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Original Research

Effects of superoxide anion on intracellular Ca^{2+} concentration in rabbit pulmonary arterial smooth muscle cells

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KEY WORDS calcium; cell separation; vascular smooth muscle; pulmonary artery; free radicals; xanthines; xanthine oxidase; adenosine triphosphate; thapsigargin

ABSTRACT

AIM: To study the effect of superoxide anion on the Ca^{2+} homeostasis in smooth muscle cells isolated from the rabbit pulmonary artery. **METHODS**: Intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) was investigated using cell suspension of freshly isolated smooth muscle cells from rabbit pulmonary artery (PASMC). Fura-2 fluorescent ratio obtained at 340 nm and 380 nm wave lengths was measured as an indicator of $[Ca^{2+}]_i$. **RESULTS**: ATP 30 $\mu mol \cdot L^{-1}$ induced a transient increase in the ratio (Ca^{2+} transient). Thapsigargin, an inhibitor of sarcoplasmic Ca^{2+} ATPase, induced a phasic increase in the ratio due to Ca^{2+} leak from intracellular store sites, but not the sustained increase, thereby suggesting the absence of Ca^{2+} release-activated Ca^{2+} entry (CRAC) mechanism in PASMC. When PASMC were exposed to superoxide anion by the pretreatment with xanthine and xanthine oxidase (X/XO) for 30 min, sustained component of ATP-induced Ca^{2+} transient was elevated. The ratios at 5 and 10 min after ATP application ($\Delta ratio_{5\ min}$ and $\Delta ratio_{10\ min}$) were increased from 0.091 ± 0.022 to 0.149 ± 0.048 ($P < 0.05$) and from 0.021 ± 0.020 to 0.117 ± 0.047 ($P < 0.01$), respectively. But, thapsigargin-induced $[Ca^{2+}]_i$ transient was not affected by X/XO. **CONCLUSION**: Superoxide anion makes ATP-induced Ca^{2+} transient sluggish, and does not affect Ca^{2+} leak pathway in PASMC.

INTRODUCTION

The elevation of intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) in vascular smooth muscle is the initial step of vascular contraction[1]. Main Ca^{2+} sources for the contraction of smooth muscle are Ca^{2+} entry through voltage-dependent Ca^{2+} channel and Ca^{2+} release by inositol trisphosphate from intracellular store sites. Alteration of these Ca^{2+} movement would result in the disorder of vascular functions. We have reported that superoxide anion affected Ca^{2+} homeostasis by inhibiting Ca^{2+} release-activated Ca^{2+} entry (CRAC) and plasmalemmal Ca^{2+} ATPase, and by accelerating Ca^{2+} leak from intracellular store in vascular endothelium[2]. Oxidative stress including superoxide anion has been known as a cause of various vascular diseases such as hypertension[3], coronary disease[4], and diabetic vascular complications[5]. Therefore, the investigation of the effects of oxidative stress on $[Ca^{2+}]_i$ movement of vascular smooth muscle would be of importance in vascular pathophysiology. Such studies would

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be especially important in pulmonary artery because it is located in a highly aerobic environment thereby being exposed to an oxidative stress continuously. The aim of the present study was to investigate the effect of superoxide anion on the Ca^{2+} homeostasis in rabbit pulmonary artery smooth muscle cells (PASMC).

MATERIALS AND METHODS

Isolation of PASMC Albino rabbit (♂, 1.2 - 1.5 kg) was anesthetized with pentobarbital and exsanguinated. Pulmonary artery was excised in Krebs' solution. Smooth muscle layer was incubated in low Ca^{2+} solution containing papain 0.3 $g \cdot L^{-1}$ at 4 ℃ for 20 h. After the further incubation in the same solution but with dithiothreitol 0.3 $mmol \cdot L^{-1}$ at 37 ℃ for 10 min, the tissues were gently agitated to harvest the single cells. Cells were collected by centrifugation and kept at 4 ℃ in low Ca^{2+} solution containing 0.025 % bovine serum albumin.

Measurement of $[Ca^{2+}]_i$ $[Ca^{2+}]_i$ was measured using Fura-2. Cell suspension was incubated with membrane permeable Fura-2, Fura 2-AM (Dojindo Laboratories, Kumamoto, Japan) at 28 ℃ for 20 min and following 20 min at 37 ℃. The final concentration of Fura-2 was 1 $\mu mol \cdot L^{-1}$. After centrifugation, cells were suspended in normal Krebs' solution at a density of 2×10^9 cells $\cdot L^{-1}$. Fura 2-loaded cell suspension (500 μL) was put in a cuvette and measured at 340 nm and 380 nm. Emitted light of 500 nm (F340 and F380) was monitored by a spectrofluorometer (CAF-110, Japan Spectroscopic, Tokyo), and Fura 2-fluorescent ratio (F340/F380) was calculated as an indicator of $[Ca^{2+}]_i$. Because the relative values of ratio elevation was enough for the purpose of this study, we did not perform $[Ca^{2+}]_i$ calibration. Background fluorescence was not subtracted. All experiments were performed at 37 ℃.

