EFFECTS OF E-1020, A NEW CYCLIC AMP-SPECIFIC PHOSPHODIESTERASE INHIBITOR, ON CYCLIC AMP AND CYTOSOLIC FREE CALCIUM OF CULTURED VASCULAR SMOOTH MUSCLE CELLS

MICHIKUNI OHOKA, M.D., MASAOKI HONDA, M.D., SHIGEFUMI MORIOKA, M.D.
SHIGENORI ISHIKAWA, M.D., KAZUAKI NAKAYAMA, M.D.
YUKIO YAMORI, M.D., AND KATSUTOSHI MORIYAMA, M.D.

The purpose of this study is to clarify the pharmacological effects of E-1020, a new cAMP-specific phosphodiesterase (PDE) inhibitor, on cAMP or cytosolic free calcium (Ca) of cultured vascular smooth muscle cells (VSMC). VSMC were separated from the thoracic aorta of 2- to 3-month-old Wistar Kyoto rats (WKY). cAMP was measured by using the ^{125}I -cAMP radioimmunoassay kit. The cytosolic free Ca of VSMC was measured by using the fluorescence Ca indicator, Fura-2/acetoxymethyl ester (Fura-2/AM). As a result, E-1020 increased the cAMP of VSMC in a dose-dependent manner, and about two fold increase in cAMP was observed by 10^{-4}M E-1020. E-1020 decreased the cytosolic free concentration of Ca in a dose-dependent manner, and a significant decrease in cytosolic free Ca was observed by $>10^{-6}\text{M}$ E-1020. These effects of E-1020 on cAMP and cytosolic free Ca were enhanced in the presence of low concentration (10^{-8}M) of DL-noradrenaline. That is, a more prominent decrease in cytosolic free Ca than that of E-1020 alone was observed. Our results show that E-1020 decreases cytosolic free Ca of VSMC in parallel with increase in cAMP, and thereby suggest a potential vasodilating activity in vivo.

RECENTLY a new group of agents, phosphodiesterase (PDE) inhibitors, has been developed, and clinically and pharmacologically investigated to evaluate their therapeutic potentials for treatment of congestive heart failure. Common properties of these agents are positive inotropic effects on the heart and vasodilating activity!^{1,2} The combination of inotropic effects and after-load reduction for treatment of congestive

heart failure has proven to be of value³ and therefore much interest has focused on these agents!^{1,2,4-10} The mechanism of action of these agents is inhibition of PDE with an increase in cAMP. Cytosolic free Ca are thought to be increased via a cascade of reactions in the heart resulting in increased contractility of the heart!^{1,11-13} On the other hand, cytosolic free Ca of vascular smooth muscle cells (VSMC) are thought to be decreased resulting in vasodilation!¹⁴⁻¹⁹ However, there is no direct evidence that PDE inhibitor decreases cytosolic free Ca of VSMC. Moreover, other mechanisms which may play an

Key words:

E-1020
Cyclic AMP-specific phosphodiesterase inhibitor
Cultured vascular smooth muscle cell
Cytosolic free calcium

(Received May 19, 1989; accepted December 4, 1989)

Department of Internal Medicine, *Department of Pathology Shimane Medical University, Izumo, Shimane, Japan
Mailing address: Michikuni Ohoka, M.D., The 4th Department of Internal Medicine, Shimane Medical University, 89-1, Enya-cho, Izumo, Shimane 693, Japan

Molecular formula: $C_{14}H_{10}N_4O \cdot HCl \cdot H_2O$

Molecular weight : 304.81

Fig.1. Chemical structure of E-1020.

additional role may exist; therefore the exact mechanisms of action of these agents on the heart and vascular system are not well clarified yet.

E-1020 (1, 2-dihydro-6-methyl-2-oxo-5-(imidazo [1, 2- α] pyridin-6-yl)-3-pyridine carbonitrile hydrochloride monohydrate) is a new cAMP-specific PDE inhibitor, and is thought to have positive inotropic effects on the heart and vasodilating activity. In the present study we focused on its vasodilating activity, and investigated its pharmacological effects on VSMC by using cultured VSMC obtained from the thoracic aorta of Wistar-Kyoto rats (WKY) and the Ca-sensitive indicator, Fura-2/acetoxymethyl ester Fura-2/AM. To our knowledge, this is the first paper to examine the effect of a cAMP-specific PDE inhibitor on the kinetics of cytosolic free Ca of VSMC by Fura-2/AM and show direct evidence that the cAMP-specific PDE inhibitor, E-1020, decreases cytosolic free Ca in parallel with increasing cAMP content. The possible therapeutic potential of this agent is also discussed in this paper.

