Mass Analysis of 1,2-Diacylglycerol in Cultured Rabbit Vascular Smooth Muscle Cells
Comparison of Stimulation by Angiotensin II and Endothelin

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We compared the effects of angiotensin II and endothelin on mass levels of 1,2-diacylglycerol, an endogenous activator of protein kinase C, in cultured rabbit vascular smooth muscle cells with the effects of these vasoconstrictors on contractile responses of rabbit aortic strips. At a high concentration (1 μM), both angiotensin II and endothelin induced a biphasic formation of 1,2-diacylglycerol with an early transient phase and a late sustained phase. At this high concentration, angiotensin II caused a transient contraction followed by a gradual relaxation, whereas endothelin caused a slowly developing and sustained contraction. At a low concentration (EC₅₀ for the early phase of 1,2-diacylglycerol formation), angiotensin II also induced a biphasic formation of 1,2-diacylglycerol and caused a transient contraction, but endothelin induced a monophasic formation of 1,2-diacylglycerol with only an early peak. Despite a rapid decrease of 1,2-diacylglycerol, endothelin at this low concentration still caused a sustained contraction. At both the high and low concentrations, the 1,2-diacylglycerol level was sustained higher for angiotensin II, whereas the tension during the late tonic phase of contraction was greater for endothelin. These results suggest that the unique persistent nature of endothelin-induced contraction is not attributed simply to the stimulatory effect of this peptide on the 1,2-diacylglycerol/protein kinase C pathway.

In vascular smooth muscle cells (VSMC), several vasoconstrictors such as angiotensin II, vasopressin, and noradrenalin (α₁-action) have been shown to induce the phospholipase C-mediated hydrolysis of phosphoinositides. This agonist-induced phospholipase C reaction results in the generation of two intracellular messengers, inositol 1,4,5-trisphosphate (IP₃) and 1,2-diacylglycerol (DAG) (for reviews, see References 5–7). IP₃ mediates intracellular mobilization of Ca²⁺ and plays a crucial role in the early transient phase of vasoconstrictor-induced contraction of VSMC, whereas DAG activates protein kinase C. The definitive roles of protein kinase C in contraction of VSMC are not clear at present, but it has been proposed that protein kinase C is involved in the late tonic phase of vasoconstrictor-induced contraction of VSMC (for review, see Reference 8).

The novel potent vasoconstrictor peptide endothelin has been isolated from the conditioned medium of cultured porcine aortic endothelial cells. Endothelin causes a slowly developing and sustained contraction of vascular strips, which is unique among endogenous vasoconstrictors. Although specific binding sites for endothelin have been found on the surface of VSMC, the molecular mechanism of the action of endothelin is at present controversial. Originally, endothelin was proposed to be an endogenous agonist for voltage-dependent Ca²⁺ channels because it shares structural homologies with neurotoxins, which affect ion channels, and its action can be inhibited by Ca²⁺ channel antagonists. Subsequently, this peptide was shown to induce the phospholipase C-mediated hydrolysis of phosphoinositides. Moreover, recent studies have suggested that protein kinase C plays a
more important role in the endothelin-induced contraction than in the other vasoconstrictor-induced contractions.\textsuperscript{14,16,17} Indeed, protein kinase C-activating phorbol esters cause a slowly developing and sustained contraction of vascular strips, which is very similar to the endothelin-induced contraction.\textsuperscript{16,19}

As the first step in investigating the role of protein kinase C in the contraction of VSMC, it is important to examine the changes of the DAG level in VSMC during the action of vasoconstrictors because the activity of protein kinase C is regulated by DAG.\textsuperscript{5,6} However, all of the previous studies on the changes of the DAG level in VSMC have used cells labeled with radioactive fatty acids and thus provided only qualitative information.\textsuperscript{12,17,20} Preiss et al.\textsuperscript{21} have recently reported a highly sensitive radioenzymatic assay capable of quantitating the absolute mass of DAG present in crude lipid extracts of tissue culture cells. In the present studies, we have used this radioenzymatic assay to examine quantitative changes in the mass level of DAG after stimulation of VSMC with vasoconstrictors. We have compared the effects of angiotensin II and endothelin on the DAG levels to test the hypothesis that, as compared with other vasoconstrictors, endothelin induces the activation of the DAG/protein kinase C pathway with unique potency, resulting in a persistent contraction.

