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Potassium channel openers inhibit ATP-induced cytosolic free calcium increase in cultured rabbit aortic smooth muscle cells[1]

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KEY WORDS potassium channel; adenosine triphosphate; vascular smooth muscle; calcium; glibenclamide

AIM: To study the effects of potassium channel openers (PCO) on cytosolic free calcium ($[Ca^{2+}]_i$) changes and their possible mechanisms in vascular smooth muscle cells (VSMC). **METHODS**: Cultured rabbit aortic VSMC were treated with Fura-2 AM 2.5 $\mu mol \cdot L^{-1}$ at 37 ℃ for 50 min. The PCO were pinacidil (Pin), nicorandil (Nic), lemakalim (Lem), and RP 49356 (RP). $[Ca^{2+}]_i$ level was measured by fluorospectrometer. **RESULTS**: $[Ca^{2+}]_i$ increase induced by K^+ 30 $mmol \cdot L^{-1}$ was weakly inhibited by Pin, Nic, Lem, and RP (441 ± 23, 455 ± 48, 451 ± 22, 370 ± 31 *vs* 544 ± 40 $nmol \cdot L^{-1}$, $P < 0.01$). ATP (0.1 $mmol \cdot L^{-1}$)-induced peak and sustained $[Ca^{2+}]_i$ increase were inhibited by these agents in a concentration-dependent manner. The effects of Pin, Lem, and RP were completely canceled (peak phase: 549 ± 39, 540 ± 30, 564 ± 13 *vs* 541 ± 39 $nmol \cdot L^{-1}$; sustained phase: 413 ± 25, 364 ± 16, 377 ± 11 *vs* 380 ± 8 $nmol \cdot L^{-1}$), but that of Nic was only partially blocked (peak phase: 453 ± 31 *vs* 541 $nmol \cdot L^{-1}$; sustained phase: 348 ± 19 *vs* 380 ± 8 $nmol \cdot L^{-1}$, $P < 0.01$) by glibenclamide (Gli, 10 $\mu mol \cdot L^{-1}$). Pretreated with the Pin, Nic, Lem, and RP (10 $\mu mol \cdot L^{-1}$), the peak $[Ca^{2+}]_i$ elevation induced by ATP was reduced in the Ca^{2+}-free solution (129 ± 17, 142 ± 21, 136 ± 14, 114 ± 9 *vs* 258 ± 32 $nmol \cdot L^{-1}$, $P < 0.01$). **CONCLUSION**: Pin, Nic, Lem, and RP inhibited ATP-induced $[Ca^{2+}]_i$ increase, associated with decreases of both Ca^{2+} release from intracellular store and Ca^{2+} influx from extracellular store.

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The cytosolic free Ca^{2+} concentration ($[Ca^{2+}]_i$) is a major factor which determines the tone and activity of vascular smooth muscle[1,2]. Because high $[Ca^{2+}]_i$ in vascular smooth muscle cells (VSMC) has been implicated in the pathogenesis of hypertension. By K^+ efflux, cell membrane hyperpolarization, and further voltage-dependent Ca^{2+} channel blockade, potassium channel openers (PCO) reduce Ca^{2+} influx. PCO also abolish the refill of Ca^{2+} from the Ca^{2+} store, and then decrease the frequencies of polarization and contraction of vascular smooth muscle cells[3-5]. PCO possess the potential uses in antihypertension and other vascular disorders[6,7].

Seven subgroups of ATP-sensitive PCO were classified[4]. In the present study, the effects of 4 structural types of PCO — pinacidil (Pin), nicorandil (Nic), lemakalim (Lem), and RP 49356 (RP) on $[Ca^{2+}]_i$ changes in vascular smooth muscle cells were investigated.

MATERIALS AND METHODS

Drugs Trypsin, edetic acid, egtazic acid, bovine albumin, ATP (Sigma Co, USA). Pin, Nic, RP, Lem, and glibenclamide (Gli) were made by Leo Pharmaceutical Products Ltd (Denmark), Fuzhou Medical School of PLA, Center de Recherche de Vitro-Alfortville (France), SmithKline Beecham Pharmaceuticals (UK), and Tianjin Institute of Pharmaceutical Industries, respectively. Pin, Lem, RP, and Gli were dissolved in 0.001% Me_2SO; other agents were dissolved in distilled water.

