中国药理学报 *Acta Pharmacologica Sinica* 1990 Sep; 11 (5) : 450-454

尼卡地平和氯丙嗪对培养的主动脉平滑肌细胞增殖和细胞内钙调素的作用

陆志强、王道生 (苏州医学院药理学教研室, 苏州 215007, 中国)

Effects of nicardipine and chlorpromazine on proliferation and intracellular calmodulin in cultured aortic smooth muscle cells

LU Zhi-Qiang, WANG Dao-Sheng(*Department of Pharmacology, Suzhou Medical College, Suzhou 215007, China*)

ABSTRACT Effects of Ca^{2+} and calcium antagonists nicardipine (Nic) and chlorpromazine (CPZ) on the proliferation, DNA synthesis and intracellular calmodulin (CaM) levels in cultured rabbit aortic smooth muscle cells were investigated. Maximal response of cell to Ca^{2+} stimulation occurred in the normal medium with $CaCl_2$ 1.4 mmol / L, whereas $CaCl_2$ 0.02 - 0.6 mmol / L, Nic (2.5 - 40.0 μmol / L) and CPZ (5.0 - 20.0 μmol / L) inhibited the cell proliferation in a concentration-dependent manner. The additive effects of Nic and CPZ were observed. There was a transient surge of intracellular soluble CaM level at 9h in late G_1 phase of the cell cycle just before the initiation of DNA synthesis. Not only the initiation of DNA synthesis but also the CaM surge were prevented by adding Nic or CPZ (20 - 40 μmol / L) to media.

These results suggest that an appropriate extracellular Ca^{2+} concentration was required for aortic smooth muscle cells to grow normally. Since calcium antagonists influenced the intracellular Ca^{2+} pool by blockade of calcium channel and they inhibited the CaM function directly by anticalmodulin activity or indirectly through altered intracellular Ca^{2+} metabolism, intracellular Ca^{2+} and CaM levels may be involed in the effects of Nic and CPZ on the proliferation and DNA synthesis.

KEY WORDS nicardipine; chlorpromazine; cultured cells; cell cycle; calcium chloride; calmodulin; aorta

提要 体外培养的主动脉平滑肌细胞的增殖明显依赖于细胞外液中的游离 Ca^{2+}浓度. Nic 和 CPZ 都剂量依赖性地抑制细胞增殖. 在周期同步化细胞培养中, Nic 和 CPZ 不仅抑制了 DNA 的合成, 而且也明显降低了细胞 G_1 期中钙调素含量的升高. 提示 Nic 和 CPZ 对细胞内 Ca^{2+}和钙调素的调节, 导致对细胞增殖和 DNA 合成的抑制.

关键词 尼卡地平; 氯丙嗪; 培养的细胞; 细胞周期; 氯化钙; 钙调素; 主动脉

Received 1990 Jan 17 Accepted 1990 Jun 7

Ca^{2+}和钙调素(calmodulin, CaM)直接调控细胞的增殖[1]. 动脉平滑肌细胞增殖是引起动脉粥样硬化病变的一个关键因素[2]. 近年来人们已经在不同的动脉粥样硬化动物模型上证实钙拮抗剂具有防治作用[3]. 本文旨在用体外培养的动脉平滑肌细胞, 研究钙拮抗剂尼卡地平 (nicardipine, Nic)和氯丙嗪 (chlorpromazine, CPZ)对细胞增殖的抑制作用, 以及探讨它们对细胞周期不同时相中 CaM 含量的影响.

MATERIALS AND METHODS

兔主动脉平滑肌细胞培养

1 原代培养 新西兰兔, ♂, 体重 1.5 ± SD 0.4kg, 于无菌条件下取胸主动脉段, 按"组织块接种法"[4]将中层平滑肌组织小块移入培养瓶, 加入 Eagle's MEM 培养液(含 20% 小牛血清, 10 mmol / L Hepes, pH 7.0-7.2), 密闭于 37℃下静置培养. 每周换液两次, 待细胞融合成致密单层时进行传代.

2 传代培养 融合细胞采用 0.125% 胰蛋白酶和 0.02% EDTA 混合消化液(pH 7.5)处理分散后, 换入含 10% 小牛血清的新鲜培养液. 调整细胞密度(5×10^5 cells / dish)后将细胞悬液分装入培养瓶. 传代后细胞约经 7-10 d 可达融合单层, 即可再次传代.

细胞增殖的测定 采用比色法[5]进行.

