ISSN 0253-9756 Acta Pharmacol Sin 中国药理学报 2000 Oct; 21 (10): 871 – 876
http://www.chinaphar.com

Original Research

Prostaglandins but not nitric oxide are endothelium-derived relaxing factors in the trout aorta

Virginia M MILLER[1], Paul M VANHOUTTE[2]
(*Departments of Surgery, and Physiology and Biophysics, Mayo Clinic and Foundation, 200 First Street SW, Rochester, MN 55905, USA*; [2]*Institut De Recherches Internationales Servier, 6, place des Pléiades, 92415 Courbevoie Cedex, France*)

KEY WORDS acetylcholine; calcium; ionophores; *Rana catesbiana*; methylene blue

ABSTRACT

AIM: To identify the type of prostanoids produced by endothelial cells of trout aorta and to determine whether or not the smooth muscle responds to nitric oxide. **METHODS**: Ventral aortas, with and without endothelium from rainbow trout (*S gairdneri*), were incubated in a buffered salt solution. **RESULTS**: Addition of the calcium ionophore A23187 caused a significant increase in prostaglandin E's and a consistent increase in the stable metabolite of prostacyclin (6-keto-prostaglandin $F_{1\alpha}$) in the incubation media only when the endothelium was present. This production was inhibited by methylene blue (10 μmol/L). In rings of trout aorta without endothelium suspended for the measurement of isometric force in organ chambers, prostacyclin and prostaglandin E_1 but not prostaglandin E_2 caused concentration-dependent decreases in tension when the rings were contracted with acetylcholine. The smooth muscle did not relax to nitric oxide but did so to sodium nitroprusside. Relaxations to the latter nitrovasodilator were not inhibited by methylene blue. Descending aorta without endothelium from frogs relaxed in a concentration-dependent manner to nitric oxide. **CONCLUSION**: Predominant endothelium-derived relaxing factors in trout aorta are prostaglandins, the synthesis of which can be inhibited by methylene blue. A phylogenetic appearance of nitric-oxide sensitive mechanism for vasodilatation, perhaps is associated with the transition from water to air respiration.

INTRODUCTION

Unlike mammalian arteries, acetylcholine does not cause endothelium-dependent relaxations in the ventral aorta of the trout[1-3]. However, endothelium-dependent relaxations to the calcium ionophore A23187 are present[1,3]. These relaxations are abolished by inhibitors of cyclooxygenase and methylene blue but not arginine analogs suggesting that prostaglandins and not nitric oxide mediate the endothelium-dependent relaxations to the ionophore in these animals[2,3,6].

The present experiments were designed to determine whether or not prostaglandins are synthesized by the trout aorta and, if so, how the aortic smooth muscle responds to those prostaglandins. Further, experiments were designed to determine whether or not trout aortic smooth muscle relaxes to nitric oxide. Responses to nitric oxide of the frog aorta were obtained for comparison.

METHODS

Animals and tissue preparation The ventral aorta from rainbow trout (*Salmo gairdneri*, 1 kg) and the descending aorta from the American bullfrog (*Rana catesbiana*, 0.2 kg) were used in these experiments.

Trout were obtained from a commercial supplier and used in experiments the day the shipment was received. Experiments were conducted from December through September. Frogs were maintained in the laboratory (22 ℃ – 25 ℃; *ad lib* access to food, water and dry basking area) for one week prior to use.

[1] Project supported in part by grants from the National Institutes of Health HL 31183 and the Mayo Clinic and Foundation.
[2] Correspondence to Dr Virginia M MILLER, PhD.
Phn 1-507-284-2290. Fax 1-507-266-2233.
E-mail miller.virginia@mayo.edu
Received 2000-04-04 Accepted 2000-06-27

ISSN 0253-9756 Acta Pharmacol Sin 中国药理学报 2000 Oct; 21 (10)
E-mail aps@mail.shcnc.ac.cn Phn/Fax 86-21-6474-2629

All animals were killed by pithing. Aortas were removed immediately and placed in chilled (4 ℃) buffered salt solution (mmol/L composition: NaCl 100; $NaHCO_3$ 30; $MgCl_2$ 3; $CaCl_2$ 3; KCl 2; glucose 5.6) control solution[2]. Aortas were cleaned of connective tissue and cut into rings (5 mm in length); care was taken not to touch the intimal surface. In some rings the endothelium was removed deliberately by rubbing gently the luminal surface with a tapered cotton swab wetted with control solution[2]. Rings were then studied in organ chambers either for the determination of prostaglandin-production or for the measurement of isometric force.

