH
[^{3}H]Nitrendipine/nifedipine		
Control	$1.8 \pm 0.2 \times 10^{-9}$ M	0.95 ± 0.06
plus STR 10^{-5} M	$1.2 \pm 0.1 \times 10^{-9}$ M*	0.89 ± 0.04
[^{3}H]BAY K 8644/(−)-S-BAY K 8644		
Control	$2.9 \pm 0.3 \times 10^{-9}$ M	0.55 ± 0.05
plus STR 10^{-5} M	$1.1 \pm 0.5 \times 10^{-9}$ M*	0.62 ± 0.04

The results are presented as the mean ± S.E.M. of four experiments. The Hill coefficient (nH) was calculated from the slope of the plot $\ln[(B_{max} - B)/B]$ versus [displacer], M, utilizing a linear regression program (r > .98; BMDP Statistical Software, Los Angeles, CA). The concentrations of [^{3}H]nitrendipine and [^{3}HBAY K 8644 in the assay were 120 pM and 0.5 nM, respectively.

*Significantly different from control, $p < 0.05$, unpaired Student's t-test.

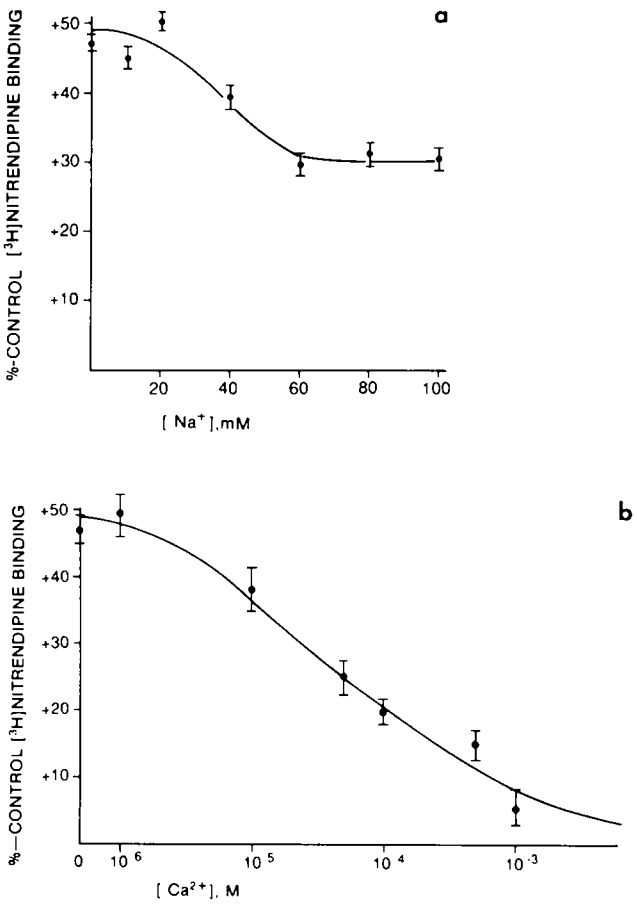

FIG. 6. The effects of cations on the enhancement of [^{3}H]nitrendipine binding to mouse brain membranes by STR. Mouse brain membranes were incubated in the absence and presence of (a) increasing concentrations of sodium and (b) calcium in the absence and presence of STR 10^{-5} M. The %-control [^{3}H]nitrendipine binding was obtained by dividing the difference in binding in the presence and absence of STR by that in the absence of STR at all concentrations of cations. The IC_{50} for the inhibition of STR's effect by calcium is $5.0 \pm 2.0 \times 10^{-5}$ M (mean ± S.E.M. of four experiments).

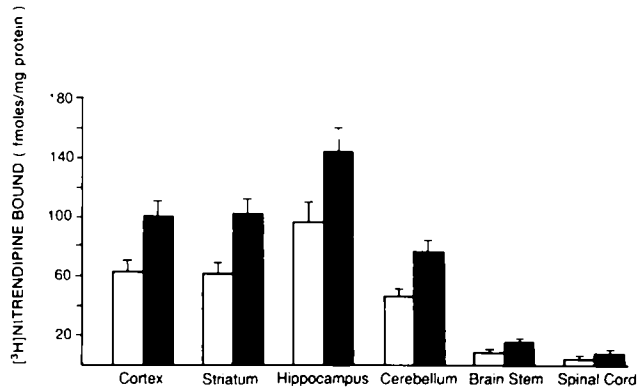

FIG. 7. Distribution of STR's enhancement of [^{3}H]nitrendipine binding to rat brain. The enhancement of [^{3}H]nitrendipine binding by STR (10^{-5} M) was investigated in different rat brain regions. The concentration of [^{3}H]nitrendipine present in the binding assay was 120 pM. The enhancement of [^{3}H]nitrendipine by STR in all brain regions was significant, $p < 0.05$, unpaired Student's t-test.

