Tachykinins and Calcitonin Gene-Related Peptide Enhance Release of Endogenous Glutamate and Aspartate from the Rat Spinal Dorsal Horn Slice

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The effects of dorsal root stimulation and of substance P (SP), neurokinin A (NKA), and calcitonin gene-related peptide (CGRP) on the basal release of 9 endogenous amino acids, including glutamate (Glu) and aspartate (Asp), have been investigated using the rat spinal cord slice–dorsal root ganglion preparation and high-performance liquid chromatography with fluorimetric detection. High-intensity repetitive electrical stimulation of a lumbar dorsal root produced a Ca^{2+} -dependent increase in the basal release of Asp, Glu, glycine (Gly), serine (Ser), and threonine (Thr). Low concentrations of SP (2×10^{-7} M) caused a selective increase in the rate of basal release of Glu, whereas higher concentrations ($1\text{--}5 \times 10^{-6}$ M) produced, in addition, an increase in the basal release of Asp. The SP-induced increase of Glu persisted in the absence of external Ca^{2+} , but the effect was blocked by (D-Arg¹, D-Pro², D-Trp^{7,9}, Leu¹¹)-SP, an SP analog claimed to be an antagonist of the synthetic SP. NKA ($5 \times 10^{-7}\text{--}10^{-6}$ M), a related tachykinin coexpressed with SP in primary sensory neurons, enhanced the basal release of Gly. CGRP (10^{-7} M) caused a significant, largely Ca^{2+} -independent increase in the basal release of Glu and Asp and a decrease in asparagine. SP and CGRP potentiated the electrically evoked release of Glu and Asp. Neonatal capsaicin treatment did not significantly alter the basal efflux of 9 endogenous amino acids from the spinal slices, but it prevented the dorsal root stimulation-evoked release of Asp, Glu, Gly, and Thr and the SP-induced increase in the basal release of Glu. However, the effect of CGRP was not significantly modified by the capsaicin treatment. These results indicate that tachykinins (SP and NKA) and CGRP are capable of modulating the basal and electrically evoked release of endogenous Glu and Asp, and these actions may provide an important mechanism by which the peptides contribute to the regulation of the primary afferent synaptic transmission. The enhancement of the basal and the dorsal root stimulation-evoked release of Glu and Asp by tachykinins and CGRP may have important physiological implications for strengthening the synaptic connections in the spinal dorsal horn.

Immunohistochemical techniques have demonstrated that neurons contain multiple chemical substances that may act as neurotransmitters or neuromodulators. However, the physiological significance of this phenomenon, especially for information transfer in the nervous system, is not well understood.

Synaptic transmitters released during activation of primary sensory neurons in the dorsal horn of the rat spinal cord may elicit both fast and slow excitatory responses in a single neuron (Murae and Randic, 1984; Urban and Randic, 1984). Dicarboxylic amino acids, glutamate (Glu) and aspartate (Asp), appear to be the major candidates for the fast excitatory neurotransmitters in the mammalian CNS (Watkins and Evans, 1981; Mayer and Westbrook, 1987), including the spinal dorsal horn (Galindo et al., 1967; Salt and Hill, 1983; Kangrga et al., 1988; Gerber and Randic, 1989a). Tachykinins, substance P (SP) and neurokinin A (NKA), and calcitonin gene-related peptide (CGRP) appear to be functionally involved in the slow primary afferent synaptic transmission (Urban and Randic, 1984; Randic et al., 1986; Ryu et al., 1988a; Gerber and Randic, 1989b).

Immunocytochemical studies have shown that about 70% of both large and small DRG neurons are labeled for Glu (Wanaka et al., 1987), and Glu immunoreactivity has been detected within myelinated and unmyelinated primary afferent terminals in the superficial dorsal horn (Weinberg et al., 1987; Westlund et al., 1989a). Ca^{2+} -dependent release of Glu from electrically activated primary afferent fibers has been demonstrated (Roberts and Mitchell, 1972; Roberts, 1974; Takeuchi et al., 1983; Kawagoe et al., 1986). L-Glu binding sites are found in high densities in the superficial laminae of the rat spinal dorsal horn (Greenamyre et al., 1984; Monaghan and Cotman, 1985). Glu was found to excite and depolarize almost all spinal dorsal horn neurons *in vivo* (Curtis et al., 1960; Watkins and Evans, 1981) and a proportion of dorsal horn neurons *in vitro* (Zieglgänsberger and Puil, 1973; Schneider and Perl, 1985).

SP and NKA are also present in a proportion of small DRG neurons and in numerous terminals in the superficial laminae of the spinal dorsal horn (Höckfelt et al., 1975; Barber et al., 1979; Kanazawa et al., 1984; Dalsgaard et al., 1985). On electrical or chemical stimulation, Ca^{2+} -dependent release of SP and NKA from activated primary afferent fibers has been demonstrated both *in vitro* (Otsuka and Konishi, 1976; Gamse et al., 1979; Hua et al., 1986) and *in vivo* (Yaksh et al., 1980; Brodin et al., 1987). SP binding sites have been demonstrated autoradiographically in the spinal dorsal horn (Mantyh et al., 1984a), and a correlation has been observed between the distribution of ³H-SP binding sites and the ability of SP to stimulate phosphatidylinositol turnover (Mantyh et al., 1984b). SP depolarizes

Received Oct. 23, 1989; revised Jan. 5, 1990; accepted Jan. 8, 1990.

We wish to thank Drs. J. S. A. Larew, V. Miletic, and G. Pavlakovic for their help and critical comments. Support was provided by grants from USPHS (NS 26352), NSF (BNS 8418042), and the USA Department of Agriculture.

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dorsal horn neurons (Henry et al., 1975; Randic and Miletic, 1977).

The presence of CGRP-like immunoreactivity (CGRP-LI) in rat DRG neurons and the spinal dorsal horn has been demonstrated (Gibson et al., 1984). Both CGRP binding sites (Henke et al., 1985) and CGRP-LI (Gibson et al., 1984) exist in high concentrations in the superficial layers of the spinal dorsal horn. Release of CGRP from rat primary sensory neurons in response to capsaicin (Franco-Cereceda et al., 1987; Diez Guerra et al., 1988) and electrical stimulation (Saria et al., 1986) has been shown.

