Effects of Calmodulin Antagonists on Norepinephrine Release and Vascular Responsiveness in Rat Mesenteric Vasculature

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SUMMARY

This study was designed to investigate the role of calmodulin in adrenergic neurotransmission of resistance vessels. The effects of various calmodulin antagonists (trifluoperazine, W-7, calmidazolium, chlorpromazine, fluphenazine) on the vascular responsiveness and norepinephrine overflow from adrenergic nerve endings were examined in perfused rat mesenteric vasculature preparations. Pressor responses to electrical nerve stimulation or exogenous norepinephrine were inhibited dose-dependently by each calmodulin antagonist. Norepinephrine overflow from the sympathetic nerve endings during electrical nerve stimulation was also suppressed by calmodulin antagonists. These results indicate that calmodulin antagonists affected both pre- and post-synaptic sites of adrenergic neurotransmission, suggesting that calmodulin is involved both in neurosecretion and vascular smooth muscle contractions in peripheral resistance vessels.

Additional Indexing Words:
Calmodulin Calmodulin antagonist Trifluoperazine W-7 Calmidazolium Chlorpromazine Fluphenazine Norepinephrine release Vascular responsiveness Mesenteric vasculature

It is widely accepted that calcium (Ca++) plays a key role in both norepinephrine secretion from sympathetic nerve endings and vascular muscle contractions.1,2 A rise in the intracellular concentration of Ca++ is considered to be an important factor in the initiation of norepinephrine release. Douglas proposed the Ca++-hypothesis of stimulus-secretion coupling, suggesting that the norepinephrine secreted by exocytosis and muscle contractions are similar.3

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Received for publication July 18, 1986.
Manuscript revised October 27, 1986.
It has been also demonstrated that many Ca\textsuperscript{++}-dependent cellular functions are regulated by a Ca-binding protein, calmodulin. In vascular smooth muscle, calmodulin is reported to phosphorylate the myosin light chain kinase, and induce the vascular muscle contractions.\textsuperscript{41-61} Calmodulin is also present both in the brain and peripheral nerve terminals in similar concentrations.\textsuperscript{71} However, little is known about the role of calmodulin in the synaptic function and neurotransmitter release in the vascular beds. This study was undertaken to examine the role of calmodulin in norepinephrine release and vascular responsiveness by using various types of calmodulin antagonists.

**Materials and Methods**

Male Wistar rats (body weight 300–400 g) were used for the experiment. The mesenteric vasculature was isolated under pentobarbital anesthesia (40 mg/kg, intraperitoneal injection) according to the method of Castellucci et al.\textsuperscript{81} After the abdominal cavity was opened, the superior mesenteric artery was isolated. A cannula was inserted into the mesenteric artery and it was flushed with heparinized saline. Four main branches of the mesenteric artery were used; other branches were ligated. The preparation was perfused with modified Ringer-Locke solution (mmol/l: NaCl 120.7, KCl 5.9, CaCl\textsubscript{2} 2.5, MgCl\textsubscript{2} 1.3, NaH\textsubscript{2}CO\textsubscript{3} 15.5, NaH\textsubscript{2}PO\textsubscript{4} 1.2 and glucose 11.5, pH 7.4, 37°C). The solution was bubbled with a 95% O\textsubscript{2}-5% CO\textsubscript{2} mixture and maintained at a flow rate of 0.8 ml/min with a peristaltic pump (Harvard apparatus, model 1200). The perfusion pressure was recorded via a side arm with a pressure transducer connected to a polygraph (Nihon Kohden, model CP-620G). Platinum electrodes were placed around the periarterial plexus of the mesenteric artery. After a 30 min stabilization period, electrical nerve stimulation or exogenous norepinephrine application were performed. Nerve stimulation was carried out at 15 Hz for 1 min with biphasic rectangular pulses of 5 msec duration at supramaximal voltage (40 V), using an electrical stimulator (Nihon Kohden, model SEN-3201). Exogenous 1-norepinephrine (3.3 μg) was given as a single injection in 0.1 ml of the buffer into the arterial cannula. The pressor responses to electrical nerve stimulation or exogenous norepinephrine were determined as an increase in the perfusion pressure.