MATERIALS AND METHODS

Drugs

E-1020 was kindly provided by Eisai Co., Ltd., Tokyo, Japan. Its chemical structure is shown in Fig. 1. Fura-2/AM was purchased from Wako Chemical, Ltd., Osaka, Japan. Cremophor^R EL was from Nakarai Techque, Inc., Tokyo, Japan. DL-noradre-

naline was from Sigma Chemical Co., ST. Louis, MO 63178, USA. Collagenase was from Worthington Biochemical Co., Freehold, NJ 07728, USA. Trypsin was from Difco Laboratories, Detroit, Michigan, USA. DL-noradrenaline was prepared as $10^{-3}M$ stock solution in 1mM and was diluted by physiological medium X (133 mM NaCl, 3.6 mM KCl, 0.4 mM $MgCl_2$, 16 mM D-glucose, 3 mM HEPES, 1% W/V BSA). Radioimmunoassay kit for cAMP was purchased from Amersham International plc, Amersham, UK.

Cell culture

Primary cultures of VSMC were obtained from WKY (2 to 3 months old) by the explantation method of Ross with some modifications.²⁰⁻²² 1 – 2 mm² sections of tunica media were then transferred into tissue culture flasks (Falcon) containing Medium 199 with 10% FCS. These flasks were incubated at 37°C in a 95% air/5% CO₂ humid atmosphere. After 3- to 4-week-incubation, VSMC were migrating confluent. Then these VSMC were trypsinized for 5 min at 37°C with PBS (-) containing 0.25% trypsin. After adjusting cell concentration to $5 \times 10^5/ml$, VSMC were subcultured in Medium 199 with 10% FCS. The medium was exchanged every 3 days. After repeated passages (Passage 3–9), cultured VSMC were used for the following experiments.

Measurement of cAMP

Abbreviation used in this paper: cAMP = Adenosine cyclic 3', 5'-monophosphate; FCS = Fetal calf serum; BSA = Bovine serum albumin; PBS=phosphate buffer saline

Fig.2. Cyclic AMP content of VSMC by treatment with E-1020. Cultured VSMC were treated with 10^{-4} M E-1020 for 30 sec, 1, 3, 5, 10, 15 min. Results were expressed as mean % changes $\pm$ SD of 5 different experiments. % changes were calculated by the following formula: % changes = $(A-B)/B \times 100$.
 A: cAMP with 10^{-4} M E-1020 at each time.
 B: cAMP with vehicle at each time (30 sec, 1, 3, 5, 10, 15 min; 19.0 ± 9.1 , 15.3 ± 6.4 , 16.2 ± 6.1 , 17.2 ± 11.3 , 15.8 ± 5.3 , 17.4 ± 7.6 pmol/mg protein).

Fig.3. Dose response of cAMP content. Cultured VSMC were treated with various concentrations of E-1020 (10^{-10} - 10^{-4} M). Results were expressed as mean % changes $\pm$ SD of 5 different experiments. % changes were calculated by the following formula: % changes = $(C-D)/D \times 100$.
 C: cAMP with various concentration of E-1020
 D: cAMP with vehicle (17.2 ± 11.3 pmol/mg protein).

The cAMP content of VSMC was determined by a radioimmunoassay kit. Briefly, VSMC were plated in 35 mm tissue culture dishes at a cell density of 10^5 /ml (2×10^5 cells/dish). After 7 days, cells reached confluence, and then were washed twice with assay buffer 1 (133 mM NaCl, 3.6 mM KCl, 1.0 mM CaCl_2 , 0.3 mM MgCl_2 , 16 mM D-glucose, 3 mM HEPES, pH 7.4). Various concentrations of drugs were added, and incubated for 30 sec, 1, 3, 5, 10, 15 min at

37°C . After washing twice with ice-cold assay buffer 1, 0.3 N perchloric acid was added to plates, which were then left for 30 min on ice. Then cells were scrubbed with a rubber policeman, and transferred to tubes, and centrifuged at 2000 rpm for 15 min at 4°C . The extracts were stored at -20°C until assay. Before assay, 1 ml of extract was mixed with 0.1 ml of 3N KHCO_3 to neutralize extracts, and acetylated and assayed in duplicates.