TABLE 5

THE EFFECT OF STR ON [^{3}H]NITRENDIPINE BINDING TO UNTREATED AND FOURPHIT-TREATED MOUSE BRAIN MEMBRANES

Treatment Condition	[^{3}H]Nitrendipine Binding (fmol/mg protein)
Untreated:	
Control	82.7 ± 7.3
plus STR 10^{-5} M	119.5 ± 7.9*
FOURPHIT-Treated:	
Control	124.9 ± 9.0†
plus STR 10^{-5} M	132.6 ± 12.7

The results are presented as the mean ± S.E.M. of four experiments. FOURPHIT-treated membranes were incubated with 10^{-5} M drug for 30 min followed by centrifugation at 24,000 × g. The supernatant was aspirated off and the membrane pellet resuspended in 80 vol. of 5 mM tris-HCl buffer and used for [^{3}H]nitrendipine binding. The concentration of [^{3}H]nitrendipine in the binding assay was 120 pM. Untreated membranes were incubated with an aliquot of buffer in place of FOURPHIT with all subsequent steps being identical [see (3) for further details].

Significantly different *from corresponding control in treatment group, †from control in untreated group, $p<0.01$, unpaired Student's t-test.

with specific DHP binding sites and will be discussed later.

An unexpected finding was the inhibition of $^{45}Ca^{2+}$-uptake by (−)-S-BAY K 8644 in the presence of STR (Fig. 3). The inability to attain a maximal inhibition and the markedly different inhibitory pattern from that of nifedipine suggest another, perhaps non-DHP site of interaction at VDCC for (−)-S-BAY K 8644. (−)-S-BAY K 8644 has been found to stimulate calcium uptake into synaptosomes and evoke contractile activity in smooth muscle at nanomolar concentrations while higher micromolar concentrations reverse these effects suggesting that a non-DHP site of interaction may in part mediate the actions of (−)-S-BAY K 8644 (26,27).

In cardiac muscle, STR reduced the rate of atrial contraction and blocked the positive chronotropic, but not the positive ionotropic effects of BAY K 8644 at concentrations comparable to those pharmacologically active in vascular smooth muscle (13). VDCC play a key role in determining both the rate and force of cardiac contractions (18). The 'T'-subtype of VDCC (8) controls the rate of contraction, while the 'L'-subtype controls the force of contraction (18). The selective block of atrial chronotropy by STR suggest its inhibiting 'T'-type VDCC. Evidence suggests that STR blocks both sodium and potassium channels in a number of tissues and possesses local anesthetic properties similar to procainamide (20,21). Since sodium and potassium channels are critical in controlling the activation of VDCC, the effects of STR at these channels might account for its selective action on atrial chronotropy. However, the complete block of the positive chronotropic effects of (−)-S-BAY K 8644 by STR implies that 'L'-type VDCC may possibly play an important role in controlling the rate of atrial contractions.

PCP produced comparably similar effects to STR in the atria. Like STR and at similar concentrations, PCP has been shown to block sodium and potassium channels (1,24) and modify the properties of DHP binding sites (2–4). Thus, STR and PCP share similar molecular properties at ion channels which contribute to a selective decrease in the rate, but not the force of atrial contraction. This does not apply to neuronal calcium channels, where PCP but not STR was a potent blocker of their activity (S. K. O'Neill and G. T. Bolger, unpublished observations). Thus, both common and unique molecular properties for PCP and STR exist in their interactions with VDCC.

A further evaluation of the effects of STR on DHP binding sites revealed several important characteristics. STR produced a biphasic concentration-dependent effect on [^{3}H]nitrendipine binding to mouse brain membranes. The concentrations of STR that affected binding are similar to those pharmacologically active in this and prior studies at calcium, sodium and potassium channels. The biphasic effects of STR on binding suggests that multiple sites of interaction are present on neuronal membranes. Similar observations were made for PCP (2,3), where conclusive evidence exists demonstrating multiple neuronal sites to regulate DHP binding and potentially calcium channels. The possibility that STR acts at many sites on or near VDCC may explain its ability to both stimulate and inhibit the activity of smooth muscle VDCC (13). The effects of STR on DHP binding sites appear to be dependent on the presence of endogenous tissue factors, since extensive washing of mouse brain membranes increased the potency of STR to enhance [^{3}H]nitrendipine binding. Extensive washing of membranes reduced the efficacy of PCP to enhance [^{3}H]nitrendipine binding consistent with previous observations (2). While the nature of the endogenous brain factor(s) remains to be determined, it is clear that the enhancement of DHP binding by PCP and STR is differentially sensitive to the presence of endogenous factors.