Measurement of prostaglandin-synthesis One aorta from a single trout was placed in 2 mL of control solution at 25 ℃ in organ baths and aerated with 95 % O_2 + 5 % CO_2. After a 15 – 20-min incubation period, the fluid was removed and replaced with fresh control solution for an additional 30-min incubation. Acetylcholine (0.3 μmol/L) was then added to the organ bath for 5 min followed by the addition of calcium ionophore A23187 (0.3 μmol/L) for 10 min. These incubation conditions duplicated those in which relaxations to the ionophore were observed[2]. Some tissues were incubated with methylene blue (10 μmol/L) during the 30-min incubation period. The incubation fluid was then collected in a polystyrene tube and frozen for subsequent analysis. Aorta with and without endothelium from different animals in the absence and presence of methylene blue, stimulated with acetylcholine or a combination of acetylcholine plus A23187, were studied in parallel.

Prostaglandins of the E series and 6-keto prostaglandin $F_{1\alpha}$ were measured by radioimmunoassay (NEN Research Products, DuPont Co, Wilmington, DE[4]). In preliminary experiments, it was found that acetylcholine, A23187 and methylene blue did not interfere with the assay for the measurement of the prostaglandins.

Measurement of isometric force Rings of trout or frog aorta without endothelium were suspended in an organ chamber between a clip and a force transducer (Gould UTC-2) for measurement of isometric tension by two stainless steel wires (0.008 in) inserted into the lumen of the vessel. The organ chambers were filled with control solution bubbled with 95 % oxygen and 5 % carbon dioxide at 25 ℃. Each ring was stretched to an initial tension of 500 mg and allowed to equilibrate for 60 min. If rings developed spontaneous tone, all subsequent drug-induced changes in tension were measured from the tension attained after the equilibration period[2]. Rings were then contracted with acetylcholine (0.3 μmol/L, trout aorta) or norepinephrine (0.3 μmol/L, frog aorta). Once the contraction stabilized, the calcium ionophore (0.3 μmol/L) was added to the chamber. The removal of the endothelium was confirmed by the absence of a relaxation to the ionophore. Rings were washed with control solution and once tension returned to resting levels, rings were incubated with indomethacin (10 μmol/L) or with indomethacin plus methylene blue (10 μmol/L) for 40 min. Rings were then contracted with acetylcholine (trout) or norepinephrine (frog) and once the contractions stabilized, cumulative concentration-response curves were obtained to either nitric oxide, sodium nitroprusside, prostaglandin I_2, E_1, or E_2.

Chemicals and drugs The following drugs were used (Sigma Chemical Co, St Louis, MO): acetylcholine chloride; calcium ionophore A23187; dimethylsulfoxide (Me_2SO); indomethacin; methylene blue; *L*-norepinephrine bitartrate; prostaglandin E_1, E_2, and I_2; sodium nitroprusside. The calcium ionophore was dissolved in Me_2SO (final bath concentration 8 mmol/L); indomethacin was dissolved Na_2CO_3 (final bath concentration 20 μmol/L); prostaglandin E's were dissolved in 40 % ethanol (final bath concentration 0.2 %) and diluted with distilled water. The tissues did not demonstrate responses to the solvents alone. All other drugs were dissolved in distilled water.

To make the nitric oxide solution, a glass bulb, fitted with a silicon injection septum, was filled with nitric oxide from a cylinder (Union Carbide, Chicago, IL). A volume of nitric oxide was removed with a gas syringe and injected into a second glass bulb containing 100 mL distilled water which had been bubbled with helium for approximately 3 h. Using this procedure, stock solutions of nitric oxide were obtained at concentrations of 40 μmol/L, 0.4 mmol/L, and 4 mmol/L[5].

Calculations and statistical analysis All data are expressed as $x \pm s_x$; n equals the number of animals studied. For organ chamber studies, data are expressed as a percent change in tension from the contraction to acetylcholine (trout) or norepinephrine (frog). Statistical evaluation of the data was by Student's t-test for either paired or unpaired observations. When more than two means were compared, a one-way analysis of variance was used. If a significant F value was found, Scheffe's test for multiple comparisons was used to identify differences among means. Values were considered to be statistically different when P was less than 0.05.

