Influence of Heat Stimulation on the Amount of Calcitonin Gene-Related Peptide and Neurokinin A in the Subcutaneous Space of the Rat Hind Instep

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ABSTRACT — The release of tachykinins and calcitonin gene-related peptide (CGRP) into subcutaneous perfusates was investigated. Immersion of the hind paw of rats in water at 47°C for 30 min led to a marked increase of immunoreactive CGRP (iCGRP) release as well as immunoreactive substance P release, but no detectable increase of immunoreactive neurokinin A release. Neonatal pretreatment with capsaicin or section of the sciatic and the saphenous nerves significantly inhibited the heat stimulation-induced release of iCGRP.

There is much evidence that primary afferent neurons that transfer information about pain to the spinal cord may participate in inflammatory responses through the release of some vasoactive substances from the peripheral terminals of small-diameter afferent fibers (1). Substance P (SP), an undecapeptide present in sensory neurons localized around the small vessels in the skin (2), is thought to be one of the substances that cause relaxation of arterioles and increase blood flow (3). In this connection, we have demonstrated that primary afferent neurons containing SP play an important role in thermal inflammatory reactions through cooperation with mediators such as bradykinin (4, 5). Recently another tachykinin named neurokinin A (NKA), which is chemically related to SP, was discovered in the central and peripheral nervous systems, especially in unmyelinated nerve fibers and the dorsal horn of the spinal cord (6). NKA and SP are formed from a common precursor (7) and coexist in the same nerve fiber in several tissues of the rat: the spinal cord, dorsal root ganglia and skin (dermis and epidermis) (8).

Another neuropeptide, calcitonin gene-related peptide (CGRP), is also abundant in nerve fibers associated with blood vessels (9). CGRP has been shown to coexist with SP in regions of the peripheral nervous system such as the varicose sensory axons supplying the skin (9). SP and CGRP have been found in the fluid of blisters in inflamed skin (9). In addition, Brain et al. (10) demonstrated that CGRP is a potent vasodilator and has a marked enhancing effect on edema induced by other mediators in inflammatory reactions. These facts led us to consider that mutual cooperation of vasoactive substances coexisting in the small-diameter afferent fibers might be involved in the regulation, development and maintenance of thermal inflammatory reactions. We, therefore, examined whether NKA and CGRP as well as SP were released into the subcutaneous space in response to noxious heat stimulation.

Male Sprague-Dawley rats (120–150 g) were anesthetized with urethane (780 mg/kg, i.p.), and a double polyethylene tube about 5 cm in length (the inner tube was 5 mm longer external one) was introduced into the sub-
cutaneous space of the instep. Perfusion was carried out at a rate of 0.1 ml/min with saline containing peptidase inhibitors, bestatin (0.03 mg/ml) and captopril (0.1 mM), through the inner tube using a peristaltic pump. The perfusate in successive 10-min periods was collected in test tubes placed in an ice bath. Each 10-min sample was lyophilized and then radioimmunoassayed (RIA) for SP, NKA and CGRP according to the methods described previously (11, 12). The antiserum of SP did not cross-react with CGRP, but cross-reacted with NKA (< 1%) and neurokinin B (NKB) (< 0.2%). The detection limit of SP was 1 fmol. On the other hand, the detection limit of NKA was 10 fmol. The NKA antiserum showed no cross-reactivity with SP or CGRP, but cross-reacted with kassinin (1.5%) and NKB (5.4%). The CGRP RIA was carried out by a slight modification of the method recommended by Amersham (U.K.). CGRP antiserum raised in rabbits against human CGRP showed 35% cross-reactivity with rat CGRP, but no cross-reactivity with SP, NKA or NKB. The detection limit of CGRP was 3 fmol.

For noxious stimulation, 1 hr after the start of perfusion, the paw on the perfused side was immersed in a water-bath (at 47°C for 30 min) and then returned to room temperature (25°C). Capsaicin at a dose of 50 mg/kg was injected s.c. into newborn rats within a week after birth, as described previously (4). Control rats were treated with the vehicle only. Two months later, animals showing at least 40% increase in latency in response to noxious thermal stimulation compared with control rats were used for the experiments. In the other set of experiments, the saphenous and sciatic nerves, containing sensory afferent fibers terminating in the hind paw, were sectioned unilaterally and animals were used for experiments 7 days later to allow time for degeneration of the peripheral portions of severed nerves. Control rats underwent sham operations. To determine the influence of heat stimulation (47°C) on peptide release, the average amount of neuropeptides released in three 10-min fractions obtained in the 30 min period before, during and after heat stimulation were determined, and that before heat stimulation was used as the control value (100%).