There is evidence that SP, NKA, and CGRP may modulate primary afferent neurotransmission by acting both at presynaptic (Kawagoe et al., 1986; Ryu et al., 1988a, b; Kangrga et al., 1989a, b) and postsynaptic sites (Murae and Randic, 1984; Murae et al., 1989a, b). Although the coexistence of SP and Glu in some small primary afferent neurons (Battaglia et al., 1987) and their terminals in the superficial dorsal horn (DeBiasi and Rustioni, 1988), and the coexistence of SP, NKA, and CGRP in a proportion of capsaicin-sensitive DRG neurons (Nagy et al., 1981; Gibson et al., 1984; Franco-Cereceda et al., 1987; Diez Guerra et al., 1988) has been reported, our understanding of physiological implications of this phenomenon is still unclear. An important, but as yet not systematically investigated site at which coexistent peptides could modulate excitatory amino acid function, and in this way contribute to primary afferent synaptic transmission, is found presynaptically in the control of basal and depolarization-evoked release of Glu and Asp. In an attempt to determine whether SP, NKA, and CGRP modulate the release pattern of excitatory amino acids, we have investigated the efflux of 9 endogenous amino acids, including Glu and Asp, from the superfused spinal cord slices of the rat in response to electrical stimulation of dorsal roots, administration of neuropeptides (SP, NKA, and CGRP), and chronic treatment of rats with capsaicin. Preliminary reports of some aspects of this work have been published (Kangrga et al., 1989a, b).

Materials and Methods

Horizontal slices were obtained from Sprague-Dawley rats of both sexes (23–45 d old) by using a technique that has been described in detail elsewhere (Murae and Randic, 1983; Gerber et al., 1989). Briefly, after the animal was anesthetized with ether, a segment of the lumbosacral (L5–S1) spinal cord was dissected out and sectioned along the longitudinal axis with a Vibratome to yield one 300–400- μ m-thick horizontal slice with dorsal rootlets and dorsal root ganglia attached. In some experiments, a part of the sciatic nerve was left in contact with a dorsal root ganglion. The slice was incubated for 1 hr in oxygenated (95% O₂ + 5% CO₂) control solution (in mM): NaCl, 124; KCl, 5; KH₂PO₄, 1.2; CaCl₂, 2.4; MgSO₄, 1.3; NaHCO₃, 26; glucose, 10; pH 7.4 at 30 $\pm$ 1°C. The use of a high-K⁺ solution during cutting and incubation of the slices seemed to improve their viability as assessed electrophysiologically in the same preparation. After the incubation, a slice was placed in one compartment of the 2-compartment chamber and perfused with oxygenated modified Krebs solution (containing 1.9 mM KCl, all other salts were unchanged) at 0.5 ml/min. The dorsal roots and dorsal root ganglia were placed into the second compartment and immersed under the mineral oil. Lubriscal (Thomas Scientific) was used to ensure a leakproof and electrical isolation between the 2 compartments. A lumbar dorsal rootlet was placed on the 2 pairs of bipolar platinum electrodes: the distal pair was used for electrical stimulation of primary afferent fibers and the proximal pair for recording the compound action potentials. The stimulation parameters were selected to activate both low-threshold, fast-conducting myelinated fibers (A β) and the high-threshold, slower-conducting myelinated (A δ) and unmyelinated (C) fibers (25–30 V, 0.02–1.0 msec at 3–10 Hz). The compound action potentials were mon-

itored throughout the periods of stimulation and stored on diskettes of a digital oscilloscope (Nicolet, model 4092). Samples of perfusate (0.5 ml) were collected at regular 3–10 min intervals before, during, and after stimulation of the dorsal roots or application of tachykinins and CGRP. Samples were kept frozen at –80°C until derivatization and chemical analysis. Quantification of 9 endogenous amino acids contained in the spinal perfusate was achieved by reversed-phase high-performance liquid chromatography (HPLC) with fluorimetric detection following precolumn derivatization with *O*-phthalaldehyde (OPA) 2-mercaptoethanol reagent (Lindroth and Mopper, 1979). OPA 2-mercaptoethanol derivatives were produced by taking 25 μ l OPA reagent solution and mixing with 25 μ l amino acid mixture (standards or sample). After 1 min, 150 μ l of the mixture was injected onto the chromatographic column for analysis. Hydroxylysine (30 μ M) was added to each sample as an internal standard and/or reference injections of amino acid standards were injected periodically. Chromatography was performed on a 15 cm, Adsorbosphere-OPA-HR column (Alltech Associates) using a pH 5.9 sodium acetate–tetrahydrofuran/methanol gradient. Fluorescence was detected with a Kratos FS 950 fluorimeter. The amino acids measured came off the column in the following order: Asp, Glu, asparagine (Asn), serine (Ser), glutamine (Gln), glycine (Gly), threonine (Thr), alanine (Ala), and GABA followed by hydroxylysine. Results reported are the averages of duplicate runs with each run lasting 31 min. SP (10^{-7} – 5×10^{-6} M, Cambridge Research Biochemicals, CRB), NKA (5×10^{-7} – 10^{-6} M, CRB), and rat CGRP (10^{-8} – 10^{-6} M, CRB) were applied into the slice perfusate for 5 min. When slices were perfused with 44 mM K⁺, the NaCl concentration of the Krebs solution was adjusted to maintain osmolarity. The calcium dependence of the dorsal root stimulation-evoked and peptide-evoked amino acid efflux was investigated by the removal of calcium ions from the Krebs solution. Statistical significance has been assessed relative to control conditions by use of either a paired or unpaired Student's *t* test, as appropriate. Levels of significance are indicated as follows: **p* < 0.01; ***p* < 0.005.

Thirteen rats of both sexes taken from 2 different litters were injected subcutaneously with 50 mg/kg capsaicin (Sigma) in vehicle [10% ethanol, 10% Tween (vol/vol) in 0.9% (wt/vol) saline] 48 hr after birth. Eight control littermates received equal volumes of vehicle alone. After a survival time of 23–45 d, the animals were subjected to the experimental procedure described above.