Reagents ATP, thapsigargin[2], xanthine, and xanthine oxidase were from Sigma. Modified Krebs' solution was used in the present experiment containing NaCl 132.4, KCl 5.9, $CaCl_2$ 1.5, $MgCl_2$ 1.2, glucose 11.5, HEPES 11.5 $mmol \cdot L^{-1}$, and pH was adjusted to 7.4 by NaOH. For the cell dispersion and storage we used low Ca^{2+} ($CaCl_2$ 0.16 $mmol \cdot L^{-1}$) Krebs' solution where $CaCl_2$ was replaced with egtazic acid (EGTA) 0.92 $mmol \cdot L^{-1}$.

Statistics Pooled data were analyzed using worksheet program, Excel (Microsoft). Values were expressed as $\bar{x} \pm s$ and compared with t test.

RESULTS

Effect of superoxide anion on ATP-induced $[Ca^{2+}]_i$ increase In control experiments, cells were incubated with xanthine 100 $\mu mol \cdot L^{-1}$ for 30 min. An application of ATP at a final concentration of 30 $\mu mol \cdot L^{-1}$ induced a steep increase in fluorescent ratio in PASMC. The peak amplitude of the net ratios increase ($\Delta ratio_{peak}$) was 0.24 ± 0.08 (n = 5). Elevated ratio declined slowly, and the net ratios increased at 5 and 10 min after ATP application ($\Delta ratio_{5\ min}$ and $\Delta ratio_{10\ min}$) were 0.091 ± 0.022 and 0.021 ± 0.020, respectively (n = 5, Fig 1A&C). The time constant of the elevated ratio could not be fitted by a single exponential decay in PASMC.

When the cells were exposed to xanthine (X) 100 $\mu mol \cdot L^{-1}$ and xanthine oxidase (XO) 10 $U \cdot L^{-1}$ for 30 min, There was no difference in the resting values of fluorescent ratio between control (X alone) and X/XO-treated cells (control 2.08 ± 0.23, X/XO 2.34 ± 0.22; $P > 0.05$). X/XO treatment did not affect the amplitude of the ATP-induced increase in the ratio ($\Delta ratio_{peak}$ = 0.16 ± 0.05, n = 5, $P > 0.05$). However, X/XO treatment prolonged

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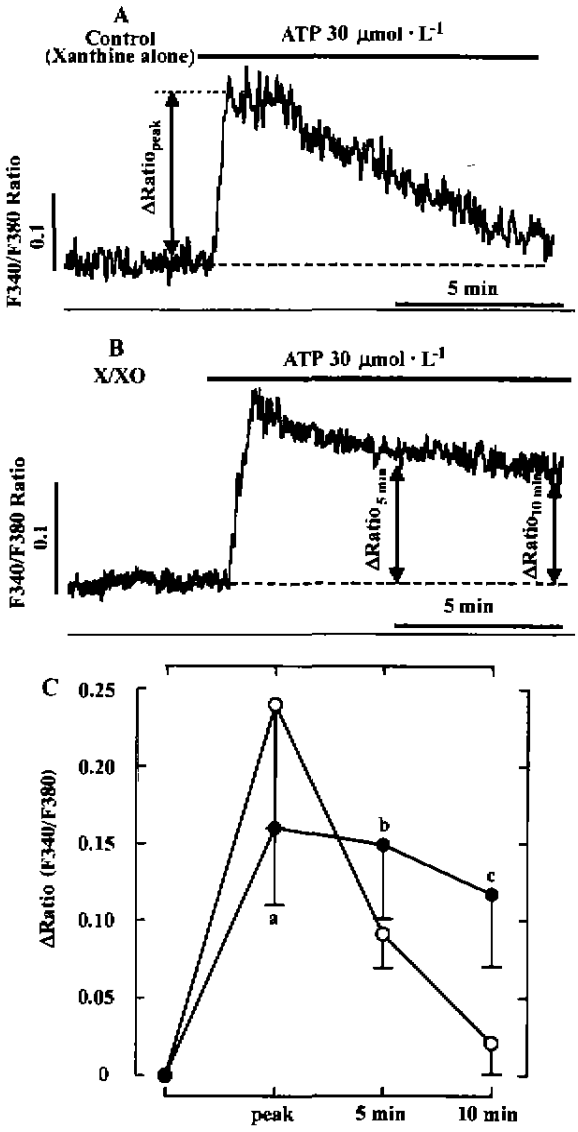