细胞接种于 24 孔细胞培养板. 细胞固定后经 0.1%结晶紫染色细胞 30 min，脱色后每孔各加入 0.2% Triton X-100 2 ml，使细胞内结合染料重新溶入溶液，于 721 型分光光度计(590 nm)上比色. 测定其吸收率.

细胞周期同步化 采用低血清乏营养法[6]. 培养细胞在 0.4%血清的培养液中孵育 72 h，大部分细胞被阻抑于细胞周期的 G_0 期，此时更换 10-20%血清的培养液，细胞即可受刺激从 G_0 期释出，同步进入细胞增殖周期的 G_1 期.

同步化细胞的[^{3}H]TdR 参入与测定 同步化细胞在接受不同处理后的不同周期时相加入[^{3}H]TdR (74 kBq / ml)，孵育 2 h 后用适量 0.9% NaCl 清洗，细胞经酸消化后加入 0.6% PPO-甲苯闪烁液.均相液闪标本用双道液体闪烁计数器(FJ-353G1 型)测定.

培养主动脉平滑肌细胞内可溶性 CaM 的提取与测定 收集培养细胞，在冰冷的匀浆缓冲液(含 Tris-HCl 50 mmol / L，EGTA 1 mmol / L，2-mercaptoethanol 1 mmol / L，NaCl 0.15 mol / L，pH 7.5) 作超声破膜处理，匀浆液经加热(95℃，3 min)和离心(10 000 × g，30 min)后取上清液测 CaM 含量. 采用酶联免疫测试法[7]测定 CaM 的含量，并制备 CaM 标准曲线. 试剂配制及操作步骤均按药盒说明书进行.

Nic 为中国药科大学合成. CPZ 为 Sigma 公司产品. [^{3}H]胸腺嘧啶核苷([^{3}H]TdR)由中国科学院原子能研究所提供. CaM 酶联免疫测试药盒为徐州医学院生化研究室惠赠.

RESULTS

培养的主动脉平滑肌细胞的形态、生长性状和对细胞外 Ca^{2+}浓度的依赖性 培养的单层细胞呈长梭状，胞浆丰富，境界清楚，核卵圆或圆形，细胞与细胞间多呈平行生长，束状排列，密集和稀疏处相互交错呈"峰谷状"，显示平滑肌细胞的典型生长特征. 从细胞生长曲线看，在传代接种后细胞逐日成倍增殖，8 d 后可形成致密单层(Fig 1).

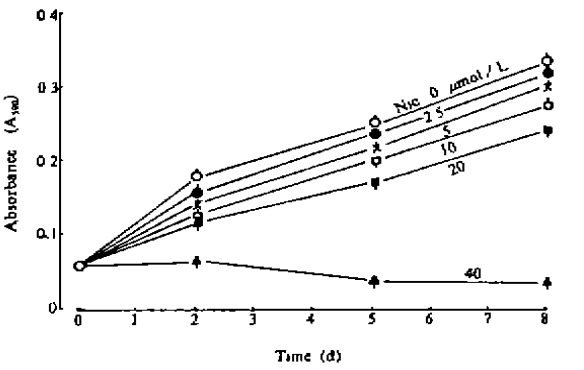

Fig 1. Effect of nicardipine (Nic) on growth of aortic smooth muscle cells $n=4$, $\bar{x} \pm SD$.

正常细胞生长液中的游离 Ca^{2+}浓度为 1.4 mmol / L 时细胞增殖能力达到最高. 随着胞外 Ca^{2+}浓度降低(含 10%小牛血清的 Ca^{2+}-Free Eagle 培养液，用适量 EGTA 调节 Ca^{2+}浓度). 细胞增殖明显受抑. 当 Ca^{2+}浓度为 20 μmol / L 时可造成细胞的脱落死亡. 而 Ca^{2+}浓度增至 5 mmol / L，细胞生长也明显受抑(Tab 1).

Tab 1. Influences of $CaCl_2$ on the growth of aortic smooth muscle cells. $n=4$, $\bar{x} \pm SD$. $^*P>0.05$, $^{***}P<0.01$ *vs* **normal $CaCl_2$ concentration 1.4 mmol / L.**

$CaCl_2$ (mmol / L)	Absorbance (A_{590})
0.02	0.022 ± 0.003***
0.2	0.141 ± 0.004***
0.6	0.151 ± 0.006***
1.4	0.201 ± 0.008
2.0	0.203 ± 0.006*
5.0	0.137 ± 0.006***

钙拮抗剂对细胞增殖的抑制作用

1 Nic 的作用 对照组细胞培养 8 d，其增殖近 6 倍. 在加入 Nic 后 2，5，8 d，细胞的增殖呈剂量依赖方式(2.5-40.0μmol / L)均

的增殖呈剂量依赖方式(2.5–40.0μmol / L)均受到 Nic 的明显抑制(Fig 1). 在细胞处于对数生长期的 d 5, 20 μmol / L Nic 的抑制率为 59 ± 5%.

