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Na^+ channel block prevents the ischemia-induced release of norepinephrine from spinal cord slices

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Abstract

The principal finding of the present study with rat spinal cord slices was the novel demonstration of the $[Ca^{2+}]_o$-independent effect of ischemia on norepinephrine release and its antagonism by tetrodotoxin and low temperature (10°C). Our finding that tetrodotoxin antagonized the effects of glucose deprivation on norepinephrine release in a $[Ca^{2+}]_o$-independent way suggests that Na^+ channel block alone, i.e., the prevention of Na^+ accumulation, may account for the protective action. Low temperature completely prevented the effect of ischemia on norepinephrine release but did not change the release associated with axonal activity. This finding is in good agreement with the observation that small changes in brain temperature critically determine the extent of neuronal injury from ischemia and suggests that both $[Ca^{2+}]_o$-independent release and cell injury are associated with the norepinephrine membrane carrier. It is suggested, therefore, that drugs able to attenuate the increase in $[Na^+]_i$ during ischemia may be useful agents to protect against ischemic damage if given before the insult. © 1998 Elsevier Science B.V.

Keywords: Spinal cord; Ischemia; Hypoxia; Norepinephrine release; Carrier-mediated release

1. Introduction

A growing body of evidence suggests that the neurotoxicity of the excitatory amino acid, glutamate, contributes to the brain damage associated with several acute insults, including hypoxia, ischemia, hypoglycemia, prolonged seizures and trauma (Coyle and Puttfarcken, 1993; Ross, 1996; Doolette, 1997; Matsuoka et al., 1997; Figiel and Kaczmarek, 1997). In addition, it has been shown that the excessive release of dopamine and norepinephrine during ischemia has an injurious effect in the striatum and hippocampus, respectively (Globus et al., 1989, 1988). Activation of β-adrenoceptors by norepinephrine released by ischemia from terminals originating in the locus coeruleus (Yang et al., 1996; Zhang et al., 1997) results in an increase of cAMP formation, which results in an increase of $[Ca^{2+}]_i$, thereby triggering cell death. These results suggest that neuronal damage following ischemia may be modulated by excessive accumulation and interaction of a variety of neurotransmitters released synaptically and non-synaptically (Vizi, 1984; Vizi and Lábos, 1991) during ischemia (Globus et al., 1989).

Secondary damage to the spinal cord after injury is due, in part, to delayed neurochemical changes (Richard et al., 1990; Simon et al., 1984; Tsuji et al., 1994; Martin et al., 1994; Vizi et al., 1996), including the release of glutamate (Richard et al., 1990), reduction of ATP level (Tsuji et al., 1994), and accumulation of $[Ca^{2+}]_i$ which is believed to be toxic to nervous tissue (Demediuk et al., 1989; Wahl et al., 1989).

Norepinephrine potentiates NMDA receptor-mediated injuries in the spinal cord (Wohlberg et al., 1987), and an elevation of extracellular concentrations of excitatory amino acids has been shown after spinal cord injury (Demediuk et al., 1989; Richard et al., 1990). Because spinal cord ischemia and the resultant paraplegia are devastating sequelae in nearly 40% of patients undergoing emergency repairs and dissections, it seemed interesting to explore the effect of ischemia on norepinephrine release from spinal cord and to study the possibility of preventing the effect of ischemia on the release of norepinephrine.

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2. Materials and methods

Male Sprague–Dawley rats (200–300 g) were used in the present study (Ko et al., 1997). Rats were housed two per cage and were kept under a 12-h dark/light cycle with free access to food and water. All procedures with animals were approved by the local Animal Care Committee and were in strict accordance with the NIH's 'Guide for the Care and Use of Laboratory Animals'.

2.1. Tissue preparation for neurotransmitter release studies

Generally, four to six rats were used per experiment. For dissection of the spinal cord, the rat was lightly anesthetized with diethyl ether and decapitated immediately. Next, after surgical exposure of the spine, the vertebral column was sectioned at the C7 and T6 level. This section of the vertebral column was freed from the attached ribs and muscles and the end tip of an infant feeding tube was used to extrude the spinal cord slowly and gently out of the vertebral canal. Spinal cord tissue was next cleansed of blood vessels and attached arachnoid membranes and rinsed in Krebs buffer, pH 7.4, then sliced into 0.4-mm thick transverse sections with a McIlwain tissue slicer, and the tissue was resuspended in Krebs solution (in mmol/l: NaCl, 113; KCl, 4.7; $CaCl_2$, 2.5; KH_2PO_4, 1.2; $MgSO_4$, 1.2; $NaHCO_3$, 2.5; and glucose, 11.5) and aerated with 95% O_2 and 5% CO_2. When Ca^{2+}-free solution was used, $CaCl_2$ was omitted from the Krebs solution and 1 mM EGTA was added.