Cell culture VSMC were isolated from the thoracic aorta of 8-wk-old rabbit by outgrowth of the explant method[8]. Briefly, the endothelium was carefully removed by abrasion and tunica media was peeled out from the adventitia. Tunica media segments were cut into 1 - 2 mm^2 explants and then cultured in a CO_2 incubator at 37 ℃ in RPMI 1640 medium (Gibco Laboratories, NY, USA) supplemented with 2 g $NaHCO_3$ and 20% newborn calf serum (NCS), penicillin (100 $kU \cdot L^{-1}$) and streptomycin (100 $mg \cdot L^{-1}$). The medium was changed every 3 d. After 2 - 3 wk of incubation, confluent cells were dispersed by brief exposure to

Hanks' medium containing 0.1 % (w/v) trypsin and 0.02 % (w/v) edetic acid. The cells were counted in hemocytometer and replated in tissue culture flasks containing 10 % NCS. Cultured VSMC were confirmed by electron microscopy[8]. Cells in 8th to 10th passages were used in the experiment.

$[Ca^{2+}]_i$ measurement After 5-d culture, VSMC were washed twice with Hank's solution, digested by using 0.1 % trypsin-containing 0.02 % edetic acid, and then washed again. The cells were suspended by loading solution: NaCl 137, KCl 5.37, $MgSO_4 \cdot 7H_2O$ 0.57, Na_2HPO_4 0.42, KH_2PO_4 0.44, glucose 11, HEPES 10, $CaCl_2$ 1.8 mmol $\cdot L^{-1}$ and 0.2 % (w/v) bovine albumin. Cell number was adjusted to 5×10^8 cells $\cdot L^{-1}$, then incubated with Fura-2 AM (2.5 μmol $\cdot L^{-1}$, final concentration) at 37 ℃ for 50 min. After incubation, cell was centrifuged (400 g, 4 min), washed and suspended with measurement solution (composition similar to loading solution but no $CaCl_2$). Cell viability (>95 %) was tested by trypan blue. Cell suspension was placed in detective chamber of fluorospectrometer (RF-5000, 2 wavelength, Japan). K^+, ATP, PCO, and Gli were added. $[Ca^{2+}]_i$ was determined[9].

Data analysis All data were expressed as $\bar{x} \pm s$, and t test was used for statistical analysis.

RESULTS

ATP-induced $[Ca^{2+}]_i$ increase $[Ca^{2+}]_i$ increase induced by ATP 0.1 mmol $\cdot L^{-1}$ showed a peak and a sustained phase. In the absence or presence of Ca^{2+} 1 mmol $\cdot L^{-1}$, $[Ca^{2+}]_i$ of VSMC were 125 ± 18 ($n = 34$) or 172 ± 22 nmol $\cdot L^{-1}$ ($n = 15$), respectively. $[Ca^{2+}]_i$ biphasic increase of VSMC induced by ATP was inhibited with a concentration-dependent manner by pretreatment with Pin, Nic, Lem, and RP in the presence of extracellular Ca^{2+} (Fig 1).

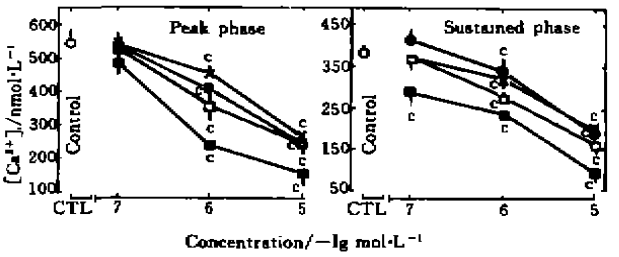

Fig 1. Effects of Pin (●), Nic (×), Lem (□), and RP (■) on ATP (0.1 mmol $\cdot L^{-1}$)-induced increase of cytosolic Ca^{2+} in Fura-2 AM-loaded cultured rabbit aortic smooth muscle cells. ATP added 0.001 % Me_2SO as control (○); $n = 4$ in all groups. $^cP < 0.01$ vs control.

In the absence of extracellular Ca^{2+}, $[Ca^{2+}]_i$ of VSMC was 70 ± 20 nmol $\cdot L^{-1}$ ($n = 15$). $[Ca^{2+}]_i$ peak elevation induced by ATP was reduced after pretreatment with Pin, Nic, Lem, and RP (10 μmol $\cdot L^{-1}$), but the $[Ca^{2+}]_i$ sustained increase was not affected by these agents (Tab 1).