\section*{Methods}

\section*{Materials and Chemicals}

Normal rat kidney cells were kindly supplied by Dr. E.M. Scolnick (Merck Sharp \& Dohme Research Labs., West Point, Pennsylvania). The method of isolation of VSMC from the thoracic aortas of male Japanese White rabbits has been described previously.\textsuperscript{22} Angiotensin II was a generous gift from CIBA-GEIGY Ltd. (Basel, Switzerland). Synthetic porcine endothelin was obtained from Peptide Institute Inc. (Minoh, Japan). A radioenzymatic assay kit for DAG and $[\gamma^32P]_{\text{ATP}}$ (2 Ci/mm) were obtained from Amersham (Buckinghamshire, UK). Other materials and chemicals were obtained from commercial sources.

\section*{Assays for 1,2-Diacylglycerol and Lipid Phosphorus}

For analyses of the mass amount of DAG and total lipid phosphorus, VSMC in secondary cultures were seeded in 60 mm dishes at a density of $6 \times 10^5$ cells/dish in Dulbecco’s modified Eagle’s medium (DMEM) containing 10\% fetal calf serum. After 3 days, the cells were washed twice with DMEM and incubated with serum-free DMEM for 48 hours. The cells were washed twice with DMEM and stimulated with angiotensin II or endothelin at 37ºC for the indicated periods of time. The incubation was terminated by aspiration of the culture media and immediate addition of 1 ml cold methanol. The cells were scraped into this methanol and added to 1 ml chloroform. The dishes were washed with an additional 1 ml cold methanol, which was also added to the chloroform. The lips were then extracted by using the method described by Bligh and Dyer,\textsuperscript{23} except that 1 M NaCl was used instead of water. Two aliquots (1.5 ml and 0.2 ml) of the final organic phase (1.9 ml) were dried separately under a stream of nitrogen and used for analyses of the mass amount of DAG and total lipid phosphorus, respectively. The mass amount of DAG was analyzed with the DAG assay kit using the method developed by Preiss et al.\textsuperscript{21} Assays were performed according to the manufacturer’s instructions with thin-layer chromatography to separate phosphatidic acid converted from DAG. Total lipid phosphorus was measured as described by Rouser et al.\textsuperscript{24}

\section*{Contraction Studies}

Thoracic aortas were isolated from male Japanese White rabbits, quickly cleaned of surrounding tissues and cut into 2 mm wide and 20 mm long helical strips with the intima denuded. Contractions were measured as an increase in isometric tension as described previously.\textsuperscript{25} The contractile responses to vasoconstrictors were standardized by tension developed in response to 20 mM KCl to minimize the individual variability in vascular reactivity.

\section*{Statistical Analysis}

All values expressed are mean±SEM. Comparisons between two means were performed with Student’s $t$ test. A value of $p<0.05$ was taken to indicate a significant difference.

\section*{Results}

First, we confirmed the accuracy of our assay method for DAG by quantitating the amount of DAG present in normal rat kidney cells, which had been measured by Preiss et al.\textsuperscript{21} Normal rat kidney cells, cultured under the conditions described previously,\textsuperscript{21} contained $0.42±0.08$ nmol DAG/100 nmol lipid phosphorus (mol \%), which was in accordance with the reported value.\textsuperscript{21} Then, we analyzed quantitative changes in the mass level of DAG after stimulation with either angiotensin II or endothelin in rabbit VSMC. Quiescent cultures of rabbit VSMC contained $0.28±0.02$ mol \% DAG. Stimulation of these cells with angiotensin II (1 \greekmu{}M) induced a biphasic formation of DAG as shown in Figure 1. The early phase was rapid and transient, peaking at 15 seconds. The amount of DAG after a 15-second incubation with angiotensin II was $0.89±0.08$ mol \%, representing a 3.2-fold rise. The late phase reached the maximal level at 5 minutes and decayed slowly. The amount of DAG after a 5-minute incubation with angiotensin II was $1.11±0.10$ mol \%, which was a 4.0-fold increase of the resting level. Endothelin (1 \greekmu{}M) also induced a biphasic formation of DAG with an early phase peaking at 30 seconds and a late phase peaking at 5 minutes. The amounts of DAG 30 seconds and 5 minutes after the addition of endothelin were $0.74±0.08$ and $0.87±0.10$ mol \%, respectively. The DAG levels were consistently higher for
angiotensin II even from 5 minutes to at least 30 minutes after stimulation. Figure 2 shows the dose-response curves of angiotensin II and endothelin for the increase in DAG. EC50 values for the early and late phases of angiotensin II–induced DAG formation were almost the same (53 ± 13 nM vs. 49 ± 20 nM, NS), but those of endothelin-induced DAG formation were significantly different (1.0 ± 1.4 nM vs. 40 ± 16 nM, <0.01).