In our experiments ischemia was simulated by having spinal cord slices in Krebs solution lacking glucose, equilibrated with 95% nitrogen, 5% carbon dioxide. For low temperature (10°C) a cooling system was used and the temperature was kept constant with a thermostat.

2.2. Tissue labeling with radioactive precursors

In our studies of norepinephrine release, we used the procedure of tissue labeling with [^{3}H]norepinephrine as described by Umeda et al. (1997). In brief, the slices were incubated for 30 min in 2 ml of Krebs solution containing [^{3}H]norepinephrine at a concentration 10 μCi, gassed with a mixture of 95% O_2 and 5% CO_2 at 37°C. After the incubation, the slices were washed 3 times with 5 ml of Krebs solution and placed in a 100-μl chamber for superfusion (Vizi et al., 1985). They were superfused with Krebs solution at 37°C at a flow rate of 0.5 ml/min. After 60 min of preperfusion, 19 3-min fractions were collected with a fraction collector. One or two supramaximal field stimulations (40 V, 3 Hz, 1 ms, for 60 s) were applied.

When the effect of hypoxia was studied, tissues were subjected to hypoxic conditions (95% N_2 + 5% CO_2) starting at the sixth collection period. In the case of hypoxia combined with glucose deprivation (ischemia), glucose was omitted from the Krebs solution. When the effect of hypoglycemia was to be studied, glucose was omitted from the Krebs solution.

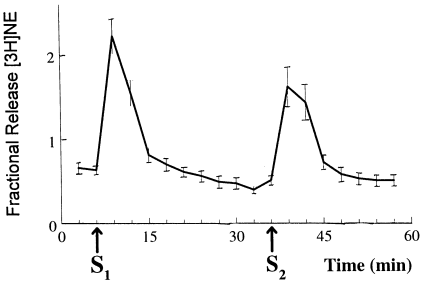

Fig. 1. Effect of electric field stimulation on the release of [^{3}H]norepinephrine from spinal cord slices. Radioactivity of 3-min fractions is expressed as fractional release (% of total tissue radioactivity at the start of the collection period). The slices were stimulated twice (S_1 and S_2).

A 0.5-ml aliquot of the superfusate or 0.1 ml of the tissue supernatant was added to 2 ml of scintillation cocktail (Ultima Gold, Packard). At the end of each experiment, the slices were kept overnight in 1 ml of tissue solubilizer (Soluvable, NEN) at room temperature and centrifuged, and the radioactivity of the tissue was measured in aliquots of the supernatant by liquid scintillation counting (Packard Tricarb).

Radioactivity released was expressed in terms of disintegrations per second per gram of tissue (Bq/g). The fractional release (FR) was also expressed in as a percentage of the total tritium content present in the tissue at the beginning of the collection. A desk-top computer was used for calculations.

2.3. Statistical analysis

The effects of the treatments (hypoxia, hypoglycemia, tetrodotoxin, lidocaine, 4-aminopyridine) and their combinations on the resting (fractional) release of [^{3}H]norepinephrine in the presence/absence of [Ca^{2+}] or at various experimental temperatures were studied by three-way analysis of variance (ANOVA) with one repeated measure factor (before or during treatment). Post-hoc Tukey tests

Table 1
Effect of hypoxia or/and hypoglycemia on the resting release of [^{3}H]norepinephrine in the presence or absence of [Ca^{2+}]

	Fractional release of [^{3}H]norepinephrine (%)	
	[Ca^{2+}]	no [Ca^{2+}]
Control	0.605 ± 0.177 ($n = 4$)	0.619 ± 0.221^{NS} ($n = 4$)
Hypoxia	0.703 ± 0.217^{ns} ($n = 6$)	0.635 ± 0.087^{NS} ($n = 4$)
Hypoglycemia	1.460 ± 0.405^{a} ($n = 6$)	3.266 ± 0.058^{b} ($n = 4$)
Ischemia	2.039 ± 0.550^{a} ($n = 6$)	8.851 ± 1.881^{b} ($n = 6$)

The slices were exposed to hypoxia or/and glucose-free conditions for 30 min in the presence or absence of [Ca^{2+}]. According to post-hoc Tukey test, $^{a}P < 0.001$, $^{ns}P > 0.05$ as compared to the [Ca^{2+}] control group above; and $^{b}P < 0.001$, $^{NS}P > 0.05$ as compared to the corresponding [Ca^{2+}] groups in the left column.

