中国药理学报 *Acta Pharmacologica Sinica* 1991 Jul ; 12 (4) : 308−311

Effects of anisodamine and dauricine on proliferation, DNA synthesis, and calcium influx in bovine anterior cerebral arterial smooth muscle cells in culture[1]

ZENG Guo-Qian, RUI Yao-Cheng (*Department of Pharmacology, College of Pharmacy, Second Military Medical University, Shanghai 200433, China*)

ABSTRACT The *in vitro* culture of bovine anterior cerebral arterial smooth muscle cells (BACASMC) was first established in our laboratory. Anisodamine and dauricine inhibited the proliferation, DNA synthesis and calcium influx in the cells in dose-dependent manners. At 0.01 mmol · L^{-1}, both drugs inhibited the proliferation by 17.6% and 8.3%, the DNA synthesis by 11.9% and 56.8%, the calcium influx by 26.6% and 31.4%, respectively. The results indicated that anisodamine and dauricine might have prospect in the prevention and treatment of cerebrovascular diseases.

KEY WORDS anisodamine; dauricine; cerebral arteries; cultured cells; vascular smooth muscle; calcium channel blockers; DNA

Smooth muscle cells (SMC) make up the media of mammalian arteries and are the principal cells of artherosclerotic lesions[1]. Many investigators have studied the effects of a lot of factors on proliferation and DNA synthesis of vascular SMC, but the emphasis was only put on the big or middle arteries[2,3]. The information attained for cerebrovascular SMC was little. Anisodamine is an analog of atropine, first isolated in China. Dauricine is a derivative of isoquinoline. Our previous work showed that both anisodamine and dauricine inhibited prostaglandins and leukotrienes production in rats and mice[4,5], and that dauricine also suppressed platelet-activating factor (PAF) release from mouse peritoneal macrophages[6], suggesting their effects on cardiovascular system. In the present work the authors established the *in vitro* culture of bovine anterior cerebral arterial smooth muscle cells (BACASMC) for the first time, and studied the effects of anisodamine and dauricine on the proliferation, DNA synthesis and calcium influx in BACASMC in culture so as to search for effective drugs for the prevention and treatment of cerebrovascular diseases (CVD).

MATERIALS AND METHODS

Newborn calves were obtained from abattoir and their anterior cerebral arteries were cut off under sterile conditions.

Eagle's MEM was purchased from Gibco, USA, and lactal-bumin hydrolysate from Oxoid, England. Fetal calf serum (FCS) was obtained from Shanghai Institute of Cell biology, Chinese Academy of Sciences. [^{3}H]TdR and $^{45}CaCl_2$ were purchased from Shanghai Institute of Nuclear Research, Chinese Academy of Sciences, and their specific activity is 111 GBq · mol^{-1} and 155 GBq · mol^{-1}, respectively.

Culture of BACASMC The anterior cerebral artery was placed in 0.5% lactalbumin hydrolysate. After the adventitia and the intima were carefully removed with a sterile stainless forceps, the artery was cut into pieces of 1−2 mm^3 and put in a culture flask with the inside sticking to the bottom for incubation for 5−7 d. Around 10 d the free smooth muscle cells derived from the explants teemed in most of the culture flask bottom. Then the cells were subcultured with 0.25% trypsin. The following experiments were

Received 1990 Sep 15 Accepted 1991 Mar 23
[1] Project supported by the National Natural Science Foundation of China, No 3880742

performed using 5th–11th generations of the BACASMC.

Determination of BACASMC proliferation The proliferation of BACASMC in culture was measured by a modification of the reported methods[7,8]. The subcultured cells were seeded in 96-well culture plates (1×10^4 cells / well). After 3 d, the media in wells were replaced by fresh complete MEM and drugs. After 24 h incubation, the cells were stained in formaldehyde and crystal violet for 20 min, and then washed with distilled water. When the plates were dry naturally, 100 μl of extraction solution consisting of sodium citrate and alcohol was added to each well to extract the crystal violet. After 30 min the absorbance of each well was determined at 595 nm with type-721 spectrophotometer.

Determination of DNA synthesis in BACASMC The DNA synthesis in BACASMC was measured by the method of Castellot *et al*[9]. After the cells were cultured for 12 h [^{3}H]TdR was added(18.5 kBq / well) for the DNA synthesis. After 12 h the cells were washed twice with Hanks' balanced salt solution (Modified), digested with 0.25% trypsin for 3 min, and then collected on type-49 glassfiber filters through filtration. The filters were counted in a FJ-2107 scintillation counter.