K^+-induced $[Ca^{2+}]_i$ increase $[Ca^{2+}]_i$ increase by K^+ 30 mmol $\cdot L^{-1}$ was slowly achieved at plateau about 5 min. This $[Ca^{2+}]_i$ increase was slightly inhibited by pretreatment with Pin, Nic, Lem, and RP (Tab 1).

Blockade effect of Gli $[Ca^{2+}]_i$ of VSMC showed no change during incubation with Gli in the presence of extracellular Ca^{2+}. Gli did not affect $[Ca^{2+}]_i$ increase induced by ATP. The effects of Pin, Lem, and RP were completely, but the effect

Tab 1. Effect of four PCO (10 μmol $\cdot L^{-1}$) on ATP (0.1 mmol $\cdot L^{-1}$)-induced biphasic increase of $[Ca^{2+}]_i$ in the absence of extracellular Ca^{2+}, on added KCl (30 mmol $\cdot L^{-1}$)-induced $[Ca^{2+}]_i$ increase, and on ATP (0.1 mmol $\cdot L^{-1}$)-induced biphasic increase of $[Ca^{2+}]_i$ after added glibenclamide (10 μmol $\cdot L^{-1}$) in the presence of extracellular Ca^{2+} (1 mmol $\cdot L^{-1}$) in Fura-2/AM loaded cultured rabbit aortic smooth muscle cells. ATP added 0.001 % Me_2SO as Control. $n = 5$ in all groups. $^aP > 0.05$, $^cP < 0.01$ vs control.

	$[Ca^{2+}]_i$/nmol $\cdot L^{-1}$				
	No extracellular Ca^{2+}		Added KCl/ 30 mmol $\cdot L^{-1}$	Added glibenclamide	
	Peak phase	Sustained phase		Peak phase	Sustained phase
Control	258 ± 32	116 ± 27	544 ± 40	541 ± 39	380 ± 8
Pinacidil	129 ± 17^c	104 ± 14^a	441 ± 23^c	549 ± 39^a	413 ± 25^a
Nicorandil	142 ± 21^c	107 ± 14^a	455 ± 48^c	453 ± 31^c	348 ± 19^c
Lemakalim	136 ± 14^c	106 ± 12^a	451 ± 22^c	540 ± 30^a	364 ± 16^a
RP 49356	114 ± 9^c	84 ± 6^a	370 ± 31^c	564 ± 13^a	377 ± 11^a

of Nic was only partially, inhibited by Gli (1 μmol $\cdot L^{-1}$) on ATP-induced $[Ca^{2+}]_i$ increase (Tab 1).

DISCUSSION

Compared with the inhibitory effects on ATP-induced $[Ca^{2+}]_i$ increase, the blockade extent of the Pin, Nic, Lem, and RP on 30 mmol $\cdot L^{-1}$ K^+-induced $[Ca^{2+}]_i$ increase was weaker in the current study. It is suggested that the 4 PCO exhibited the cell membrane hyperpolarization, reduced the possibility of opening of voltage-dependent Ca^{2+} channels[10]. Because of polarization by lower concentration of K^+, the effect of Pin, Nic, Lem, and RP on K^+-induced $[Ca^{2+}]_i$ increase did not exert completely[11].

Sulfuriods, for an example, glibenclamide have the ability to cancel the effects of PCO on heart and vascular smooth muscle attributed to block ATP-sensitive K^+ channels. Our study showed that the inhibitory effects of Pin, Lem, and RP on ATP-induced $[Ca^{2+}]_i$ increase were completely abolished but that of Nic was only partially blocked by glibenclamide. These facts implied that the effect of Pin, Lem, RP, and Nic possess the opining the ATP-sensitive K^+ channel, whereas Nic might have other mechanisms. Due to the structure containing nitro-group, Nic promoted nitro oxide formation in the tissues or cells, and suppressed the agonists-induced $[Ca^{2+}]_i$ increase[12]. Interestingly, recent study indicated that nitroglycerin which also containing nitro-group, had not the ability to decrease $[Ca^{2+}]_i$[13]. Further studies need to be done to determine the mechanisms of Nic on ATP-induced $[Ca^{2+}]_i$ elevation.