Fig.4. Cytosolic free calcium of VSMC by treatment with E-1020. Fura-2/AM loaded VSMC (10^6 cells) were treated with 10^{-5} M E-1020, then cytosolic free Ca was monitored for over 420 sec. Concentration of free Ca in the presence of vehicle at each time was similar, therefore % changes were calculated by the following formula: % changes = $(E-F) / F \times 100$.

E: $[Ca^{2+}]_i$; concentration of cytosolic free Ca after treatment with 10^{-5} M E-1020. t: time

F: $[Ca^{2+}]_i$; concentration of cytosolic free Ca before treatment with E-1020. Concentration of free Ca before treatment with E-1020 of 5 different experiments was 146.5 ± 7.9 nM. Results were expressed as mean % changes $\pm$ SD of 5 different experiments.

Measurement of cytosolic free calcium

VSMC cultured to reach confluence in 75 cm² flasks were incubated in serum-free medium for 24–48 hours before experiments. VSMC were trypsinized for 5 min at 37°C by PBS(-) with 0.125% trypsin. VSMC were washed once with medium X. After adjusting cell concentration to 10^6 /ml in medium X, 5 μ l of 1 mM Fura-2/AM and 2 μ l of Cremophor^R EL were added to 1ml of cell suspension (10^6 cells), and incubated for 30 min at 37°C with continuous gentle shaking. After washing cells once with medium X, Fura-2/AM loaded cell suspensions were kept on ice until they were employed in measurement of Ca^{2+} -Fura-2 fluorescence. Fluorescence was recorded with a Hitachi F-4000 Fluorescence spectrophotometer (Hitachi, Ltd, Tokyo, Japan). For standard setting excitation, 340 and 380 nm wavelengths of light were used; standard emission wave was 505 nm. One ml aliquot of Fura-2/AM loaded cell suspension was placed in a cuvette and was stirred continuously with a magnetic stir bar. The temperature was maintained at 37°C. Cytosolic free Ca concentrations ($[Ca^{2+}]_i$) were obtained by the method described by Grynkiewicz et al, with some modifications^{21–23}

Protein assay

Protein concentration of extracts from VSMC for the assay of cAMP was measured by the Folin-Lowry method²⁴

Data analysis

All data are expressed as mean $\pm$ SD. Student's t test was applied for the statistical analysis.

RESULTS

Time course study of cAMP content

Time course study for cAMP content of VSMC was carried out before evaluation of the effect of various concentration of E-1020 on cAMP content of VSMC (Fig. 2). That is, VSMC were treated with 10^{-4} M E-1020 for 30sec, 1, 3, 5, 10, 15 min. Then cAMP content of VSMC was measured. As a result, treatment of VSMC with E-1020 for 30 sec–5 min increased cAMP content by 80–90% compared with control (treatment with vehicle). However, cAMP content treated with E-1020 for more than 5 min resulted in gradual decrease, and returned to almost control level by the treatment of VSMC with E-1020 for 15 min.

Dose response of cAMP content

Fig.5. Dose response of cytosolic free calcium. Fura-2/AM loaded VSMC (10^6 cells) were treated by various concentrations of E-1020 (10^{-10} – 10^{-5} M), then cytosolic free Ca was measured. Results were expressed as mean % changes $\pm$ SD of 5 different experiments. % changes were calculated by the following formula: $(G-H)/H \times 100$.

G: $[Ca^{2+}]_i$ 30 sec; concentration of cytosolic free Ca after treatment with various concentrations of E-1020.

H: $[Ca^{2+}]_i$ 0; concentration of cytosolic free Ca before treatment with E-1020. Concentration of free Ca before treatment with E-1020 of 5 different experiments was 140.5 ± 17.1 nM.