For SP and CGRP immunohistochemistry, lumbar spinal cords from vehicle-injected and capsaicin-treated rats were fixed with Zamboni's fixative. Serial, 50- μ m-thick transverse sections were cut through the spinal cord with a Vibratome. These sections were then processed for SP- or CGRP-like immunoreactivity with the Sternberger's peroxidase–antiperoxidase (PAP) method and commercially available SP (Incstar Corp.) or CGRP (Peninsula Labs.) antisera at a dilution of 1:7000 and 1:3000, respectively. Details of the PAP procedure have been published (Coffield et al., 1986). Following the PAP incubation, the sections were incubated for 7–10 min in 0.05% 3,3'-diaminobenzidine hydrochloride (DAB, Sigma) in 0.1 M PBS and 0.01 M hydrogen peroxide (H₂O₂) to obtain the specific immunoreactive label distinguished by a reddish-brown chromogen. The DAB-reacted sections were mounted on gelatin-coated slides, dehydrated in ethanol, cleared in xylene, and coverslipped with Permount for light microscopic analysis. As a control for ligand specificity, spinal cord sections were processed without the addition of primary antisera. In this case, no immunoreactivity was seen.

Results

Basal release of endogenous amino acids from spinal dorsal horn slices

The efflux of 9 endogenous amino acids—Asp, Glu, Asn, Gln, Gly, GABA, Ser, Thr, and Ala—from the spinal cord slices into the Krebs perfusion-medium occurred, and the mean basal amino acid concentrations in the spinal cord slice perfusate are presented in Table 1. The 9 amino acids studied are the most prominent ones to be detected in the spinal slice perfusate. The amount of Gln released was the highest, at least 5–6 times that of the next 3 relatively abundant amino acids, Ala, Gly, and Ser. Glu, GABA, and Thr were present in moderate amounts, whereas lower levels of Asp and Asn were found (Table 1).

No significant differences in the basal release of 9 endogenous

Table 1. Amino acid concentrations in the perfusate of the horizontal spinal cord slices ($\mu\text{M}/5$ min collection period)

Amino acid	Control ($n = 12$)	Zero Ca^{2+} ($n = 3$)	Capsaicin ($n = 13$)
Aspartate	0.25 ± 0.04	0.21 ± 0.06	0.16 ± 0.03
Glutamate	0.51 ± 0.09	0.59 ± 0.15	0.66 ± 0.09
Asparagine	0.10 ± 0.02	0.21 ± 0.04	0.16 ± 0.03
Glutamine	7.20 ± 1.40	5.30 ± 2.81	6.94 ± 0.99
Glycine	1.21 ± 0.20	1.30 ± 0.17	1.20 ± 0.28
GABA	0.68 ± 0.15	0.61 ± 0.30	0.92 ± 0.15
Serine	1.10 ± 0.15	1.09 ± 0.21	1.39 ± 0.10
Threonine	0.60 ± 0.07	0.56 ± 0.15	0.89 ± 0.23
Alanine	1.29 ± 0.27	1.66 ± 0.60	1.82 ± 0.32

Results are presented as means $\pm$ SEM of the basal concentrations for 25 experiments conducted in duplicate. Number of observations shown in parentheses.

amino acids were detected when the spinal slices were perfused with either nominally zero Ca^{2+} -containing medium ($n = 3$; Table 1) or in those obtained from rats neonatally treated with capsaicin ($n = 13$; Table 1).

Effects of dorsal root stimulation

Electrical stimulation of a lumbar dorsal root produced a significant increase in the rate of basal efflux of several endogenous amino acids (Fig. 1, Table 2). Thus, in 6 different slices, high-intensity repetitive stimulation (25 V pulses of 20–100 μsec duration applied at 3–5 Hz for 5 min) of a lumbar dorsal root produced a significant increase in the basal release of Asp (to $160.7 \pm 9.6\%$), Gln (to $143.7 \pm 9.5\%$), Gly (to $130.0 \pm 9.6\%$), Ser (to $145.3 \pm 14.4\%$), and Thr (to $141.8 \pm 8.5\%$), whereas the levels of Asn, Glu, GABA, and Ala were elevated only to smaller degrees (Fig. 1A). The stimulation-evoked increase in the basal release of amino acids was rarely maintained for more than one collection period of 5 min. The higher increase in the rate of stimulation-evoked release of Asp than of Glu, and the increase in the efflux of endogenous GABA, Gly, Ser, Thr, Ala, and Asn in the spinal slice perfusate are reported here for the first time.

In order to evaluate whether the increased release of endogenous amino acids following activation of primary afferent fibers was likely to be of neuronal origin, experiments were carried out with altered levels of Ca^{2+} ions in the perfusing medium. The dependence of the dorsal root electrically evoked increase in the basal release of endogenous amino acids upon the presence of Ca^{2+} ions in the external medium was investigated by perfusing the slices ($n = 3$) with nominally zero Ca^{2+} medium. The results obtained in one of those experiments are illustrated in Figure 1B. When Ca^{2+} ions were omitted from the perfusing medium, the synaptic transmission was blocked and the stimulation-evoked increase in the basal release of Glu and Asp was absent (Fig. 1B, Table 2).

SP and NKA modulate the basal and electrically evoked release of Glu and Asp

Perfusion of spinal cord slices with lower concentrations of SP (2×10^{-7} M for 5 min) caused a selective and significant increase (to $254.3 \pm 62.0\%$; $n = 8$) in the rate of basal release of Glu (Fig. 2A). With higher concentrations of SP (10^{-6} – 5×10^{-6} M; $n = 3$), in addition to Glu, a significant and dose-dependent increase in the basal release of Asp was observed (Fig. 2B). Thus,

Figure 1. Effects of depolarizing stimuli and Ca^{2+} on the rate of basal efflux of 9 endogenous amino acids. Histogram of the release of 9 endogenous amino acids in response to electrical stimulation of a lumbar dorsal rootlet (25 V, 0.02–0.1 msec, 5 Hz, 5 min) relative to their basal efflux determined from first 2 collection periods prior to the first period of stimulation. The results are expressed as the mean percentage of the basal efflux $\pm$ SEM for 6 experiments conducted in duplicate. *A*, Electrical stimulation of a lumbar dorsal rootlet produced a significant increase in the concentrations of Asp ($160.7 \pm 9.6\%$), Glu ($143.7 \pm 9.5\%$), Gly ($130.0 \pm 9.6\%$), Ser ($145.3 \pm 14.4\%$), and Thr ($141.8 \pm 8.5\%$), whereas the levels of Asn, Glu, GABA, and Ala were elevated to a smaller degree. *B*, The stimulation-evoked increase in the basal efflux of Glu, Asp, Asn, Gly, and Ser (solid columns, $n = 2$) was reduced or blocked in a zero- Ca^{2+} solution (hatched columns, $n = 3$). Statistically significant results in this and other figures are indicated: $**p < 0.005$, 23- to 33-d-old rats.

for instance in a single experiment, the increase in the basal efflux of Asp elicited by 10^{-6} and 5×10^{-6} M SP amounted to 150 and 270% of their control levels, respectively. With higher concentrations of SP, the increased efflux of Glu and Asp was frequently maintained for 2–3 consecutive 5 min collection periods. The averaged values for the SP-caused increase in the release of Glu and Asp from spinal slices are summarized in Table 2. The levels of the 7 other endogenous amino acids determined in the spinal slice perfusate were not modified in a consistent manner by SP (Fig. 2B).