Determination of $^{45}CaCl_2$ influx into BACASMC The $^{45}CaCl_2$ influx into BACASMC was measured according to the method of Lee *et al*[10]. After the cells were incubated for 3 d the media in 96-well plates was discarded and 0.2 ml buffer solution containing drug and 18.5 kBq of $^{45}CaCl_2$ were added into each well. Having been incubated at 37℃ for 40 min the buffer solution was discarded followed by rapid washing 5 times with cold buffer solution. Then 100 μl Triton X-100 (0.2%, vol / vol) was added. The radioactivity was counted in a scintillation counter (FJ-2107).

Statistics Statistical analysis was made by *t* test. All values are $\bar{x} \pm SD$.

RESULTS

Growth of BACASMC BACASMC were seeded at the density of 1×10^4 cells / well and reached the maximal amounts at 72 h BACASMC grew logarithmically

Tab 1. Effects of anisodamine and dauricine on the proliferation, DNA synthesis, and $^{45}CaCl_2$ influx in cultured BACASMC. $n = 6$ **for proliferation and $^{45}CaCl_2$ influx tests,** $n = 12$ **for Hank's synthesis tests ($n = 19$, 6 for control and verapamil, respectively).** $\bar{x} \pm SD$. $^{*}P > 0.05$, $^{**}P < 0.05$, $^{***}P < 0.01$ *vs* **Hanks's solution.**

Drugs	mmol · L^{-1}	Absorbance	[^{3}H]TdR, dpm	$^{45}CaCl_2$, dpm
Hanks' solution		0.60 ± 0.02	12 746 ± 2 166	5 964 ± 400
Anisodamine	0.001	0.52 ± 0.08**	13 510 ± 1 500*	
	0.01	0.51 ± 0.06***	11 224 ± 1 590**	4 380 ± 428***
	0.1	0.43 ± 0.03***	10 328 ± 1 152***	4 044 ± 420***
	1			3 716 ± 224***
Dauricine	0.001	0.59 ± 0.13*	8 840 ± 1 254***	
	0.01	0.55 ± 0.5**	5 502 ± 1 344***	4 092 ± 276***
	0.1	0.35 ± 0.10***	2 550 ± 786***	3 796 ± 576***
	1			3 216 ± 400***
Verapamil	0.1	0.35 ± 0.02***	6 464 ± 1 080***	4 092 ± 778***
A-23187	0.01			7 624 ± 1 060***

during 24–72 h, and had a linear relationship with the corresponding absorbance when the cell quantity ranged $5\times 10^3 - 3\times 10^4$. The correlation coefficient $r=0.986$. That was the basis of determinating cell growth.

Effects of anisodamine and dauricine on proliferation Both anisodamine and dauricine suppressed the proliferation of BACASMC in dose–dependent (0.001–0.1 mmol · L^{-1}) manner (Tab 1). At 0.1 mmol · L^{-1} both drugs inhibited the growth by 28.3% and 41.7%, respectively. On the same condition calcium antagonist verapamil also significantly inhibited BACASMC growth at 0.1 mmol · L^{-1} with an inhibitory rate of 41.7%.

Effects of anisodamine and dauricine on DNA synthesis When DNA synthesis was assayed using [^{3}H]TdR incorporation as described above, cultured BACASMC utilized [^{3}H]TdR to synthesize DNA. Tab 1 showed that dauricine suppressed the DNA synthesis at the concentrations of 0.001 – 0.1 mmol · L^{-1} and anisodamine inhibited the DNA synthesis at 0.01 and 0.1 mmol · L^{-1}, but not at low concentration (0.001 mmol · L^{-1}). Verapamil showed an inhibitory effect on the DNA synthesis at 0.1 mmol · L^{-1}.

Effects of anisodamine and dauricine on influx of $^{45}CaCl_2$ into BACASMC The uptake of $^{45}CaCl_2$ by BACASMC increased with the incubation time and attained a plateau at 40 min. Tab 1 showed that anisodamine and dauricine suppressed $^{45}CaCl_2$ influx into BACASMC in concentration–related manner at 0.01 – 1 mmol · L^{-1}. At 0.1 mmol · L^{-1} the influx was inhibited by 32.2% and 36.4%, respectively. Verapamil inhibited $^{45}CaCl_2$ influx into the cells by 31.4%. whereas calcimycin (A–23187), a calcium ionophore, increased $^{45}CaCl_2$ influx into BACASMC by 27.8% at 0.01 mmol · L^{-1} on the same condition.

DISCUSSION

Proliferative arterial smooth muscle cells play a key role in the course of atherosclerosis and are believed to have direct relevance to CVD. As a second messenger Ca^{2+} is distributed broadly in living things and plays a pivotal role in the regulation of cell proliferation[11,12]. Calcium antagonists inhibiting proliferation of smooth muscle cells have been reported recently[13,14]. In this report the authors established the *in vitro* culture of BACASMC for the first time and modified the methods of reference[7,8] for the determination of cell number in monolayer cultures and applied it to the measure of proliferation of BACASMC. The results showed the inhibitory effects of anisodamine and dauricine on the proliferation, DNA synthesis and $^{45}CaCl_2$ influx into the cells. Verapamil, a calcium antagonist, also inhibited the proliferation, DNA synthesis, and $^{45}CaCl_2$ influx in BACASMC. Indicating that the inhibitory effects of anisodamine and dauricine on the proliferation and DNA synthesis of BACASMC might result from the inhibition of calcium influx into BACASMC.