For the measurement of norepinephrine release from the sympathetic nerve endings in the vascular beds, the perfusate was collected in tubes containing a mixture of EGTA (90 mg/ml) and glutathione (60 mg/ml) (20 μl/ml of the perfusate) for a 3 min period before and after the electrical nerve stimulation. The latter collecting period commenced with a 1 min stimulation period. Norepinephrine was absorbed on alumina, extracted in 200 μl
of 0.1 mol/l perchloric acid, and assayed by high pressure liquid chromatography with an electrochemical detector.\textsuperscript{9-11} The norepinephrine overflow elicited by nerve stimulation was defined as the difference between pre- and post-stimulatory periods, and then calculated as ng per g wet tissue weight of the mesenteric preparation.

The calmodulin antagonists were perfused for 9 min before nerve stimulation or exogenous norepinephrine administration, and the effects of calmodulin antagonists were expressed as a percentage of the control pressor responses or norepinephrine release obtained in the absence of the drugs. The calmodulin antagonists used in this experiment were trifluoperazine (Sigma Chemical Co., Ltd.), N-(6-aminohexyl)-5-chloro-1-naphthalenesulfonamide (W-7) (Rikaken Co., Ltd.), calmidazolium (Janssen Pharmaceutical Co., Ltd.), chlorpromazine (Wako Chemical Co., Ltd.), fluphenazine (Wako Chemical Co., Ltd.). All values were expressed as mean±SEM. Statistical significances were determined by Student’s paired t-test. A p-value<0.05 was considered significant.

\textbf{RESULTS}

The initial perfusion pressure of the mesenteric preparations was 20.8±0.8 mmHg (n=46). The pressor responses and norepinephrine release induced by electrical stimulation (15 Hz) were 77.5±5.5 mmHg (n=24) and 1.06±0.05 ng/g of wet weight (n=23). The pressor response to exogenous norepinephrine (3.3 μg) was 72.7±5.3 mmHg (n=22). The pressor responses and norepinephrine release induced by electrical nerve stimulation were completely abolished in the presence of guanethidine (2.0×10^{-6} M) in the perfusion medium,\textsuperscript{12} implying that periarterial stimulation effects were neuronal. Additionally, the pressor responses and norepinephrine release were constant for seven repeated stimuli, as shown previously.\textsuperscript{12} The experiment

\begin{figure}[h]
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\caption{Effects of trifluoperazine on pressor responses to electrical nerve stimulation (supramaximal voltage: 40 V, 5 msec rectangular pulses, 15 Hz frequency, 1 min duration) in perfused mesenteric vasculature preparations from rats. (1) control response without trifluoperazine. (2) trifluoperazine 1.7×10^{-8} M. (3) trifluoperazine 3.3×10^{-8} M. (4) trifluoperazine 6.6×10^{-8} M. (5) after wash-out of trifluoperazine.}
\end{figure}
was performed during the stable period of each preparation.

Fig. 1 shows the effects of trifluoperazine on the pressor responses to nerve stimulation. The basal perfusion pressure was not affected by trifluoperazine in the concentrations used in this experiment. Trifluoperazine inhibited the electrically-induced pressor responses dose-dependently. The pressor responses recovered almost to the control level when trifluoperazine was washed out from the perfusion medium (Fig. 1).

The pressor responses to exogenous norepinephrine were also inhibited by trifluoperazine, but the attenuation was less than that observed for electri-
Fig. 3. Effects of calmodulin antagonists on norepinephrine overflow during electrical nerve stimulation in mesenteric vascular preparations of rats. Values are expressed as the percent of the control norepinephrine overflow in the absence of the drugs. The numbers of the preparations are shown for each condition.

cal stimulation in each drug concentration (Fig. 2). Other calmodulin antagonists (W-7, calmidazolium, chlorpromazine, fluphenazine) also inhibited the pressor responses to either nerve stimulation or exogenous norepinephrine (Fig. 2).

The changes of the norepinephrine release in the presence of calmodulin antagonists are shown in Fig. 3. As in the case of the pressor responses, the norepinephrine release during nerve stimulation (15 Hz) was reduced by each calmodulin antagonist (IC₅₀ value: trifluoperazine 2.6×10⁻⁸ M, W-7 4.4×10⁻⁸ M, calmidazolium 5.8×10⁻⁸ M, chlorpromazine 3.9×10⁻⁷ M, fluphenazine 2.4×10⁻⁸ M).
Calcium has a major role in neurotransmission in the cardiovascular system, especially in neurotransmitter release and in vascular smooth muscle contractions. The importance of Ca\(^{++}\) in neurotransmitter release has been demonstrated by many authors.\(^1\)-\(^3\) The term "stimulus-secretion coupling" suggests that the Ca\(^{++}\)-influx across the cell membrane is an inevitable concomitant of the stimulus, and is necessary for transmitter release. Our previous reports also showed that Ca\(^{++}\)-antagonists, such as verapamil and diltiazem, affected both pre- and post-synaptic sites of resistance vessels and caused a decrease in electrically-stimulated norepinephrine release from the adrenergic nerve endings distributed in rat mesenteric arteries, in addition to direct effects on vascular smooth muscle.\(^{11},^{12}\)