ATP-induced $[Ca^{2+}]_i$ increase was majority due to Ca^{2+} release from inositol 1, 4, 5-trisphosphate (IP_3) sensitive intracellular Ca^{2+} store and it might also attribute to Ca^{2+} influxes from the extracellular site at the sustained phase[14]. Ito *et al*[15] indicated that Lem selectively inhibited IP_3 synthesis and this effect of Lem was blocked by glibenclamide. In our experiment, $[Ca^{2+}]_i$ elevation by ATP was decreased by Pin, Nic, Lem, and RP in the absence of extracellular Ca^{2+}, which suggested that the action of the 4 PCO be associated with decreasing Ca^{2+} release from intracellular store. On the other hand, compared with the decrease magnitude of $[Ca^{2+}]_i$ increase by ATP in the presence of extracellular Ca^{2+}, these agents might also inhibit Ca^{2+} influx from extracellular site.

In conclusion, Pin, Nic, Lem, and RP significantly inhibited $[Ca^{2+}]_i$ increase induced by ATP. This effect was blocked by glibenclamide. The action of Nic possesses multiple mechanisms. Pin, Nic, Lem, and RP inhibit both Ca^{2+} influx from extracellular site and Ca^{2+} release from intracellular store.

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钾通道开放剂降低血管平滑肌细胞内游离钙浓度[1]

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关键词 钾通道；腺苷三磷酸；血管平滑肌；钙；格列苯脲

目的：研究 PCO—Pin，Nic，Lem 及 RP 对 VMSC 内$[Ca^{2+}]_i$的改变及其可能机制。**方法**：VSMC 加入 Fura-2 AM 2.5 μmol·L^{-1} 37 ℃ 下孵育 50 min，$[Ca^{2+}]_i$用荧光分光光度计检测。**结果**：4 种 PCO 能较弱地抑制 K^+ 30 mmol·L^{-1}诱导的$[Ca^{2+}]_i$增加，但明显抑制 ATP 0.1 mmol·L^{-1}诱导的$[Ca^{2+}]_i$峰相及持续相增加，且呈剂量依赖性。格列苯脲完全阻断 Pin，Lem 及 RP 的作用，只部分抑制 Nic 的作用。无钙液中先给 4 种 PCO，能显著抑制 ATP 诱导的$[Ca^{2+}]_i$峰相增加。**结论**：4 种 PCO 均抑制 ATP 诱导的$[Ca^{2+}]_i$增加，此作用与减少细胞外钙内流及细胞内钙释放有关。

重组人生长激素获准上临床

珠海经济特区恒通生物工程制药公司拳头产品重组人生长激素(γ-HGH)于 1996 年 1 月正式获得卫生部药政管理局的临床批文。

重组人生长激素主要适用于垂体性侏儒症和多种原因引起的矮小症，还可用于严重烧伤、艾滋病、延缓衰老等。该产品经中国科学院上海细胞生物学研究所研制成功，1992 年 6 月通过了中科院院级技术鉴定和国家中试工艺验收。同年 9 月转让于恒通生物工程制药公司。该产品在美国莱曼兄弟公司(Lehman Brothers)发表的 2000 年 114 种畅销药物预测中排名 11。

目前恒通生物工程制药公司生产的重组人生长激素质量基本上达到国际同类产品的质量水平。侏儒症患者平均每月身高增长达 1 cm - 1.5 cm，最好可达 2 cm。此外，根据美国威斯康星大学 Rudman 教授对 61 - 81 岁的健康人注射 6 个月 HGH 后，精力较以前充沛，健康状况明显好转，从对肌肉、脂肪、皮肤、骨骼等组织进行相应的各项生理测定表明：明显年轻 10 - 20 岁。由此可见重组人生长激素具有可观的前景。据欧美及日本预测 2000 年，世界销售额可达 20 亿美元。如进一步开发其用于烧伤、外科、老年病等潜在市场，总销售额可达 70 亿美元。而根据不完全统计我国近年市场需求量，销售额可达 63 亿元。

恒通生物工程制药公司拳头产品重组人生长激素的生产及临床应用的成功是我国基因工程技术在科研成果产业化过程中的一次突破性成功范例，填补了我国生物工程制药的一项空白。国产重组 HGH 的问世，不仅从根本上克服了国外药品价格昂贵、来源不足、数量有限的问题，而且也为无数侏儒症患者，无数渴望健康的中老年人带来福音。