In the experiment shown in Figure 3, we compared contractile responses of rabbit aortic strips to angiotensin II and endothelin. Angiotensin II (1 μM) caused an early transient contraction reaching a plateau at 5 ± 1 minute and then slowly relaxing to a late tonic phase, whereas endothelin (1 μM) caused a slowly developing and sustained contraction that reached a stable plateau at 33 ± 4 minutes and lasted for at least 1 hour. Under these conditions, the maximum tension reached and the tension during the late tonic phase of contraction were consistently greater for endothelin.

In the experiments shown in Figures 1 and 3, we used maximal concentrations of angiotensin II and endothelin to compare the maximal effects of these two vasoconstrictors on both DAG formation and contraction. In the experiments shown in Figure 4, we compared the effects of angiotensin II and endothelin at their EC50 for the early phase of DAG formation. The time course of an angiotensin II (50 nM)–induced biphasic formation of DAG was similar to that of the angiotensin II (1 μM)–induced reaction. Angiotensin II at this low concentration also caused an early transient contraction of aortic strips that reached a plateau at 4 ± 1 minute and was followed by a gradual relaxation. Endothelin (1 nM) induced an early phase of DAG formation with a peak at 30 seconds. However, a late phase of DAG formation was not detected and the DAG level returned to nearly the basal level within 10 minutes. Despite the rapid decrease of DAG, endothelin at this low concentration still caused a slowly developing and sustained contraction of aortic strips. Under these conditions, the tension during the late tonic phase of contraction was greater for endothelin, although the maximum tension reached was greater for angiotensin II.

Discussion

Using VSMC labeled with [3H]arachidonic acid, Griendling et al20 showed that angiotensin II induces a biphasic formation of DAG with an early transient
smooth muscle cells (VSMC) were stimulated by angiotensin II. Induced by low concentrations of angiotensin II and endothelin, DAG formation and contractile responses to angiotensin II (50 nM) or endothelin (1 nM) were measured and standardized as described in Methods. Results are presented as the mean±SEM of four separate experiments. (●—●) angiotensin II; (O—O) endothelin. *Significantly greater than endothelin (p<0.05). Panel B: Contractile responses. Contractile responses of helical strips to angiotensin II (50 nM) or endothelin (1 nM) were measured and standardized as described in Methods. Results are presented as the mean±SEM of six separate experiments. (●—●) angiotensin II; (O—O) endothelin. *Significantly greater than endothelin (p<0.01). †Significantly greater than angiotensin II (p<0.01).

Figure 4. Line graphs showing time courses of 1,2-diacylglycerol (DAG) formation and contractile responses induced by low concentrations of angiotensin II and endothelin. Panel A: DAG formation. Quiescent cultures of vascular smooth muscle cells (VSMC) were stimulated by angiotensin II (50 nM) or endothelin (1 nM) for various periods of time at 37°C. Mass level of DAG was assayed as described in Methods. Results are presented as the mean±SEM of six separate experiments. (●—●) angiotensin II; (O—O) endothelin. *Significantly greater than endothelin (p<0.01). †Significantly greater than endothelin (p<0.005). Panel B: Contractile responses. Contractile responses of helical strips to angiotensin II (50 nM) or endothelin (1 nM) were measured and standardized as described in Methods. Results are presented as the mean±SEM of six separate experiments. (●—●) angiotensin II; (O—O) endothelin. *Significantly greater than endothelin (p<0.01). †Significantly greater than angiotensin II (p<0.01).

endothelin as well as angiotensin II induces a biphasic formation of DAG in this cell type.