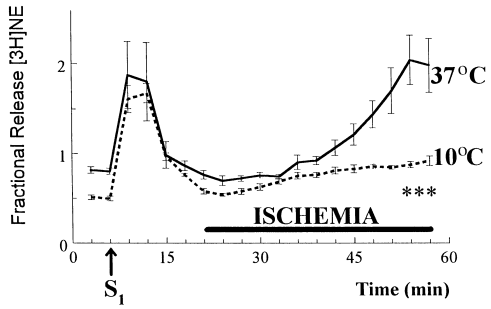

Fig. 2. Low temperature prevents the effect of ischemia on the release of [^{3}H]norepinephrine. The slices were stimulated, then exposed to ischemia for 36 min. Radioactivity of 3-min fractions expressed as fractional release. According to post-hoc Tukey test, $***P < 0.001$ as compared to the 37°C control.

were used for multiple-comparison procedures. $P < 0.05$ was considered significant. All data are presented as the means ± S.E.M.

3. Results

3.1. Effect of hypoxia or / and hypoglycemia

After loading of the slices with [^{3}H]norepinephrine, and perfusion for 90 min, the average uptake of radioactivity was 1258.55 ± 59.79 kBq/g ($n = 95$) and the average resting release in a 3-min collection period was 6.90 ± 0.86 kBq/g, i.e., 0.63 ± 0.08% ($n = 8$) of the total radioactivity. When the preparation was stimulated (3 Hz, 180 shocks), it responded with a significant increase of [^{3}H]norepinephrine release, 30.45 ± 3.61 kBq/g (Fig. 1). The amount of [^{3}H]norepinephrine released during the second stimulation was 25.96 ± 2.88 kBq/g, producing an FRS_2/FRS_1 ratio of 0.80 ± 0.06 ($n = 6$). The release was $[Ca^{2+}]_o$-dependent: $[Ca^{2+}]_o$ withdrawal and the presence of EGTA (1 mM) completely prevented the stimulation-evoked release (data not shown).

The 30-min exposure of the tissue to hypoxic conditions had no effect on the resting release, but enhanced the stimulation (3 Hz, 180 shocks) evoked release ($FRS_2/FRS_1 = 1.36 \pm 0.06$, $n = 6$, $P < 0.01$). However, when the glucose was withdrawn from the Krebs solution, the resting release was enhanced. This effect was even more pronounced when hypoxia was combined with glucose deprivation (Table 1).

3.2. Effect of $[Ca^{2+}]_o$-removal

Since Ca withdrawal prevents Na_i^+ from being exchanged for Ca_o^{2+}, resulting in fast accumulation of $[Na^+]_i$, and since the Na^+–Ca^{2+} exchange is a general mechanism of Ca^{2+} entry for many types of neuronal injury (LoPachin and Lehning, 1997; Stys and LoPachin, 1997), the $[Ca^{2+}]_o$ dependence of the effect of hypoxia or/and hypoglycemia on norepinephrine release was also tested. Removal of $[Ca^{2+}]_o$ had no effect alone, but significantly potentiated the resting release of norepinephrine evoked by hypoglycemia or ischemia but not that by hypoxia (Table 1).

3.3. Prevention of the effect of ischemic condition

Fig. 2 shows that, in contrast to observations at 37°C, ischemia was not able to release norepinephrine when the temperature was reduced to 10°C. Unlike the resting release, the stimulation-evoked release was not affected by low temperature (10°C): FRS_2/FRS_1 was 1.05 ± 0.06 ($n = 6$; $P > 0.05$).