According to the above results, cAMP content of VSMC in response to treatment of various concentrations of E-1020 was examined by treatment of VSMC with E-1020 for 5 min. As shown in Fig. 3, cAMP content of VSMC increased in a dose-dependent manner. Increment of cAMP of VSMC by treatment of 10^{-5} M, 10^{-4} M E-1020 was 72.5%, 90.3%, respectively. The cAMP content of VSMC treated with more than 10^{-5} M E-1020 was significantly higher than that of control ($p < 0.05$). These results, shown in Fig. 2 and Fig. 3, demonstrate that E-1020 increases cAMP content of VSMC.

Time course study of cytosolic free calcium

Then we examined whether or not E-1020 can affect cytosolic free Ca of VSMC. At first, cytosolic free Ca of VSMC treated with 10^{-5} M E-1020 was monitored for over 420 sec by a fluorescence spectrophotometer after loading Fura-2/AM as described on materials & methods. As a result, cytosolic free Ca of VSMC decreased rapidly after addition of 10^{-5} M E-1020, and reached to the lowest level at 30 sec, then the level of cytosolic free Ca gradually returned to control level. The cytosolic free Ca treated with E-1020 for 30 sec was decreased

about 30% compared with that of control (Fig. 4).

Dose response of cytosolic free calcium

Then we examined dose response of cytosolic free Ca of VSMC in response to treatment of various concentrations (10^{-10} – 10^{-5} M) of E-1020 (Fig. 5). Cytosolic free Ca of VSMC decreased in a dose-dependent manner. The cytosolic free Ca treated with more than 10^{-6} M E-1020 was significantly lower than that of control ($p < 0.05$). Percent decrement of the cytosolic free Ca of VSMC treated with 10^{-6} M and 10^{-5} M E-1020 was 21.7%, 31.2%, respectively.

Changes of cytosolic free calcium in the presence of noradrenaline

So far we examined the single effect of E-1020 on cAMP content and cytosolic free Ca of VSMC. Plasma norepinephrine level is one of the factors which modulates tension and contraction of vascular smooth muscle in vivo. Moreover, plasma norepinephrine increases in congestive heart failure. Therefore, we also examined the effect of E-1020 on cytosolic free Ca in the presence of low concentration of DL-noradrenaline (Fig. 6). That is, 5 min after addi-

Fig.6. Changes of cytosolic free calcium in the presence of noradrenaline. Fura-2/AM loaded cultured VSMC (10^6 cells) were pretreated by 10^{-8} M DL-noradrenaline(NA) for 5 min, then E-1020 or vehicle was added to cell suspension, and cytosolic free Ca was monitored for over 300 sec. Results were expressed as mean % changes $\pm$ SD of 5 different experiments. % changes were calculated by the following formula: % changes = $(I - J) / J \times 100$.

I: $[Ca^{2+}]_i$; concentration of cytosolic free Ca in the presence of 10^{-8} M NA + 10^{-7} M E-1020 or 10^{-8} M NA + distilled water (DW). t: time.

J: $[Ca^{2+}]_i$; concentration of cytosolic free Ca 5 min after addition of 10^{-8} M NA. Concentration of free Ca in the presence of NA before treatment with E-1020 of 5 different experiments was 163.4 ± 20.4 nM.

*: significantly different compared with that treated with vehicle ($p < 0.05$).

tion of 10^{-8} M DL-noradrenaline to cell suspension of VSMC, a stable condition, in which cytosolic free Ca tended to be slightly higher than that without noradrenaline (146.2 ± 12.2 vs. 163.4 ± 20.4 nM), was obtained. Then 10^{-7} M E-1020 or vehicle (distilled water) was added. Thereafter, cytosolic free Ca was monitored for over 300 sec as shown in Fig. 6. As a result, E-1020 (10^{-7} M), which alone decreased cytosolic free Ca by 16.6% (Fig. 5), decreased cytosolic free Ca by 38.7%. The decrement of Ca of VSMC was augmented by about 20% in the presence of 10^{-8} M DL-noradrenaline. As for cAMP content of VSMC in 5 min after addition of E-1020 in the presence of low concentration (10^{-8} M) of noradrenaline, a representative data showed that is tended to be augmented compared with that without noradrenaline (10^{-7} M E-1020 alone vs 10^{-8} M noradrenaline + 10^{-7} M E-1020: 22.2 ± 7.0 vs 24.4 ± 12.1 pmol/mg protein). These results show that the effect of E-1020 on cytosolic free Ca was augmented in the presence of low concentration of noradrenaline, which may be a more physiological condition.