The following drugs and radiolabeled peptides were used: SP, NKA and CGRP (Peptide Institute, Inc., Japan); capsaicin (Sigma Chemical Co., U.S.A.); [125I][Tyr8]SP (New England Nuclear Co., U.S.A.); [125I][His10]-CGRP, CGRP antiserum and [3H]NKA (Amersham, U.K.); NKA antiserum (donation from Prof. H. Kamiya and Dr. Y. Takanoh, Fukuoka University, Japan).

The spontaneous release of immunoreactive CGRP (iCGRP) was stabilized 30 min after the start of perfusion and remained constant for at least 3 hr. This time course was similar to that of immunoreactive SP (iSP), which was described previously (4). In the experiments of capsaicin treatment and denervation, the basal release of iCGRP in vehicle and capsaicin group were 11.5 ± 2.4 fmol/10 min (n = 8) and 10.6 ± 1.7 fmol/10 min (n = 9), respectively; and in the sham operation and nerve section group, they were 7.2 ± 1.9 fmol/10 min (n = 12) and 4.4 ± 1.1 fmol/10 min (n = 13), respectively. The immunoreactive NKA (iNKA) level was below the detection limit of the assay. Immersion of the hind paw in water at 47°C for 30 min resulted in significant increases of iCGRP release (504 ± 78% in the capsaicin experiment (n = 8) and 413 ± 66% in the denervation experiment (n = 12)) as well as iSP (basal release: 7.0 ± 1.1 fmol/10 min (n = 41); heat stimulation-induced release: 53.1 ± 7.7 fmol/10 min (n = 40)). The increase in iCGRP release disappeared when the hind paw was returned to room temperature. The amount of iNKA released in the perfusate during heat stimulation was still below the detection limit of the assay. Pretreatment with capsaicin in the newborn rats (Fig. 1) or denervation of the saphenous and sciatic nerves (Fig. 2) prevented significantly the increase of iCGRP release evoked by noxious heat stimulation.

Concerning the origin of iSP and iCGRP released by heat stimulation, immunohisto-
chemical studies have shown that CGRP is localized not only in SP-containing neurons but also in many small and intermediate sized sensory neurons that do not appear to contain SP (13). The simultaneous increases in the release of iCGRP and iSP observed following heat stimulation, therefore, do not necessarily mean that these peptides were released from the same neurons. However, it has been known that pretreatment of newborn rats with capsaicin results in a selective and permanent degeneration of small-diameter afferent fibers with a significant decrease of the contents of neuropeptides such as SP and CGRP (14) and that, simultaneously, the capsaicin-treated animals show significantly increased latencies in response to a noxious thermal stimulus in either the tail-flick or hot-plate test. Among the small-diameter afferent fibers excited by capsaicin, there are polymodal nociceptors which respond to noxious heat. In this context, we have already shown that iSP was released from the peripheral endings of capsaicin sensitive small-diameter afferent fibers through the activation of polymodal nociceptors by heat stimulation (4). Matsuyama et al. (15), furthermore, demonstrated that capsaicin treatment of neonatal rat elicited degenerative changes in CGRP neurons containing SP, but had little if any effect on CGRP neurons lacking SP. Taking these findings into account, it may be reasonable to consider that a certain proportion of these peptides released by noxious heat stimulation originates from the same primary afferent terminals.

Besides SP and CGRP, it has been known
that a new mammalian tachykinin referred to as neurokinin A (NKA) recently found in primary afferent neurons coexists with SP and is synthesized via the same precursor (7). In the present study, heat stimulation, however, did not induce any increase in NKA release. Although since the detection limit of NKA was 10 fmol, 10-fold higher than that of SP, if the amount of NKA release evoked by heat stimulation is similar to that of SP, NKA released into the perfusates during heating should be detectable. This result therefore lead us to the idea that activation of capsaicin-sensitive primary afferent neurons may cause the differential release of coexisting peptides or that the sensitivity to noxious heat stimulation of SP neurons containing NKA may differ from that of SP neurons lacking NKA.

Acknowledgments

We would like to thank Prof. H. Kamiya and Dr. Y. Takano (Department of Pharmacology, Faculty of Pharmaceutical Sciences, Fukuoka University, Japan) for the kind gift of NKA antiserum. This work was supported by a Grant-in-Aid for Scientific Research from the Ministry of Education, Science and Culture of Japan.

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