The dependence of the SP-evoked increase in the release of Glu and Asp upon external Ca^{2+} ions was investigated by omission of this ion from the perfusing medium. When the slices ($n = 3$) were perfused with a nominally Ca^{2+} -free medium, SP (5×10^{-7} M) still elicited a significant increase in the concentration

Figure 2. SP and NKA enhance the basal efflux of endogenous Glu and Asp into the spinal perfusate. *A*, Perfusion of spinal cord slices with SP ($2-5 \times 10^{-7}$ M for 5 min) produced a significant increase in the concentration of Glu ($254.3 \pm 62.0\%$; $n = 8$; $p < 0.005$) in the perfusate, whereas the levels of other 8 amino acids were not significantly altered. *B*, Histogram of the efflux of 9 endogenous amino acids, obtained in a single experiment in response to addition of 3 different concentrations of SP to the perfusing medium: solid columns, 5×10^{-7} M; hatched columns, 10^{-6} M; dotted columns, 5×10^{-6} M. Bath application of SP in a concentration of 5×10^{-7} M markedly increased (533.6%) the basal efflux of Glu only. Higher concentrations of SP (10^{-6} and 5×10^{-6} M), in addition to Glu, caused a significant and dose-dependent increase in the basal efflux of Asp. A tendency for a decrease in the efflux of Asn and Glu was also observed. *C*, Addition of NKA (10^{-6} M, 5 min) to the perfusing medium produced a marked increase in the basal efflux of Asp (282.7%), Gly (222.2%), and Glu (170.3%), whereas the efflux of remaining 6 amino acids was not significantly changed. *A*, 24- to 33-d-old rats; *B*, 24-d-old rat; *C*, 28-d-old rat.

Figure 3. Enhancement of the basal efflux of Asp and Glu by SP is not Ca^{2+} -dependent but is blocked by a claimed SP antagonist. *A*, Perfusion of the slices with nominally zero Ca^{2+} solution containing SP (5×10^{-7} M, 5 min) produced a significant increase in the concentration of Glu ($198.2 \pm 29.7\%$; $n = 3$) and Asp ($141.9 \pm 7.0\%$; $n = 3$) in the spinal perfusate. The concentrations of remaining 7 amino acids were not significantly different from the control. *B*, Perfusion of a spinal cord slice with SP (5×10^{-7} M, 5 min) in the presence of SP antagonist, (D-Arg¹, D-Pro², D-Trp^{7,9}, Leu¹¹)-SP (2×10^{-6} M, 10 min), failed to elicit an increase in the basal efflux of either Asp or Glu in the spinal perfusate (hatched columns). The data obtained during the perfusion with SP (5×10^{-7} M) alone are presented with the solid columns (the same slice). *A*, 26- to 33-d-old rats; *B*, 25-d-old rat.

of Glu (to $198.2 \pm 29.7\%$) in all 3 slices examined, whereas the Asp increase (to $141.9 \pm 7.0\%$) was present in 2 out of 3 slices (Fig. 3*A*, Table 2). The persistence of the SP effect did not appear to be a consequence of an inadequate removal of extracellular Ca^{2+} since the dorsal root-stimulation-evoked release of Glu and Asp was virtually abolished by the removal of external Ca^{2+} ($n = 3$).

The effect of SP (5×10^{-7} M) was effectively blocked by an SP analog (D-Arg¹, D-Pro², D-Trp^{7,9}, Leu¹¹)-SP (2×10^{-6} M), a claimed antagonist of synthetic SP (Fig. 3*B*). This finding suggests that the effect of SP on the basal efflux of Glu is a true tachykinin receptor-mediated response.

The possibility that NKA, a SP-related tachykinin that is coexpressed with SP in primary sensory neurons (Dalsgaard et al., 1985), may regulate primary afferent transmission by mod-

Figure 4. SP potentiates the dorsal root stimulation-evoked efflux of endogenous Glu and Asp. Interaction between the dorsal root stimulation and the SP-evoked release of Glu and Asp was studied by using 2 different protocols (*A* and *B*). *Left panels*, Three periods of electrical stimulation (25 V, 0.02 msec, 5 Hz for 5 min; onset of stimulation marked by arrowheads) were applied. During the first and the third periods, the slice was perfused with a control Krebs solution, while during the second period, SP (5×10^{-7} M) was added to the perfusate. SP increased the electrically evoked efflux of Glu from 141 to 236% (*A*) and that of Asp from 185 to 228% (*B*) of the basal value. Characteristically, the peptide prolonged the duration of the electrically evoked efflux of the amino acids from 5 to 15 min. 29-d-old rat. *Right panels*, Electrical stimulation of the dorsal rootlet (30 V, 1 msec, 0.5 Hz for 3 min) produced about a 4-fold increase in the basal release of Glu (*A*) and about a 2.5-fold increase in Asp (*B*). Addition of SP (5×10^{-7} M for 3 min) to the perfusing medium produced a similar increase in Glu (359%) and somewhat higher release of Asp (485%). However, the electrical stimulation of the primary afferents in the presence of SP resulted in a higher increase in the basal efflux of Glu (to 1091% of the basal release) and Asp (to 743%) during the first collection period if compared to the effects of either treatment alone. The SP-enhanced efflux of Glu and Asp lasted about 12 min. 27-d-old rat.

ulating the basal release of Glu and Asp was investigated in 5 spinal slices. As shown in a single experiment illustrated in Figure 2C, NKA (10^{-6} M) produced a marked increase in the rate of basal efflux of Asp (to 282.7%), Gly (to 222.2%), and Glu (to 170.3%), whereas the efflux of the remaining 6 amino acids was not significantly altered. The summary of the effects of NKA (5×10^{-7} to 10^{-6} M) on the basal efflux of Glu and Asp is presented in Table 2.