2 CPZ 的作用 将 CPZ 加入培养液后 d 3 测定其对细胞增殖的抑制率，结果表明 CPZ 也以剂量依赖方式(5–20μmol / L)明显抑制细胞增殖. 5, 10 和 20 μmol / L CPZ 的抑制率分别为 12 ± 3%, 35 ± 2% 和 67 ± 3%. 当 CPZ 浓度达到 40 μmol / L 时可造成细胞的脱落死亡.

3 Nic 和 CPZ 的相加作用 细胞贴壁后将不同浓度的 Nic 和 CPZ(0, 5, 10, 20 μmol / L)按不同的浓度组合，共同加入正常细胞生长液中孵育 3 d 后，可观察到两药对各组细胞增殖的抑制有显著的相加作用(Tab 2).

Tab 2. Inhibition (%) of the growth of aortic smooth muscle cells by Nic and chlorpromazine. $n=4$, $\bar{x} \pm SD$. $^{**}P<0.05$, $^{***}P<0.01$ *vs* **Nic group without chlorpromazine;** $^{\#\#}P<0.05$, $^{\#\#\#}P<0.01$ *vs* **chlorpromazine group without Nic.**

Nic (μmol / L)	Chlorpromazine (μmol / L) 0	5	10	20
0	0	12 ± 3	35 ± 2	67 ± 3
5	9 ± 3	22 ± 4$^{**}_{\#\#}$	43 ± 4$^{***}_{\#\#}$	104 ± 3$^{***}_{\#\#\#}$
10	18 ± 4	30 ± 3$^{***}_{\#\#}$	52 ± 4$^{***}_{\#\#}$	109 ± 3$^{***}_{\#\#\#}$
20	26 ± 3	40 ± 4$^{***}_{\#\#}$	74 ± 3$^{***}_{\#\#\#}$	111 ± 3$^{***}_{\#\#\#}$

Nic 对同步化动脉平滑肌细胞 DNA 合成的抑制作用 在细胞同步进入增殖周期的 24 h 内，对照组细胞在第 12 h 后[^{3}H]TdR 参入量开始急剧升高，提示细胞在第 12 h 开始合成 DNA，从 G_1 期转入 S 期. 而在 Nic(20 μmol / L)处理组，[^{3}H]TdR 参入量明显降低，曲线低平，表明 Nic 可明显抑制动脉平滑肌细胞的 DNA 合成，使细胞周期从 G_1 期进入 S 期的进程受阻(Fig 2).

钙拮抗剂对同步化动脉平滑肌细胞内 CaM 含量的影响 随着动脉平滑肌细胞同步地进入细胞增殖周期，细胞内可溶性 CaM 含量也随之增加，在第 9 h 时(G_1 晚期)呈一短暂的高峰(约比 G_1 早期增加一倍)，随后细胞内 CaM 含量又降至稍低的水平(Fig 3). 当细胞与 Nic 或 CPZ(20, 40 μmol / L)孵育后，两药均可使细胞 G_1 期的 CaM 含量显著降低(Fig 4).

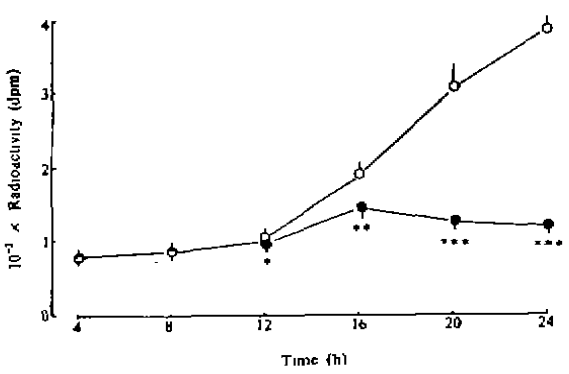

Fig 2. Effect of Nic 20 μmol / L (●) on incorporation of [^{3}H]TdR after release from G_0 phase in the synchronous cells $n=3$, $\bar{x} \pm SD$. $^{*}P>0.05$, $^{**}P<0.05$, $^{***}P<0.01$. *vs* **control (○).**