RESULTS

Production of prostaglandins In trout aorta with endothelium, the calcium ionophore A23187 increased significantly the production of prostaglandins of the E series (Fig 1). Methylene blue inhibited the increase in prostaglandin E's (Fig 1) without altering their production when incubated with tissues in the absence of the ionophore [(211 ± 43) ng/L, $n = 4$]. The stable end product of prostacyclin, 6-keto-prostaglandin $F_{1\alpha}$ increased in response to the ionophore in four of five experiments. However, because of the variability among animals, this increase was not statistically significant (Fig 1).

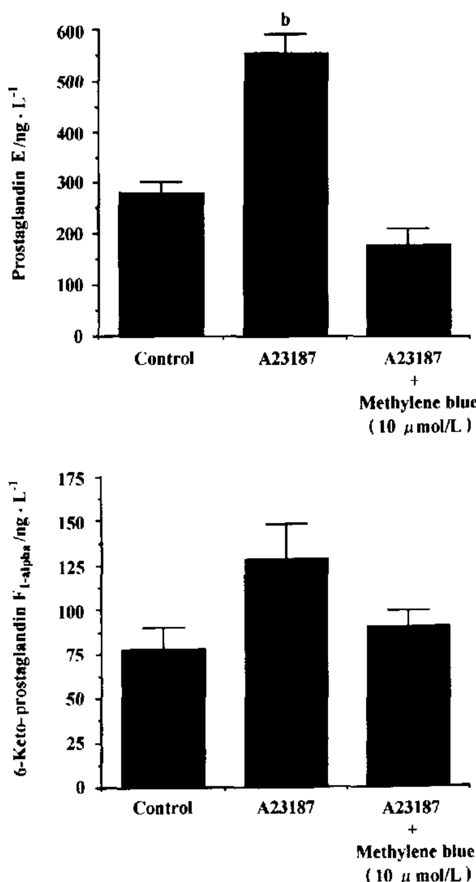

Fig 1. Production of prostaglandin E's (upper panel) and 6-keto-prostaglandin $F_{1\alpha}$ in trout aorta with endothelium incubated with acetylcholine (0.3 μmol/L) alone or in combination with calcium ionophore (0.3 μmol/L) and/or methylene blue (10 μmol/L). $\bar{x} \pm s_{\bar{x}}$. $n = 6$ in all experiments. (one way analysis of variance; Scheffe's test for post hoc analysis of means, [b]$P < 0.05$).

Calcium ionophore did not cause a statistically significant change in production of either prostaglandins of the E series or 6-keto-prostaglandin $F_{1\alpha}$ in trout aorta without endothelium (Tab 1).

Tab 1. Production of prostaglandins in trout aorta without endothelium stimulated with the calcium ionophore A23187. $n = 6$ in each group. $\bar{x} \pm s_{\bar{x}}$, ng/L.

	Control	A23187 (0.3 μmol/L)
Prostaglandin E	337.5 ± 60.6	450.2 ± 126.5
6-Keto-prostaglandin $F_{1\alpha}$	93.7 ± 14.3	70.7 ± 15.5

Responses to prostaglandins and nitric oxide In trout aortas without endothelium contracted with acetylcholine (0.3 μmol/L), prostaglandin I_2 and E_1 but not prostaglandin E_2 caused concentration-dependent decreases in tension (Fig 2). Nitric oxide caused no significant changes in tension in this tissue, while sodium nitroprusside caused modest relaxation. Methylene blue caused contraction in three of five aortas (0.4 – 0.5 g). Incubation of the rings with methylene blue did not affect the responses to sodium nitroprusside in the trout aorta (Fig 3).

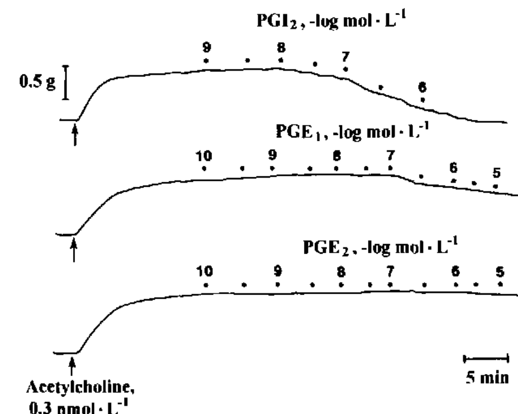

Fig 2. Tracings of responses to prostaglandin I_2 (PGI_2, top tracing), E_1 (PGE_1, middle tracing) and E_2 (PGE_2, bottom tracing) in trout aorta without endothelium contracted with acetylcholine (0.3 μmol/L). Experiments were conducted in the presence of indomethacin (10 μmol/L). Similar results were obtained in two additional experiments.