Griendling et al have also shown that angiotensin II induces the sequential phospholipase C–mediated hydrolysis of, initially, phosphatidylinositol 4,5-bisphosphate (PIP2) and phosphatidylinositol 4-monophosphate (PIP) and, subsequently, phosphatidylinositol (PI) in VSMC and have suggested that the early phase of DAG is derived from the hydrolysis of PIP2 and PIP and the late phase of DAG from the hydrolysis of PI. In the present studies, we have shown that endothelin also induces a biphasic formation of DAG. In our previous report, we showed that endothelin induces, though to a lesser extent than angiotensin II, the phospholipase C–mediated hydrolysis of phosphoinositides in a time course similar to that of the angiotensin II–induced reaction. The time courses and the dose-response curves for the two vasoconstrictors of the early phase of DAG formation and the hydrolysis of PIP2 and PIP are similar, supporting the idea that the early transient phase of DAG is a result of hydrolysis of PIP2 and PIP. The time courses of the late sustained phase of DAG formation and the hydrolysis of PI are also similar in both angiotensin II– and endothelin-stimulated VSMC. Moreover, the concentrations of angiotensin II necessary for the late sustained phase of DAG formation and the formation of inositol monophosphate are similar (data not shown). These results support the idea that the late phase of DAG is derived from the hydrolysis of PI. However, the EC50 value of endothelin for the late sustained phase of DAG formation is about 40 nM, whereas that for the formation of inositol monophosphate is about 1 nM. Together with the recent observations in other systems that the phospholipase C–mediated hydrolysis of phosphoinositides cannot account for all of the DAG formed during the action of agonists, these findings suggest that a large part of the late sustained phase of DAG, at least in the case of endothelin-stimulated cells, results from reactions other than the phospholipase C–mediated hydrolysis of PI. It is also possible that the sources of the late sustained phase of DAG formation are different between endothelin and angiotensin II. The sources of the vasoconstrictor-induced late sustained phase of DAG remain to be clarified.

Vascular contraction caused by traditional vasoconstrictors is composed of two phases: an early transient phase and a late tonic phase. The early transient phase is induced mainly by the IP3/Ca2+ pathway. Based on the observation that protein kinase C–activating phorbol esters cause a slowly developing and prolonged contraction of vascular strips, it has been proposed that protein kinase C plays a major role in the late tonic phase of vasoconstrictor-induced contraction of VSMC. Our present finding that DAG levels are sustained above the basal level for at least 30 minutes after stimulation by either angiotensin II (1 μM) or endothelin (1 μM) is consistent with this proposal. However, the
unique persistent nature of the endothelin-induced contraction suggests that the mechanism of action of this peptide may be different from that of traditional vasoconstrictors. Recently, there has been increasing evidence suggesting that protein kinase C plays a major role in the vasoconstricting action of endothelin.14,16,17 We have compared the effects of endothelin and angiotensin II at the maximal and half maximal concentrations for the early phase of DAG formation. At both concentrations, angiotensin II induces a biphasic formation of DAG in VSMC and causes a transient contraction of aortic strips. Endothelin at the maximal concentration also induces a biphasic formation of DAG in VSMC. Endothelin at the EC50 for the early phase of DAG formation, however, induces only the early phase of DAG formation. Endothelin at this low concentration still causes a slowly developing and sustained contraction of aortic strips. At both concentrations, the DAG levels are consistently greater for angiotensin II from 5 minutes after stimulation, although the isometric tension during the late tonic phase of contraction is consistently greater for endothelin. These results do not support the hypothesis that endothelin activates the DAG/protein kinase C pathway with unique potency resulting in the unique persistent nature of its contraction. Further studies are needed to clarify the molecular basis for the unique persistent nature of the endothelin-induced contraction.

References


Key Words: angiotensin II • endothelin • vascular smooth muscle cells • diacylglycerol
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