Since tetrodotoxin, a selective Na^+ channel blocker, and lidocaine, a local anesthetic (sodium and potassium channel blocker) have been shown to reduce glutamate release during ischemia (Fujitani et al., 1994) and inhibit sodium influx (Fried et al., 1995), we studied their actions

Table 2
Effect of inhibition of sodium or/and potassium channels on the effect of ischemia on the resting release of [^{3}H]norepinephrine in the presence or absence of $[Ca^{2+}]$

		Fractional release of [^{3}H]norepinephrine (%)	
		$[Ca^{2+}]$	no $[Ca^{2+}]$
Control		0.605 ± 0.177 ($n = 10$)	0.619 ± 0.221^{ns} ($n = 4$)
Ischemia		2.039 ± 0.550^{a} ($n = 6$)	8.851 ± 1.881^{c} ($n = 6$)
Ischemia +	Tetrodotoxin, 1 μM	1.298 ± 0.381^{ns} ($n = 5$)	1.866 ± 0.610^{NS} ($n = 5$)
	Tetrodotoxin, 1 μM	0.620 ± 0.182^{ns} ($n = 4$)	0.628 ± 0.240^{NS} ($n = 4$)
Ischemia +	Lidocaine, 0.1 mM	2.447 ± 0.725^{b} ($n = 4$)	5.069 ± 1.767^{c} ($n = 4$)
Ischemia +	Lidocaine, 1.0 mM	3.475 ± 0.945^{a} ($n = 4$)	2.303 ± 0.297^{d} ($n = 5$)
Ischemia +	Lidocaine, 3.0 mM	7.009 ± 0.841^{a} ($n = 4$)	5.994 ± 1.052^{c} ($n = 5$)
	Lidocaine, 3.0 mM	2.364 ± 0.744^{a} ($n = 4$)	1.847 ± 0.242^{NS} ($n = 4$)
Ischemia +	4-Aminopyridine, 100 μM	5.229 ± 0.611^{a} ($n = 4$)	
	4-Aminopyridine, 100 μM	2.700 ± 0.790^{a} ($n = 6$)	

The slices were exposed to ischemia or/and various drugs: tetrodotoxin, lidocaine, 4-aminopyridine for 30 min in the presence (or absence) of $[Ca^{2+}]$. According to post-hoc Tukey tests, $^{a}P < 0.001$, $^{b}P < 0.01$, $^{ns}P > 0.05$ as compared to the $[Ca^{2+}]$ control group above; and $^{c}P < 0.001$, $^{d}P < 0.01$, $^{NS}P > 0.05$ as compared to the corresponding 'no $[Ca^{2+}]$' control group above.

on norepinephrine release under various conditions. Tetrodotoxin significantly reduced the effects of ischemia on resting norepinephrine release, whether in the presence or the absence of $[Ca^{2+}]_o$ (Table 2).

In contrast, lidocaine at concentrations of 0.1, 1, and 3 mM increased the effect of ischemia in a concentration-dependent manner under conditions in which $[Ca^{2+}]_o$ was present at a concentration of 3 mM the release was significantly potentiated (Table 2). In the absence of $[Ca^{2+}]_o$, however, lidocaine significantly reduced the release of [^{3}H]norepinephrine evoked by ischemia. While there was a concentration-dependent action at concentrations of 0.1 and 1 mM, at 3 mM lidocaine was significantly less effective than at 1 mM (Table 2). Lidocaine itself at a concentration of 3 mM enhanced the release of norepinephrine (Table 2).

Since lidocaine also blocks K^+ channels (Strichartz and Ritchie, 1987), the effect of 4-aminopyridine, a K^+ channel inhibitor, was also studied. 4-Aminopyridine per se, enhanced the release of [^{3}H]norepinephrine. When applied during ischemia, its effect was additive according to a three-way ANOVA (Table 2).

Idazoxan, an α_2-adrenoceptor antagonist, at a concentration of 1 μM failed to influence the resting release of [^{3}H]norepinephrine evoked by ischemia (it was 2.15 ± 0.42 in the absence and 2.07 ± 0.40 in the presence of idazoxan, $n = 5$).

4. Discussion

The principal finding of the present study with rat spinal cord slices was the novel demonstration of a $[Ca^{2+}]_o$-independent effect of ischemia on norepinephrine release and its antagonism by tetrodotoxin and low temperature. Removal of extracellular Ca^{2+} further increased the ischemia-induced release of norepinephrine. In our experiments ischemia was simulated by the lack of glucose and oxygen in the Krebs solution. Since the action potential-associated, $[Ca^{2+}]_o$-dependent release of norepinephrine was completely blocked when glucose was withdrawn (Milusheva et al., 1996), but the $[Ca^{2+}]_o$-independent spontaneous release of transmitter continued to occur, even at a much higher rate, in a temperature-dependent manner, it is suggested that, under ischemic conditions, the carrier-mediated, $[Ca^{2+}]_o$-independent release is operative.