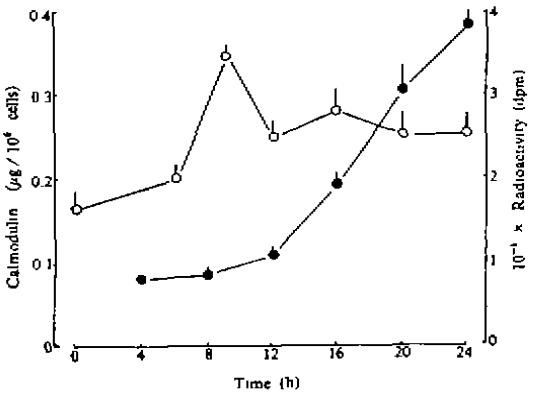

Fig 3. Calmodulin content (○) and DNA synthesis (●), in synchronous aortic smooth muscle cells during G_1 phase of cell cycle.

DISCUSSION

本实验证明，血管平滑肌细胞的生长和增殖明显依赖于细胞外游离 Ca^{2+}浓度. 随着细胞

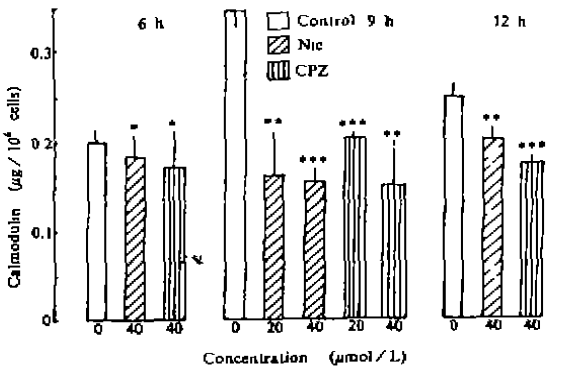

Fig 4. Effects of Nic and CPZ on calmodulin content in synchronous sortic smooth muscle cells during G_1 phase (6, 9 and 12 h). $n=3$, $^{*}P>0.05$, $^{**}P<0.05$, $^{***}P<0.01$.

外 Ca^{2+}浓度的降低，细胞的增殖受到不同程度的抑制，而钙拮抗剂 Nic 和 CPZ 也对细胞的增殖产生剂量依赖性抑制，并且在一定剂量范围内两药合用产生明显的相加或协同作用，提示两药可能在细胞的不同部位产生了对 Ca^{2+} 依赖性细胞过程的双重抑制. 许多研究证实 CaM 参与多种细胞的增殖过程[1,8,9]. 本文的动脉平滑肌细胞同步化增殖周期的实验显示在细胞从 G_0 期释放进入增殖周期后的 G_1 晚期，胞内可溶性 CaM 含量有一个显著的升高峰. 提示在细胞的 G_1 晚期 CaM 含量的增加，连同此期胞 Ca^{2+}浓度的突发性升高，可使 Ca^{2+}–CaM 复合物达到一定的浓度，进而启动一系列受 Ca^{2+}与 CaM 调控的细胞内生理生化反应，并促使细胞进入 DNA 合成期(Fig 3). Nic 和 CPZ 显著地抑制细胞 DNA 合成，使细胞从 G_1 期向 S 期的转化进程受阻. 钙拮抗剂的这种作用与它们对 G_1 期中 CaM 的影响可能有一定的内在联系. 本实验观察到 Nic 和 CPZ 均明显降低细胞 G_1 期的 CaM 含量，使 CaM 在 G_1 晚期的升高峰消失. CaM 拮抗剂对 CaM 的影响已为许多实验所证实. 钙通道阻断剂与 CaM 的关系也正在为许多学者深入探讨. Nic 竞争性抑制 CaM 敏感的和 CaM 不敏感的 cAMP 磷酸二酯酶[10]. Nic 可使犬动脉条 cAMP 水平明显增加，并证实它竞争性抑制 cAMP 磷酸二酯酶[11]，而磷酸二酯酶和腺苷酸环化酶等均受到 CaM 调控. 在整体实验中有报道硝苯啶(nifedipine)在降低 SHR 大鼠血压的同时，使血浆及主动脉组织中 CaM 含量明显降低[12].

实验结果提示钙拮抗剂 Nic 和 CPZ 通过阻断钙通道影响胞内 Ca^{2+}水平，或通过直接和／或间接地影响胞内 CaM 水平，进而导致对动脉平滑肌细胞增殖和 DNA 合成的抑制作用.