Nitric oxide and sodium nitroprusside caused concentration-dependent relaxation in frog aorta without

ISSN 0253-9756 Acta Pharmacol Sin 中国药理学报 2000 Oct; 21 (10)
E-mail aps@mail.shcnc.ac.cn Phn/Fax 86-21-6474-2629

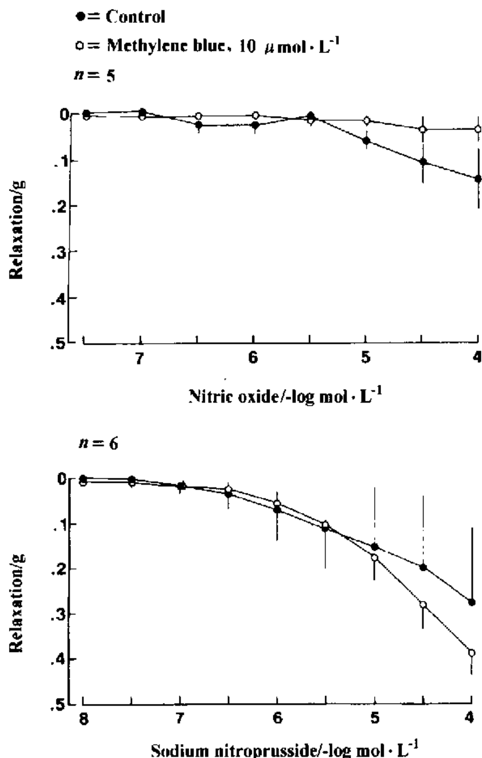

Fig 3. Responses to nitric oxide (upper panel) and sodium nitroprusside (lower panel) in trout aorta without endothelium contracted with acetylcholine (0.3 μmol/L). Experiments were conducted in the presence of indomethacin (10 μmol/L). Data are expressed as change in tension from contractions, which averaged 0.8 g ± 0.1 g ($n = 5$). Methylene blue did not affect the contraction significantly.

endothelium (Fig 4). Methylene blue significantly inhibited relaxations to nitric oxide but not to sodium nitroprusside in this tissue.

DISCUSSION

Results of this study confirm previous observations that the endothelium of the ventral aorta of trout produces vasoactive substances in response to the Ca^{2+}-ionophore A23187[2,3]. In the previous study, endothelium-dependent relaxations to A23187 were inhibited by meclofenamate, an inhibitor of cyclooxygenase. The present study extends previous observations to identify the vasoactive substances produced in response to A23187 to be prostaglandin E_1 and I_2. Since increases in prostanoids in the incubation media of aorta stimulated with the ionophore were observed only in preparations with endothelium, the endothelial cells are the most likely source of the prostanoids. The radioimmunoassay used in these studies does not distinguish between prostaglandins E_1 and E_2. However, it is likely that prostaglandin E_1 is the predominant vasoactive factor as this prostanoid caused relaxation of trout aorta while prostaglandin E_2 did not.

It is unlikely that the endothelium of trout aorta produces nitric oxide as a synthetic analog of *L*-arginine, N^G-monomethyl-*L*-arginine does not inhibit relaxations to A23187 in this tissue[6]. Results of the present study suggest that the smooth muscle of the trout aorta is insensitive to stimulation by nitric oxide. However, the smooth muscle does respond to another nitrovasodilator, sodium nitroprusside. Both nitric oxide and sodium nitroprusside cause relaxation through increased production of cyclic guanosine monophosphate by guanylate cyclase[7,8]. However, nitric oxide stimulates the soluble form of the enzyme, while sodium nitroprusside stimulates both the soluble and particulate form of the enzyme, and in addition, depolarizes the cell membrane[9-11]. Methylene blue inhibits soluble guanylate cyclase[12] but does not reduce relaxations to sodium-nitroprusside. Therefore, relaxations caused by sodium-nitroprusside in the trout aorta may result from activation of particulate guanylate cyclase and membrane hyperpolarization. The inability of methylene blue to inhibit relaxations to sodium nitroprusside in both trout and frog aorta is consistent with what has been observed in mammalian veins[13,14]. The lack of response to nitric oxide in the trout cannot be due to inadequate preparation of the nitric oxide solution as relaxations to the same preparations were observed in frog aortas. Therefore, endothelium-dependent relaxations mediated by nitric oxide sensitive-soluble guanylate cyclase may have evolved with a transition from water-gill to air-lung respirations.