DISCUSSION

The development and investigation of new inotropic agents, PDE inhibitors, for treatment of congestive heart failure is a relatively new and evolving field. While many relevant aspects of the pharmacokinetics and pharmacodynamics of these agents have been clarified^{1,2} much study remains to be done. E-1020 is a new cAMP-specific PDE inhibitor, and has a vasodilating activity, in addition to its inotropic effects on the heart^{25,26}. In the present study we focused on the vasodilating effect of this agent and examined the mechanisms for vasodilation by using cultured rat VSMC. It was found that, E-1020 increased intracellular cAMP in a dose-dependent manner, and a significant increase in cAMP was observed by $>10^{-5}$ M E-1020. Moreover, cytosolic free Ca was decreased in a dose-dependent manner, and a significant decrease in cytosolic free Ca was observed by $>10^{-6}$ M E-1020. Our results suggest that E-1020 decreases cytosolic free Ca in parallel with increasing in intracellular cAMP. As for the mechanisms for vasodilation by PDE inhibitors, the

following possibilities are postulated. (1) inhibition of Ca uptake, (2) stimulation of Ca efflux, (3) enhancement of Ca transport into sarcoplasmic reticulum and other Ca-storage sites, (4) phosphorylation of myosin light chain kinase with concomitant low sensitivity of myosin light chain kinase to Ca-calmodulin complex. We can not clarify the exact mechanisms for decrement of cytosolic Ca. Further studies are necessary to clarify the exact mechanisms for decrement of cytosolic free Ca by E-1020.

The significant changes of cAMP and cytosolic free Ca of VSMC were obtained by rather high concentrations of E-1020 compared with pharmacologically effective dose in vivo²⁶ As for this point, multiple molecular forms of PDE have been indentified in a number of tissues of different animals and human²⁷ Moreover, recent studies have shown that functional subclasses of cAMP-specific PDE exist in ventricular muscle and that intracellular localization of subclass of cAMP-specific PDE may relate to the degree of positive inotropic response to PDE inhibitors^{28,29} Therefore, the following possibilities for the different effective doses between in vivo and in vitro are speculated: (1) In the present study we used rat VSMC, so the difference of effective doses of E-1020 may be related to species difference. (2) Changes in cAMP or cytosolic free Ca of VSMC in response to E-1020 might be related to the existence and/or intracellular localization of subclass of cAMP-specific PDE ever though the existence of this subclass has not been clarified. Another possibility for the difference of effective doses between this in vitro study and an in vivo study is that tension of vascular smooth muscle in vivo is modified by catecholamine and other vasoactive substances, and that in those physiological conditions lower concentrations of E-1020 may be effective on the changes of cAMP and cytosolic free Ca. To examine this possibility, we looked at the effect of E-1020 on the change of cytosolic free Ca of VSMC in the presence of low concentration of noradrenaline (Fig. 6). We found that, in the presence of 10^{-8} M noradrenaline, cytosolic free Ca of VSMC decreased more prominently than that treated with 10^{-7} M E-1020 alone, which is 10 times lower concentration than

that of single effective dose of E-1020 (i.e. 10^{-6} M) on cytosolic free Ca of VSMC. Plasma catecholamine level in patients with congestive heart failure is high³⁰⁻³⁵ and therefore, in such conditions E-1020 may exert a more effective vasodilating activity. To evaluate the pharmacological effects of E-1020 and effective dose in vivo, action via endothelium is kept in mind because endothelium releases vasoactive substances in response to stimuli³⁶⁻³⁸ Such study is now in progress in our laboratory.

In summary, the present pharmacological study demonstrates the direct evidence that E-1020, the cAMP-specific PDE inhibitor, decreases cytosolic free Ca by increasing cAMP of VSMC, thereby suggesting a potential vasodilating activity of this agent in vivo. Pharmacological and hemodynamic effects of E-1020 in vivo are also now under investigation in our department.

Acknowledgments

We would like to express our appreciation to Yasuo Nara, PhD in the Department of Pathology in our university for technical assistance and useful discussion.

We also wish to express our appreciation to Kenichi Hayashi, PhD of Eisai Co., Ltd. for useful discussion.

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