Anisodamine has been long used as a cholinergic receptor antagonist in the treatment of endotoxic shock and visceral smooth muscle spasm, and dauricine was used clinically as an antiarrhythmic drug in China. The mechamisms of their actions, however, are still unclear. The inhibition of calcium influx into cells accounted partly for their diverse effects. On the other hand, the inhibitory effects of anisodamine and dauricine on the proliferation and DNA synthesis of BACASMC suggested that both drugs might be effective in the prevention and treatment of CVD, but the details demand

further investigation.

REFERENCES

1 Ross R, Glomset JA. Atherosclerosis and the arterial smooth muscle cell. Proliferation of smooth muscle is a key event in the genesis of the lesions of atherosclerosis. *Science* 1973; **180**: 1332

2 Myasnikov AL, Block YE. Influence of some factors on lipoidosis and cell proliferation in aorta tissue culture of adult rabbits. *J Atheroscler Res* 1965; **5**: 33

3 Stout RW, Bierman EL, Ross R. Effect of insulin on the proliferation of cultured primate arterial smooth muscle cells. *Circ Res* 1975; **35**: 136

4 Tong L, Yue TL. Effect of dauricine on rat and human platelet aggregation and metabolism of arachidonic acid in washed rat platelets. *Acta Pharm Sin* 1989; **24**: 85

5 Yue TL, Zeng GQ, Li J. Inhibition of release of prostaglandins and leukotrienes from zymosan-stimulated mouse peritoneal macrophages by anisodamine. *Chin J Pharmacol Toxicol* 1989; **3**: 91

6 Zeng GQ, Ruo YC. Inhibitory effect of dauricine on platelet activating factor released from calcimycin-stimulated mouse peritoneal macrophages. *Acta Pharmcol Sin* 1990; **11**: 346

7 Gillies RJ, Didier N, Denton M. Determination of cell number in monolayer cultures. *Anal Biochem* 1986; **159**: 109

8 Flick DA, Gifford GE. Comparison of in vitro cell cytotoxic assays for tumor necrosis factor. *J Immunol Methods* 1984; **68**: 167

9 Castellot JJ Jr, Cochran DL, Karnovsky MJ. Effect of heparin on vascular smooth muscle cells. I. cell metabolism. *J Cell Physiol* 1985; **124**: 21

10 Lee TC, Malone B, Blank ML, Snyder F. 1-Alkyl-2-acetyl-*sn*-glycero-3-phosphocholine (platelet-activating factor) stimulates calcium influx in rabbit platelets. *Biochem Biophys Res Comm* 1981; **102**: 1262

11 Whitefield JF, Boynton AL, Macmanus JP, Sikorska M, Tsang BK. The regulation of cell proliferation by calcium and cyclic AMP. *Mol Cell Biochem* 1979; **27**: 155

12 Metcalfe JC, Moore JP, Smith GA, Hesketh TR. Calcium and cell proliferation. *Br Med Bull* 1986; **42**: 405

13 Nilsson J, Sjolund M, Palmberg L, Von Euler AM, Jonzon B, Thyberg J. The calcium antagonist nifedipine inhibits arterial smooth muscle cell proliferation. *Atherosclerosis* 1985; **58**: 109

14 Orekhov AN, Tertov VV, Khashmov KA, Kudryashov SS, Smirnov VN. Evidence of antiatherosclerotic action of verapamil from direct effects on arterial cells. *Am J Cardiol* 1987; **59**: 495

山莨菪碱和蝙蝠葛碱对培养的小牛大脑前动脉平滑肌细胞增殖、DNA 合成及钙内流的影响

曾国钱、芮耀诚 (第二军医大学药学院药理教研室，上海 200433，中国)

提要 用组织块法培养了小牛大脑前动脉平滑肌细胞，山莨菪碱和蝙蝠葛碱在 0.01 mmol · L^{-1} 时对上述细胞增殖的抑制率分别为 17.6%和 8.3%，对 DNA 合成的抑制率分别为 11.9%和 56.8%，对该细胞钙内流的抑制率分别为 26.6%和 31.4%. 提示两药对脑血管病的防治有意义.

关键词 山莨菪碱；蝙蝠葛碱；脑血管；培养细胞；血管平滑肌；钙通道阻滞剂；核糖核酸