Moreover, we sought to determine whether, besides modulating the basal release, SP also modulates the dorsal root stim-

ulation-evoked release of Glu and Asp from the spinal slices. Thus, in 4 slices, the release during a stimulation period was compared with that measured during exposure of a spinal slice to SP (10^{-7} – 10^{-6} M) and electrical stimulation of a lumbar dorsal root. We found that in the presence of SP, the electrically stimulated release of Glu (Fig. 4*A*) and Asp (Fig. 4*B*), was potentiated. Characteristically, unlike the response to electrical stimulation that was usually limited to the first collection period, the SP-evoked response was prolonged, lasting 10–15 min after the first exposure to the peptide (Fig. 4).

Figure 5. *A*, CGRP enhanced the basal efflux of Asp and Glu into the perfusate of spinal slices. Perfusion of spinal cord slices with CGRP (10^{-7} M) produced a significant increase in the concentration of Glu (to $170.5 \pm 30.6\%$) and Asp (to $159.5 \pm 20.1\%$) and a decrease in Asn (to $56.2 \pm 29.2\%$), whereas the levels of the remaining 6 amino acids were not markedly changed. The results are expressed as mean percentages of the basal efflux $\pm$ SEM for 4 experiments. *B*, In 3 experiments the CGRP-evoked increase in the basal efflux of Asp and Glu (solid columns, before; dotted columns, after returning to 2 mM Ca^{2+} medium) was not significantly altered when the slices were perfused with a nominally zero Ca^{2+} medium (hatched columns). 27- to 31-d-old rats.

rCGRP modulates the basal and electrically evoked release of Glu and Asp

We found that rat calcitonin gene-related peptide (rCGRP, 10^{-7} M) caused a significant increase in the basal efflux of Glu (to 170.5 ± 30.6 ; $n = 4$) and Asp (to 159.5 ± 20.1 ; $n = 4$), and a smaller increase in Thr (Fig. 5*A*). In addition, there was a significant decrease in the basal release of Asn, whereas the levels of 5 other endogenous amino acids were increased only to a small degree. In contrast to the immediate elevation of Glu and Asp in response to electrical stimulation of primary afferents, the CGRP-caused increase of the basal release was delayed, frequently reaching a maximum 10 min after the first exposure of the slice to the peptide. At the second exposure to the same concentration of CGRP, the CGRP effect was reduced, indicating the occurrence of desensitization.

When calcium was removed from the perfusing medium, the CGRP-evoked enhancement of the basal release of Glu occurred in 2 out of 4 slices examined, whereas the increase in Asp was present, although reduced in magnitude, in 3 out of 4 slices. The average of 3 experiments where the effects of CGRP were tested in nominally zero Ca^{2+} medium is presented in Figure 5*B*.

We have recently shown that CGRP enhances calcium currents of rat dorsal root ganglion neurons and spinal excitatory synaptic transmission (Ryu et al., 1988a). As Ca^{2+} influx is intimately related to neurotransmitter release, a similar action of CGRP on voltage-sensitive Ca^{2+} channels at central terminals of primary sensory neurons, as shown for the somatic membrane of DRG neurons (Ryu et al., 1988a), could increase neurotransmitter release and facilitate excitatory synaptic transmission. In support of this hypothesis, we found that in the presence of CGRP (10^{-7} M) the electrically elicited release of Glu (Fig. 6*A*) and Asp (Fig. 6*B*) was increased. The CGRP-evoked response was prolonged, lasting about 30 min, and was oscillatory in character.

Effects of neonatal capsaicin treatment on the dorsal root- and peptide-evoked release of endogenous amino acids

The question of whether the enhanced release of Glu and Asp following electrical stimulation of dorsal roots and administration of SP, NKA, and CGRP reflects direct release from activated primary afferent fibers (axons and/or presynaptic endings) in the dorsal horn or whether it could reflect activation of sec-

Table 2. Summary of the effects of electrical stimulation of the dorsal rootlets and administration of SP, NKA, and CGRP on the levels of endogenous Asp and Glu in the perfusate of the spinal slices

	Control			Capsaicin		
	Asp	Glu	<i>n</i>	Asp	Glu	<i>n</i>
A. Electrical stimulation						
Normal Krebs	209.2 $\pm$ 26.7	168.3 $\pm$ 14.0	(13)	109.8 $\pm$ 14.2	109.6 $\pm$ 14.8	7
Zero Ca^{2+} solution	94.7 $\pm$ 10.9	101.4 $\pm$ 7.8	(3)	—	—	—
B. Peptides						
SP ($2-5 \times 10^{-7}$ M)	123.2 $\pm$ 15.3	254.3 $\pm$ 62.0	(8)	113.6 $\pm$ 18.7	104.9 $\pm$ 19.0	6
SP ($1-5 \times 10^{-6}$ M)	181.7 $\pm$ 40.1	339.3 $\pm$ 84.2	(3)	—	—	—
SP (5×10^{-7} M in 0 Ca^{2+})	141.9 $\pm$ 7.0	198.2 $\pm$ 29.7	(3)	—	—	—
NKA (5×10^{-7} to 10^{-6} M)	179.1 $\pm$ 35.1	145.3 $\pm$ 16.1	(5)	—	—	—
CGRP (10^{-7} M)	159.5 $\pm$ 20.1	170.5 $\pm$ 30.6	(4)	161.2 $\pm$ 15.0	138.8 $\pm$ 13.4	4

Results are presented as mean percentages $\pm$ SEM of the basal efflux. Dorsal root stimulation: 25–30 V, 0.02–1.0 msec, 3–10 Hz for 5 min. Number of observations shown in parentheses (*n* represents the number of slices; each slice was obtained from a different animal).

Figure 6. CGRP enhances the dorsal root stimulation-evoked efflux of endogenous Glu and Asp. *A*, Bath addition of CGRP (10^{-7} M for 5 min) increased the electrically (25 V, 0.1 msec, 5 Hz for 5 min) evoked efflux of Glu from about 257 to 486% relative to control. Characteristically, the increase in the release oscillated and the oscillations lasted about 30 min. *B*, In the same slice, CGRP also increased the efflux of Asp from 209 to 456% of control. As with Glu, the release was oscillatory in character and of prolonged duration. 29-d-old rat.

ondary or tertiary cells in the primary afferent–dorsal horn neuronal pathways was investigated in slices obtained from capsaicin-treated rats. If capsaicin were to prevent the enhancement of the stimulation-evoked and peptide-evoked basal release of Glu and Asp, it is likely that a significant proportion of released amino acids would arise from sensory endings sensitive to capsaicin.