Previous experiments have shown that endothelium-dependent relaxations to the calcium ionophore in trout aorta can be inhibited by meclofenamate and reversed by methylene blue[2]. The former is consistent with blockade of cyclooxygenase and reduction in the production of inhibitory prostanoids. The results of the present study explain that the reversal of the relaxation to the ionophore by methylene blue may also be due to decreased production of inhibitory prostanoids rather than inhibition of the mechanism of the relaxation. Methylene blue also interferes with the metabolism of arachidonic acid in cultured rabbit aortic endothelial cells and canine renal arter-

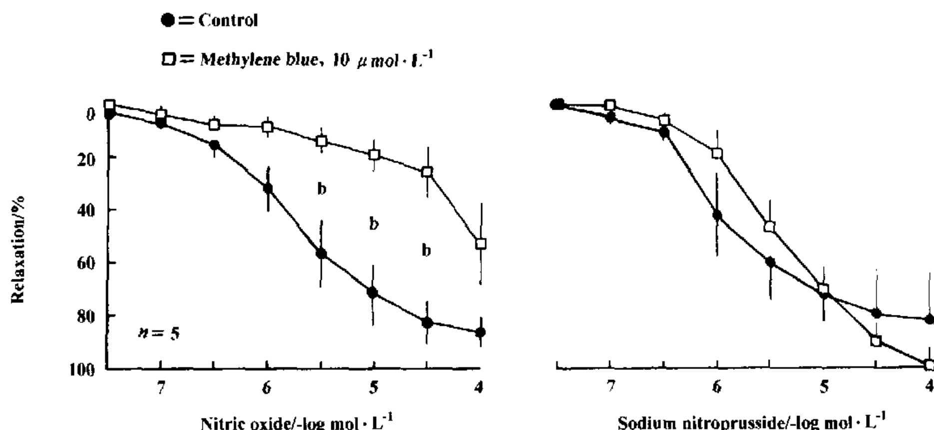

Fig 4. Relaxations to nitric oxide (left panel) and sodium nitroprusside (right panel) in frog aorta without endothelium contracted with norepinephrine (0.3 μmol/L). Experiments were conducted in the presence of indomethacin (10 μmol/L). Data are expressed as percent change in tension from contractions, which averaged 0.7 g ± 0.1 g ($n=5$). Methylene blue caused contraction in four of five rings (0.5 g ± 0.2 g, $n=5$). ${}^{b}P<0.05$ denotes significant difference in area under the curve (Student's t-test for paired observations).

ies[15,16]. The mechanism of this effect is not known.

In summary, results of this study identify the vascular endothelium as a source of vasoactive prostanoids in the trout aorta. Production of these prostanoids may represent a paracrine regulatory mechanism which mediates cardiovascular adaptations necessary for responses to seasonal variation in environmental temperature and circulating levels of reproductive hormones[17-19].

ACKNOWLEDGMENT The authors thank Mr Kevin RUD for technical assistance, Ms Sharon SANDBERG for performing the assays for prostaglandin, and Mr Robert LORENZ for drawing the figures. This work was supported in part by grants from the National Institutes of Health HL 31183 and the Mayo Clinic and Foundation.

REFRENCES

1 Furchgott RF, Zawadski J. The obligatory role of endothelium cells in the relaxation of arterial smooth muscle by acetylcholine. Nature 1980; 288: 373-6.

2 Miller VM, Vanhoutte PM. Endothelium-dependent responses in isolated blood vessels of lower vertebrates. Blood Vessels 1986; 23: 225-5.

3 Olson KR, Villa J. Evidence against nonprostanoid endothelium-derived relaxing factor (s) in trout vessels. Am J Physiol 1991; 260: R925-3.