REFERENCES

1 Hickie RA, Wei JW, Blyth LM, Wong DYW, Klaassen DJ. Cations and calmodulin in normal and neoplastic cell growth regulation. *Can J Biochem Cell Biol* 1983; 61 : 934

2 Ross R. The smooth muscle cell. II . Growth of smooth muscle in culture and formation of elastic fibers. *J Cell Biol* 1971; 50 : 172

3 Weinstein DB, Heider JG. Antiatherogenic properties of calcium antagonists. *Am J Cardiol* 1987; 59 : 163B

4 卢咏才、郭肇铮、贾绪东、刘小青、周玉琳. 家兔主动脉平滑肌细胞培养(组织块接种法). 北京中医学院学报. 1983; (1) : 61

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

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

7 Zhao SH, Yu HL, Zhang MZ, Xu YJ, Zhou X, Lu L. Calmodulin enzyme–linked immunosorbent assay. *Acta Acad Med Xuzhou* 1988; 8 : 54

8 Veigl ML, Vanaman TC, Sedwick WD. Calcium and calmodulin in cell growth and transformation. *Biochim Biophys Acta* 1983; 738 : 21

9 Cheung WY. Calmodulin: its potential role in cell proliferation and heavy metal toxicity. *Fed Proc* 1984; 43 : 2995

10 Epstein PM, Fiss K, Hachisu R, Andrenyak DM. Interaction of calcium antagonists with cyclic AMP - phosphodiesterases and calmodulin. *Biochem Biophys Res Commun* 1982; 105 : 1142

11 Sakamoto N, Terai M, Takenaka T, Maeno H. Inhibition of cyclic AMP phosphodiesterase by 2,6-dimethyl-4-(3-nitropheyl)-1,4-dihydropyridine-3,5-dicarboxylic acid 3-(2-(*N*-benzyl-*N*-methylamino)) ethyl ester 5-methyl ester hydrochloride (YC-93), a potent vasodilator. *Biochem Pharmacol* 1978; 27 : 1269

12 Wen YY, Hu P, Chen MQ, Zhang SF. Effect of nifedipine on blood pressure, calmodulin and cyclic nucleotides in plasma and aorta from spontaneously hypertensive rats. *Acta Acad Med Sin* 1987; 9 : 113

中国药理学报 *Acta Pharmacologica Sinica* 1990 Sep; 11 (5) : 454-457

Latin square *vs* twin crossover design for mouse blood glucose assay to estimate insulin potency

LIN Zhi-Gong, ZHOU Hai-Jun (*National Institute for the Control of Pharmaceutical and Biological Products, Beijing 100050, China*)

ABSTRACT According to parallel line analysis, a Latin square design was used for estimating insulin potency in mouse blood glucose assay. Four to six groups of 4 × 4 Latin squares were used to estimate 80 %, 100% and 120% standard preparations and the recovery rates were 95-106 %. Meanwhile, in comparison with twin crossover design, 13 batches of variant preparations including some beyond expiry dates were checked by Latin square design. The results showed that the potencies were about the same in two designs. The average fiducial limit rates were still less than 25% but 40% or so of animal numbers were saved. Therefore, Latin square design is a precise and accurate assay for the estimation of insulin.

KEY WORDS research design; biological assay; blood glucose; insulin

Although twin crossover design is better than (2,2), 2 doses of standard and 2 doses of preparation, or (3, 3) assay,[1] it must cut back interact effect in statistical analysis. Latin square design can get more information from fewer animals in order to compare the drug effect. The experimental design possesses more effect in statistical analysis. Each subject can be used four times. The mouse blood glucose is estimated by a sensitive glucose oxidase method, a small amount of blood sample can be taken to replicate the treatment and if the investigator is prepared to get his results, a Latin square is a satisfactory design[2]. This paper reports on the results of a Latin square design in estimation of insulin bioactivity in comparison with twin crossover design.

Received 1989 May 2 Accepted 1990 Apr 21

MATERIALS AND METHODS

Insulin 1) National biological standards, batch 780307, 27 IU / mg were prepared by our Institute; 2) International biological standards, batch 83 / 515, 26 IU / mg, were provided by WHO International Laboratory for Biological Standards; 3) preparations of insulin were provided by Shanghai Biochemical Pharmaceutical Factory.

Mice Mice of same sex weighing 22 ± 2g were used and 16, 20 or 24 mice in each test. Before the assay all the mice should be deprived of food and fed with water only.

Determination of glucose Content of glucose of 0.06 ml blood taken from the orbital venous sinus was measured by glucose oxidase reagent method [3].

Design Mice were allotted at random into 4-6 blocks. Each block was carried on