Although in the present study we found that neonatal capsaicin treatment did not markedly alter the basal efflux of 9 endogenous amino acids from the spinal slices, it prevented the dorsal root stimulation-evoked release of endogenous amino acids (Fig. 7). The stimulation-enhanced efflux of Asp, Glu, Asn, Gly, and Thr seen in vehicle-injected control animals ($n = 8$) was significantly reduced in the slices obtained from the capsaicin-treated rats ($n = 13$; Fig. 7, Table 2).

The effects of neonatal capsaicin treatment on the SP- and CGRP-enhanced basal efflux of Glu and Asp from the spinal slices of rats killed at 3–6 weeks of age are shown in Figures 8 and 9 and Table 2. The results obtained show that neonatal capsaicin treatment prevented the SP-induced increase in the concentration of Glu in the spinal perfusate (Fig. 8). This result suggests that intact unmyelinated (C) and perhaps small myelinated (A δ) primary afferent fibers may be an important source of released Glu following dorsal root stimulation or SP administration. In contrast to the SP-effect, the CGRP-induced increase in the basal levels of Glu and Asp was not prevented in the slices obtained from the capsaicin-treated rats (Fig. 9). It is of interest, however, that CGRP-caused reduction of the basal release of Asn seen in the vehicle-treated animals appears to be abolished by capsaicin treatment. The blockade of the SP effect on Glu release seen in the capsaicin-treated rats suggests that the peptide may regulate the release of the excitatory neurotransmitters by acting at presynaptic sites. On the other side, the persistence of the effect of CGRP in the capsaicin-treated rats may reflect the prevalent effect of CGRP on the release of Glu and Asp from the interneurons or descending afferents to the dorsal horn. However, since there is significant CGRP staining left after capsaicin treatment (Fig. 9, *A*, *B*; see also Diez

Guerra et al., 1988), this finding may imply that subcutaneous treatment with capsaicin is not effective in eliminating all the CGRP-LI primary afferent input.

Discussion

Dorsal root stimulation-evoked release of endogenous amino acids and the effects of capsaicin

The findings of the present study confirm previous reports that dorsal root stimulation evokes a Ca^{2+} -dependent release of Glu

Figure 7. Neonatally applied capsaicin reduced the dorsal root stimulation-evoked release of Asp and Glu. The dorsal root stimulation-evoked increase in the release of endogenous Asp, Glu, Gly, and Thr from the horizontal spinal cord slices of intact rats (solid columns) was absent in the slices obtained from the rats treated neonatally with capsaicin (hatched columns). Rats were allowed to survive 3–6 weeks after injection of Tween 80/ethanol/saline (1:1:8) vehicle or vehicle containing 50 mg/kg capsaicin. The results for control ($n = 6$) and capsaicin ($n = 7$) groups are expressed as mean percentages $\pm$ SEM of the respective basal values. Statistical difference between the release in intact and capsaicin-treated rats: ** $p < 0.005$, * $p < 0.01$. 23- to 45-d-old rats.

Figure 8. Immunoperoxidase staining of the rat lumbar spinal cord for SP-LI in rats injected with control vehicle (A) and rats treated with capsaicin neonatally (B). Scale bar, 100 μ m. C, The SP-induced increase in the basal efflux of endogenous Glu from the spinal cord slices of the intact rats (solid columns) was absent in the rats treated with capsaicin neonatally (hatched columns). The results for control ($n = 7$) and capsaicin-treated animals ($n = 6$) are presented as mean percentages $\pm$ SEM of their respective basal values. Statistical difference between the release in the intact and capsaicin-treated rats: ** $p < 0.005$. 24- to 44-d-old rats.

Figure 9. Immunoperoxidase staining of the rat lumbar spinal cord for CGRP-LI in rats injected with control vehicle (A) and rats treated with capsaicin neonatally (B). Scale bar, 100 μ m. C, The CGRP-elicited increase in the basal efflux of endogenous Glu and Asp from the spinal cord slices of intact rats (solid columns) was present in rats treated with capsaicin neonatally (hatched columns). The results in control ($n = 4$) and capsaicin-treated rats ($n = 4$) are presented as mean percentages of their respective controls $\pm$ SEM. 27- to 45-d-old rats.

from the frog (Takeuchi et al., 1983) and the newborn rat spinal cord *in vitro* (Kawagoe et al., 1986). These earlier studies also examined the evoked release of Asp and found it to be less regularly observed than for Glu and statistically insignificant. On the basis of these observations, the suggestion was made that Glu is the principal transmitter released by dorsal root stimulation (Kawagoe et al., 1986). The results from this study clearly show that besides Glu, several endogenous amino acids, including Asp, Asn, Gly, Ser, and Thr are released in significantly higher amounts upon high-intensity repetitive electrical stimulation of primary afferent fibers. It is worth noting that despite our finding that the basal efflux of Asp is only about half that of Glu, the stimulation-evoked increase in the basal release of Asp appears to be similar, or even higher, than that of Glu. Two observations indicate that the electrically evoked release of Asp does not result from nonspecific changes in permeability of neuronal membranes. First, the release of Asp was repeatable, and, following stimulation, baseline levels of released Asp completely recovered to control levels. Second, the release was blocked by perfusing solution containing zero Ca^{2+} . This is as expected for a process of calcium-dependent exocytosis. We would like to suggest, therefore, that the stimulation-evoked release of Asp is likely to be relevant for excitatory neurotransmission in the spinal dorsal horn. This suggestion is in agreement with a recent demonstration of Asp-immunoreactive axons in normal rat L4 dorsal roots (Westlund et al., 1989b). Although it is well known that L-Glu acts as a mixed agonist at both NMDA and non-NMDA excitatory amino acid receptors (Mayer and Westbrook, 1984), L-Asp appears to be selective for NMDA receptors (Watkins, 1981; Mayer and Westbrook, 1984). Several recent *in vitro* studies, using either brain slices or isolated spinal cord preparations, have demonstrated that NMDA receptors can be activated during monosynaptic and polysynaptic transmission (Dale and Roberts, 1985; Forsythe and Westbrook, 1988; Gerber and Randic, 1989a).