4 Schryver S, Sanders E, Beierwalters WH, Romero JC. Cortical distribution of prostaglandin and renin in isolated dog glomeruli. Kidney Intl 1984; 25: 512-8.

5 Palmer RMJ, Ashton DS, Moncada S. Vascular endothelial cells synthesize nitric oxide from *L*-arginine. Nature 1988; 333: 664-6.

6 Miller VM, Vanhoutte PM. Endothelium-dependent vascular responsiveness: evolutionary aspects. In: Ryan US, Rubanyi GM, editors. Endothelial regulation of vascular tone. New York: Marcel Dekker, Inc; 1992. p 3-20.

7 Gruetter CA, Gruetter DY, Lyon JE, Kadowitz PJ, Ignarro LJ. Relationship between cyclic guanosine 3′:5′-monophosphate formation and relaxation of coronary arterial smooth muscle by glyceryl trinitrate, nitroprusside, nitrite and nitric oxide: effects of methylene blue and methemoglobin. J Pharmacol Exper Ther 1981; 219: 181-6.

8 Rapoport RM, Schwartz K, Murad F. Effect of sodium-potassium pump inhibitors and membrane-depolarizing agents on sodium nitroprusside-induced relaxation and cyclic guanosine monophosphate accumulation in rat aorta. Circ Res 1985; 57: 164-70.

9 Foley DH. Diminished arterial smooth muscle response to sodium nitroprusside during Na^+-K^+ pump inhibition. Pharmacol 1984; 28: 95-103.

10 Itoh T, Kajiwara M, Kitamura K, Kuriyama N. Effects of vasodilator agents on smooth muscle cells of the coronary artery of the pig. Br J Pharmacol 1981; 74: 455-68.

11 Rapoport RM, Schwartz K, Murad F. Effects of Na^+, K^+-pump inhibitors and membrane depolarizing agents on acetylcholine-induced endothelium-dependent relaxation and cyclic GMP accumulation in rat aorta. Eur J Pharmacol 1985; 110: 203-9.

12 Ignarro LJ, Harbison RG, Wood KS, Kadowitz PJ. Dissimilarities between methylene blue and cyanide on relaxation and cyclic GMP formation in endothelium-intact intrapulmonary artery caused by nitrogen oxide-containing vasodilators and

acetylcholine. J Pharmacol Exper Ther 1985; 236: 30–36.
13 Miller VM, Vanhoutte PM. Is nitric oxide the only endothelium-derived relaxing factor in canine femoral veins? Am J Physiol 1989; 257: H1910–16.
14 Vidal M, Vanhoutte PM, Miller VM. Dissociation between endothelium-dependent relaxations and increases in cyclic GMP in systemic veins. Am J Physiol 1991; 260: H1531–7.
15 Martin W, Drazan KM, Newby AC. Methylene blue but not changes in cyclic GMP inhibits resting and bradykinin-stimulated production of prostacyclin by pig aortic endothelial cells. Br J Pharmacol 1989; 97: 51–6.
16 Okamura T, Yoshida K, Toda N. Suppression by methylene blue of prostaglandin I_2 synthesis in isolated dog renal arteries. J Pharmacol Exper Ther 1990; 254: 198–203.
17 Cetta F, Goetz FW. Ovarian and plasma prostaglandin E and F levels in brook trout (*Salvelinus fontinalis*) during pituitary-induced ovulation. Biol Reprod 1982; 27: 1216–21.
18 Hazel JR, Carpenter R. Rapid changes in the phospholipid composition of gill membranes during thermal acclimation of the rainbow trout, Salmo gairdneri. J Comp Physiol B 1985; 155: 597–602.
19 Herman CA, Zimmerman PR, Doolittle KD. Prostaglandin synthesis in goldfish heart, *Carassius auratus*. Gen Comp Endocrinol 1984; 54: 478–85.

前列腺素而非一氧化氮是鳟主动脉内皮细胞舒血管因子

Virginia M MILLER[1], Paul M VANHOUTTE[2]
(*Departments of Surgery, and Physiology and Biophysics, Mayo Clinic and Foundation, 200 First Street SW, Rochester, MN 55905, USA*; [2]*Institut De Recherches Internationales Servier, 6, place des Pléiades, 92415 Courbevoie Cedex, France*)

关键词　乙酰胆碱; 钙; 离子载体; 牛蛙; 亚甲蓝

(责任编辑　吕　静)