Scatchard analysis revealed that STR increased the affinity of [^{3}H]nitrendipine binding, but did not affect the maximum binding site capacity. More detailed kinetic analyses revealed that STR increased the affinity of the DHP binding site by producing changes in both the microassociation and microdissociation constants. Such changes are consistent with those of PCP (2) and suggest that STR acts in an allosteric manner to increase the affinity of the DHP binding site.

Both DHP calcium antagonist and agonist binding were enhanced by STR. However, direct binding and displacement studies demonstrated that STR produced a greater increase in the affinity of agonist binding than antagonist binding. The DHP calcium channel antagonist- and agonist-dependent effects of STR support the proposal that different sites are present for the binding of DHP calcium antagonist and agonist (11,26).

Both calcium and sodium ions inhibited the effects of STR on [^{3}H]nitrendipine binding to mouse brain membranes, suggesting a dependence on both monovalent and divalent cations. The inhibition of STR's enhancement of [^{3}H]nitrendipine binding by calcium occurs over the same concentration range as that for its inhibition of the enhancement of [^{3}H]nitrendipine binding by PCP (2). In contrast, sodium did not inhibit the enhancement of [^{3}H]nitrendipine binding by PCP (2). Thus, the effects of cations point to differences between STR and PCP in their interaction with DHP binding sites. Particularly interesting was the ability of STR to enhance the binding of [^{3}H]nitrendipine to mouse brain neurons, even though the extraneuronal calcium concentration was 1.8 mM. The discrepency between the effects of calcium on STR's enhancement of [^{3}H]nitrendipine binding to mouse brain membranes and mouse brain neurons may arise from a preferential interaction of STR with the DHP binding site from the cytosolic side of the membrane.

Although it is clear that an allosteric site(s) mediates the effects of STR on DHP binding in the CNS, the nature of the site(s) remains to be determined. The possibility that the well characterized CNS binding sites for STR are involved can, however, be ruled out. Firstly, the concentrations of STR that modulate DHP binding (μM) suggest that high-affinity convulsant STR binding sites in the CNS are not involved. Furthermore, glycine did not affect the potentiation of DHP binding by STR. Lastly, the distribution of STR's effect on DHP binding sites in rat brain was not selective and markedly different from the distribution of high-affinity glycine-associated STR binding sites (28). A detailed study of STR binding in brain has revealed multiple binding sites,

some of which are glycine independent (14). Such sites may represent those allosterically coupled to DHP binding sites.

The effects of STR on DHP binding sites were reminiscent of those for PCP, some similarities having already been discussed. The hypothesis that STR acted with the same site as PCP was tested using FOURPHIT, an acylator of the PCP site allosterically linked to DHP binding sites. FOURPHIT's block of STR's enhancement of DHP binding provided tacit proof that they are acting at the same site. Nonetheless, in addition to the cation dependence already mentioned, other differences in the interaction of PCP and STR on neuronal and peripheral DHP binding sites exist. PCP, but not STR, displays a brain region dependence (2), enhances [^{3}H]nitrendipine binding in brain, but not in cardiac membranes (2) and has its activity increased in the presence of endogenous brain factors [this study; (2)]. Such differences might be expected given the vastly different structures of PCP and STR, but suggest that the allosteric site with which they interact is subject to some degree of structural selectivity. While it is tempting to compare the effects of STR on DHP binding sites with its pharmacologic actions, all such comparisons must be considered highly speculatory. Worthy comparisons are the effects of STR on $^{45}Ca^{2+}$-uptake and atrium contractility and their modulation by DHPs. The inhibition of $^{45}Ca^{2+}$-uptake by nifedipine in the presence of STR may be related to the STR-dependent increase in the affinity of DHP binding sites, since it was demonstrated that STR could increase the affinity of [^{3}H]nitrendipine binding to a preparation of mouse brain neurons. In cardiac muscle, the STR-dependent changes in DHP binding sites may lead to their uncoupling from VDCC and an inability of DHPs and endogenous DHP-like compounds (22) to elicit their effects.

This study suggests that STR can interact with VDCC, in particular the 'L'-subtype, likely through binding to allosteric sites that are linked to specific DHP calcium channel regulatory sites. A further characterization of the allosteric sites associated with DHPs is warranted.

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