As shown in Figure 1A, electrical stimulation of dorsal roots also resulted in significant increases in the concentrations of Gly and Ser in the spinal perfusate. This finding may have functional implications for spinal excitatory synaptic transmission and integration of sensory information incoming to the dorsal horn since it has been shown that responses of spinal neurons to the excitatory amino acid NMDA are markedly potentiated by nanomolar concentrations of Gly (Johnson and Ascher, 1987). This, together with the demonstration that the strychnine-insensitive Gly binding site is distinct from, but associated with, the NMDA receptor (Bonhaus and McNamara, 1988) has initiated considerable interest in Gly as a modulator of NMDA-receptor-mediated synaptic transmission (Salt, 1989; Thomson et al., 1989). Although the mechanisms by which Gly acts on NMDA receptors are not well understood (Danyisz et al., 1989), Mayer et al. (1989) have recently presented evidence indicating that at least part of the enhancement of NMDA response by Gly occurs through acceleration of recovery from desensitization. D-Ser (a Gly analog) is able to substitute for Gly in preventing desensitization (Mayer et al., 1989). In this context, it is noteworthy that we have recently observed that the responses of rat dorsal horn neurons, either acutely isolated (Murase et al., 1989b) or some in the spinal slice (Gerber et al., 1989), to NMDA are augmented by 10^{-7} – 10^{-6} M Gly.

It has been shown that, besides the primary afferent fibers (Wheeler et al., 1966; Roberts, 1974; Takeuchi et al., 1983; Kawagoe et al., 1986), some descending pathways in the dorsal

horn (Stone, 1979; Rustioni and Cuenod, 1982; Potashner and Tran, 1985; Potashner and Dymzyk, 1986) and some intrinsic dorsal horn interneurons (Davidoff et al., 1967; Rustioni and Cuenod, 1982) may use Glu as a neurotransmitter. Therefore, the question of whether a proportion of the released Glu and Asp following electrical stimulation of primary afferent fibers derives from activation of primary sensory neurons or whether it could reflect activation of secondary or tertiary cells in the dorsal horn pathways cannot be satisfactorily addressed by the experiments discussed above. In order to investigate the contribution of primary sensory neuronal Glu and Asp pools to the stimulation-evoked release of these amino acids, we used neonatal treatment of rats with capsaicin, a neurotoxin known to cause degeneration of a large number of small "dark" sensory neurons (Jancso et al., 1977; Nagy et al., 1981). In this study, we show that capsaicin prevents the stimulation-evoked release of Glu, Asp, Gly, and Thr, the results suggesting a possibility that a significant proportion of the release of these endogenous amino acids is likely to arise from primary afferent fibers sensitive to capsaicin. Our finding of capsaicin sensitivity of the stimulation-evoked Glu release is in agreement with recent morphological data indicating that Glu is preferentially localized in a subpopulation of small dorsal root ganglion cells (Cangro et al., 1985; Battaglia et al., 1987), unmyelinated dorsal root axons (Westlund et al., 1989a), and synaptic terminals in the superficial laminae of the spinal cord of rats, many of which are likely to represent endings of unmyelinated (C) or small myelinated (A δ) fibers (DeBiasi and Rustioni, 1988). Thus, the morphological data and the results of the capsaicin release experiments reported in this work lend further support to the hypothesis that Glu and/or Asp is likely to be involved in the first-order transmission of cutaneous information, particularly from C and A δ primary afferents (Schneider and Perl, 1985).

Enhancement of the basal and the dorsal root stimulation-evoked release of endogenous Glu and Asp by SP, NKA, and CGRP

The results of the experiments reported here demonstrate that tachykinins (SP and NKA) and CGRP induce an apparently specific, predominantly Ca^{2+} -independent increase in the basal release of putative primary afferent transmitters, Glu and Asp. Although the enhancing and selective effect of SP on the release of endogenous Glu from the hemisectioned spinal cord of newborn rats, first observed by Kawagoe et al. (1986), has been confirmed, the different results were obtained in regard to the magnitude of the SP effect, its Ca^{2+} dependence, and the dose-related, SP-induced release of Asp. Kawagoe et al. (1986) found that the bath application of SP (5–10 μM) caused an average increase in the basal release of Glu of about 130%, the magnitude of the effect being comparable to the effect of dorsal root stimulation. The Asp release induced by SP was small and statistically insignificant. In addition, they found that the release of Glu, but not of Asp, was decreased or abolished in the perfusing medium containing low concentrations of Ca^{2+} or TTX. In contrast to the results of Kawagoe et al. (1986), we found that perfusion of spinal cord slices with lower concentrations of SP (10^{-7} M) was accompanied by a selective and marked (2- to 3-fold) increase in the rate of basal efflux of Glu. With higher concentrations of SP (10^{-6} M), however, the basal release of Asp was also augmented in a dose-dependent manner. The SP-caused increase in the release of Glu was consistently larger than that produced by the dorsal root stimulation, and the effect could be demon-

strated in the absence of external Ca^{2+} . The inconsistencies between our results and those of Kawagoe et al. (1986) may be ascribed to methodological differences presented by their use of glucose-free perfusing solution, an amino acid uptake blocker (α -methyl Asp), and the newborn-rat hemisectioned spinal cord preparation.

In relation to the results discussed above, it is noteworthy that Smullin et al. (1988), using dorsal horn dialysis probe in freely moving rats, observed a higher increase in endogenous Asp than in Glu in response to 1 mM SP administration, and no increase in the basal concentration of Asp, Glu, Gly, and taurine upon administration of 10 μM CGRP.

The data in Figure 2C show that an SP-related tachykinin, NKA, which occurs in primary sensory neurons, and in an even higher concentration than SP in the rat spinal dorsal horn (Kana-zawa et al., 1984; Brodin et al., 1986), also increases the basal release of Glu, Asp, and Gly from the rat spinal slices. Although in a few experiments the relative potencies of SP and NKA with respect to the stimulation of the basal release of Asp and Glu appear to be similar, in 8 experiments SP appeared to increase the release of Glu more, whereas NKA preferentially increased the basal release of Asp ($n = 5$). Since NKA also exhibits a potent excitatory action on spinal neurons that is depressed by an SP antagonist (D-Arg¹, D-Pro², D-Trp^{7,9}, Leu¹¹)-SP, it is likely that some of the physiological roles that have been attributed to SP in sensory neurotransmission are, in fact, mediated by NKA, or both tachykinins.