The $[Ca^{2+}]_o$-dependent release of vesicular glutamate (Adam-Vizi, 1992; Erecinska et al., 1996), vesicular dopamine (Erecinska et al., 1996; Milusheva et al., 1992, 1996) and vesicular norepinephrine (Milusheva et al., 1996) has a high requirement for energy, and is very sensitive to the intracellular ATP level. The $[Ca^{2+}]_o$-independent release of glutamate (Kauppinenen et al., 1988) and norepinephrine caused by reversal of the carrier system does not require energy and is consistent with a drop in intracellular ATP levels and the consequent inhibition of sodium pump activity, which leads to a decline in the Na^+ electrochemical gradient across the plasma membrane and accumulation of $[Na^+]_i$, resulting in release of cytoplasmic transmitter (Vizi, 1972, 1978; Vizi et al., 1985; Milusheva et al., 1996). The release of glutamate (Faden et al., 1987; Richard et al., 1990; Phillis et al., 1994) and norepinephrine (this study) in response to ischemia can be mediated by inhibition of the sodium pump (Faden et al., 1987; Milusheva et al., 1996; Silver et al., 1997).

Since neurochemical evidence has been obtained (Klarica et al., 1996) that NMDA receptor activation results in the release of norepinephrine from rat spinal cord, it seems plausible to explain the release of norepinephrine in response to ischemia as a result of the presence of excess glutamate in the vicinity of noradrenergic axon terminals. This case is supported by the finding that dizocilpine, an NMDA receptor antagonist, significantly reduces the release of norepinephrine evoked by ischemia in spinal cord slices (Nakai, T., Milusheva, E., Sato, K., Vizi E.S., unpublished). It has been shown that the release of norepinephrine associated with axonal activity in the spinal cord is modulated by presynaptic α_{2A}-adrenoceptors (Umeda et al., 1997) and that norepinephrine is able to inhibit the release of glutamate evoked by excess K (Kamisaki et al., 1993) or by capsaicin (Ueda et al., 1995) via stimulation of α_2-adrenoceptors expressed on glutamatergic axon terminals (Kamisaki et al., 1993). In contrast, in our experiments the release of [^{3}H]norepinephrine in response to ischemia was not subject to presynaptic modulation via α_{2A}-adrenoceptors, also indicating that release was not associated with exocytosis, but was due instead to a reversed operation of the carrier. Omission of glucose, an insult to cellular energy production, causes a fall in $[K^+]_i$ and a simultaneous rise in $[Na^+]_i$ (Silver et al., 1997). The pattern of these changes is consistent with inhibition of the Na^+/K^+ pump, which results in $[Ca^{2+}]_o$-independent release of transmitters (cf. Vizi, 1978; Milusheva et al., 1996), and inhibition of $[Ca^{2+}]_o$-dependent release associated with axonal activity (Milusheva et al., 1996).

Our finding that tetrodotoxin antagonized the ability of glucose deprivation to release norepinephrine in a $[Ca^{2+}]_o$-independent way suggests that Na^+ channel block alone, i.e., prevention of Na^+ accumulation, may account for the protective action. Lidocaine, a major local anesthetic in clinical use, reduced the effect of ischemia on norepinephrine release when Ca^{2+} was withdrawn (Table 2).

One unexpected finding from our studies in cooperation with Fried et al. (1995) was that lidocaine at a higher concentration (3 mM) in the presence of Ca^{2+} increased the spontaneous release of norepinephrine. Since high lidocaine concentrations block both sodium and potassium channels (Strichartz and Ritchie, 1987) and 4-aminopyridine, a K^+ channel blocker, per se enhances the release of norepinephrine and strongly potentiates the effect of is-

chemia, it seems very likely that the effect of lidocaine at higher concentrations is similar to that of 4-aminopyridine and is due to inhibition of the potassium channel. A similar observation was made with hippocampal slices (Weber and Taylor, 1994) in a microdialysis study (Fujitani et al., 1994).

Low temperature completely prevented the effect of ischemia (Fig. 2) but did not affect the release of norepinephrine associated with action potentials. This finding is in good agreement with the finding that moderate hypothermia of brain tissue can prevent or reduce the damage seen on histological examination (Busto et al., 1987).

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