It is generally accepted that calmodulin is an intracellular Ca\(^{++}\)-binding protein that modulates the effects of Ca\(^{++}\) on several important enzyme systems. The present study with calmodulin antagonists\(^{13},^{14}\) is an attempt to evaluate the contributions of endogenous calmodulin to both norepinephrine release from adrenergic nerve endings and vascular muscle contractions. In the rat mesenteric vasculature, different classes of calmodulin antagonists inhibited norepinephrine release and vascular responsiveness to endogenous and exogenous norepinephrine in a dose-dependent manner.

It has been reported that antipsychotic drugs such as trifluoperazine, fluphenazine and chlorpromazine bind to calmodulin. However, Norman et al reported that correlation between the activity of these drugs as calmodulin antagonists and as antipsychotics is poor, suggesting the involvement of other non-specific actions of the drugs.\(^{15}\) In contrast, W-7 and calmidazolium have potent inhibitory actions on phosphodiesterase activity. In particular, W-7 may penetrate the cell membranes quickly to enter the cytoplasm,\(^{16}\) and seems to be the most specific antagonist for calmodulin.\(^{13},^{14},^{17}\) Asano et al reported that W-7 and other calmodulin antagonists reduced the contractile responses of aortic strips to norepinephrine, serotonin, histamine, prostaglandin F\(_2\alpha\), angiotensin II or KCl.\(^{17}\) On the other hand, hormonal release is analogous with the electromechanical coupling that is characteristic of the muscle contraction.\(^{9}\) Kenigsberg et al reported that trifluoperazine inhibited the acetylcholine- or KCl-induced release of norepinephrine from cultured adrenal chromaffin cells.\(^{18}\) However, few studies have investigated the effects of calmodulin antagonists on norepinephrine release from sympathetic nerve endings in the vascular beds. In this study, direct measurements suggested that W-7 and other calmodulin antagonists could suppress electrically-stimulated norepinephrine release from the nerve terminals in
isolated mesenteric vasculature preparations. Therefore, calmodulin may be involved in the process of adrenergic transmission in vascular beds. There are several possible mechanisms of actions of calmodulin in neurotransmitter release.\(^{19}\) In vascular smooth muscle cells, the activation of myosin by actin is mediated by calmodulin, and the phosphorylation of myosin light chain kinase is a calmodulin-dependent process.\(^{4-6}\) Also in pre-synaptic neurons, calmodulin may mediate the effects of calcium on synaptic protein phosphorylation.\(^{7,20,21}\) DeLorenzo et al also suggested a role of calmodulin in vesicle (containing neurotransmitters)-cytoplasmic membrane interactions.\(^{7}\) Furthermore, calmodulin may regulate endogenous tubulin kinase activity in pre-synaptic nerve terminals,\(^{22}\) although the precise role of calmodulin in regulating many of the aspects of synaptic function remains to be identified.

Acknowledgments

This study was supported in part by Grant-in-Aids for Scientific Research (60570404, 6040049) from the Ministry of Education, Science and Culture of Japan, and by the Nanki Scholarship Fund. We thank Seiko Tsuda for her special assistance.

References

2. Quastel DMJ, Hackett JT, Cooke JD: Calcium; is it required for transmitter secretion? Science 172: 1034, 1971


15. Norman JA, Drummond AH, Moser P: Inhibition of calcium-dependent regulator-stimulated phosphodiesterase activity by neuroleptic drugs is unrelated to their clinical efficacy. Mol Pharmacol 16: 1089, 1979


22. DeLorenzo RJ, Gonzalez B, Goldenring J, Bowling A, Jacobson R: Ca\textsuperscript{2+}-calmodulin tubulin kinase system and its role in mediating the Ca\textsuperscript{2+} signal in brain. Prog Brain Res 56: 257, 1982