The origin of the excitatory amino acids that are released and the mechanism(s) underlying the enhancement of the release of Glu and Asp by tachykinins and CGRP have yet to be elucidated. In the present study, we have demonstrated that in contrast to the electrically evoked release of Glu and Asp from the spinal slice, which does not occur when Ca^{2+} is absent from the external medium, the enhancement of the basal release of the amino acids by SP and CGRP appears to be, in a large part, Ca^{2+} independent. Since the Ca^{2+} independence of the releasing action of peptide receptors seems to exclude the involvement of Ca^{2+} -mediated exocytosis, it is possible that peptide ligand-induced neurotransmitter release may be mediated by second-messenger systems rather than depolarization-induced calcium influx. There is evidence for the role of protein kinase C (Nishizuka, 1984; Nichols et al., 1987; Gerber et al., 1989) and the cyclic AMP system in neurotransmitter (Nestler and Greengard, 1983) and peptide release. In addition, it is of interest that CCK-8 evokes secretion of oxytocin and vasopressin from rat neural lobe, independent of external calcium, and that the CCK-8 action is blocked by an inhibitor of protein kinase C (Bondy et al., 1989). On the other hand, SP is known to increase hydrolysis of phosphoinositides in central neurons (Watson and Downes, 1983), whereas some actions of CGRP are thought to be mediated through activation of adenylate cyclase (Crossman et al., 1987; Wang and Fiscus, 1989). Two experimental results are relevant for the possible second-messenger mediation of the enhancement of the release of Glu by SP. First, Womack et al. (1988) have shown that in about one-third of cultured dorsal horn neurons, SP receptor activation increases cytosolic free Ca^{2+} via mobilization of intracellular Ca^{2+} stores, and a suggestion was made that the intracellular pathway for the action of SP may involve the generation of inositol phosphate intermediates. Second, we have recently demonstrated (Gerber et al., 1989) that perfusion of rat spinal slices with phorbol esters, the agents known to activate the calcium- and phospholipid-dependent

protein kinase C (Nishizuka, 1984, 1986), produces an increase in the basal and electrically evoked release of endogenous excitatory (glutamic, aspartic) and inhibitory amino acids (GABA, Gly).

Besides the neuronal source (axons and/or nerve terminals), the peptide-caused increase in the basal release of Glu and Asp from the glial cells should also be considered. The extracellular microenvironment of the central neurons is largely bounded by glial membranes, and glial cells have been postulated to influence the concentration of neurotransmitters in the synaptic cleft by a variety of mechanisms. Evidence exists that amino acids are released by high- K^{+} solution in a Ca^{2+} -independent manner (Drejer et al., 1982, 1983) and that astrocytes may be intimately involved in neurotransmission processes, amino acid uptake (Hertz and Schousboe, 1986; Höslí et al., 1986), and they possess receptors for amino acid transmitters (Glu, Asp, GABA) and peptides (Bowman and Kimelberg, 1984; Kettermann and Schachner, 1985; Hamprecht, 1986; Torrens et al., 1986). Binding sites for SP were detected on glial cells of the spinal cord and SP enhances accumulation of labeled inositol phosphates in cultures of cortical glial cells from the mouse (Torrens et al., 1986). In addition, synthetic human calcitonin raises intracellular concentration of cyclic AMP in rat astroglia cells (Hamprecht, 1986).

It is well established that the principal mode of inactivation of putative excitatory (Glu, Asp) and inhibitory (GABA, Gly) neurotransmitters released from nerve endings is by reuptake, using high-affinity ion- and membrane potential-dependent transport systems that are known to operate both in neuronal and glial membranes (Fonnum, 1984; Höslí et al., 1986). Thus, another potential mechanism for the enhancement of the basal release of Glu and of Asp by tachykinins and CGRP is via the electrogenic transport system, which is Ca^{2+} independent and would be expected to "release" Glu and Asp whenever cells are depolarized. It is of interest that the Gly uptake system is inhibited by the peptides, leu- and met-enkephalin (Rhoads et al., 1984).

It is well established that the release of classical neurotransmitters is controlled by autoreceptors, heteroreceptors, or receptors that are acted upon by co-localized substances, such as neuropeptides (Chesselet, 1984; Bartfai et al., 1988) and that this mechanism plays an important role in determining the amount of transmitter released per each stimulus. It was first demonstrated in the example of ACh/vasointestinal polypeptide (VIP) coexistence in the postganglionic neurons of the cat that muscarinic cholinergic autoreceptors inhibit the release of both ACh and VIP. VIP, on the other hand, enhanced the release of ACh (Lundberg et al., 1980). VIP enhancement of ACh-evoked salivation in the cat submandibular gland (Lundberg et al., 1980) was followed by demonstration of synergistic effects of ACh and VIP in promoting phosphatidylinositol turnover in the cerebral cortex, i.e., at the sites of coexistence of ACh and VIP. Synergistic effects of 5-HT, TRH, and SP in the ventral spinal cord have also been observed (Iverfeldt et al., 1986; Tremblay et al., 1986).

The finding that Glu and SP coexist in primary afferent terminals in the superficial laminae of the rat spinal dorsal horn (DeBiasi and Rustioni, 1988), coupled with the demonstrated potentiation of the basal and stimulation-evoked efflux of Glu and Asp by SP and NKA in this study, provide evidence for a role of tachykinins in the regulation of Glu and Asp release. Thus, in addition to the excitatory postsynaptic actions of tachy-

kinins on the dorsal horn neurons (Murase and Randic, 1984; Murase et al., 1989a, b) and modulation of the Ca^{2+} conductances (Ryu and Randic, 1990), the tachykinins may also serve some important presynaptic function through the regulation of the release of coexisting primary afferent transmitters. It would seem that excitatory co-release of amino acids and tachykinins could serve to interact cooperatively to result in a potentiation of depolarizing action at postsynaptic sites on dorsal horn neurons. These pre- and postsynaptic mechanisms of action of tachykinins, and other sensory peptides, may have important physiological implications for strengthening the synaptic connections in the spinal dorsal horn. Such a dual role is consistent with our present knowledge about multiple pre- and postsynaptic actions of peptides in the PNS (Lundberg et al., 1980).

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