Fig 1. Effects of ATP on $[Ca^{2+}]_i$ of rabbit pulmonary artery smooth muscle cell (PASMC) suspension. A) ATP final concentration of 30 μmol·L^{-1} was added to xanthine 100 μmol·L^{-1} treated suspension. B) Cells were treated with xanthine (X) 100 μmol·L^{-1} and xanthine oxidase (XO) 10 U·L^{-1} for 30 min before ATP. C) Increment of fluorescent ratio (Δratio) at peak level and 5 or 10 min after starting ATP in control and X/XO-treated cells ($n = 5$).
$^aP > 0.05$, $^bP < 0.05$, $^cP < 0.01$ *vs* control.

the ATP-induced increase in the ratio. Namely, ATP-induced ratio sustained at a higher level, ratio of which at 5 and 10 min after the application ($\Delta ratio_{5\ min}$ and $\Delta ratio_{10\ min}$) were 0.149 ± 0.048 and 0.117 ± 0.047, respectively (Fig 1B&C, $n = 4$; $P < 0.05$ and $P < 0.01$ compared with control, respectively). These results indicated that superoxide anion delayed ATP-induced Ca^{2+} transient.

Effect of superoxide anion on thapsigargin-induced $[Ca^{2+}]_i$ increase When thapsigargin 1 μmol · L^{-1} was applied to the control cells pretreated with xanthine 100 μmol · L^{-1}, a phasic increase in the ratio was observed and the maximal rate of rise was 0.019 ± 0.012 s^{-1} ($n = 10$; The peak value of $dratio/dt$ was measured as in the inset of Fig 2A).

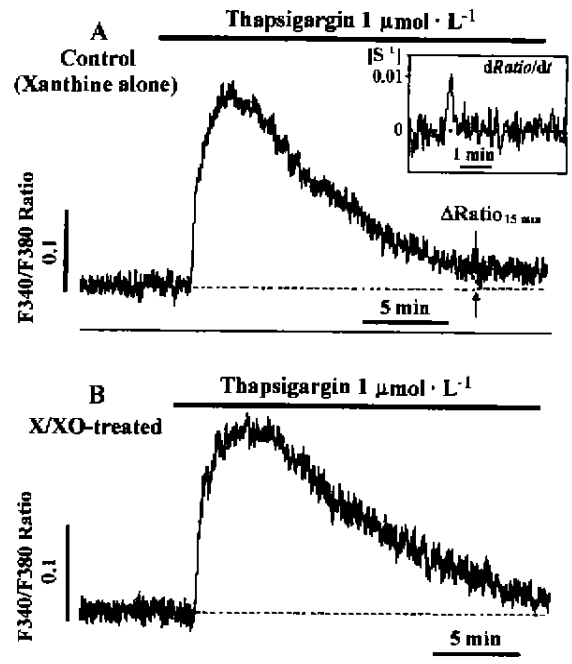

Fig 2. Effects of thapsigargin on $[Ca^{2+}]_i$ of PASMC suspension. A) Thapsigargin (1 μmol · L^{-1}) was applied to xanthine 100 μmol·L^{-1} treated suspension. The inset shows the differential curve ($dratio/dt$) of the fluorescent ratio trace between two asterisks. B) Cells were pretreated with X/XO for 30 min before the experiment. Thapsigargin-induced increase in the ratio did not show any remarkable difference from control (xanthine alone).

The net ratio increase 15 min after starting thapsigargin application ($\Delta ratio_{15\ min}$) was 0.011 + 0.028 (n = 10), thereby indicating that the store depletion did not induce apparent Ca^{2+} entry in PASMC.

When thapsigargin was applied to the X/XO-treated cells, fluorescent ratio elevated with the same maximal rate of rise (0.020 ± 0.007, n = 6; $P > 0.05$). There was no significant difference in $\Delta ratio_{15\ min}$ between treated and control PASMC (0.019 ± 0.029, n = 6, $P > 0.05$) (Tab 1). These observations suggested that thapsigargin-induced $[Ca^{2+}]_i$ transient was not affected by superoxide anion.

Tab 1. Effects of thapsigargin on $[Ca^{2+}]_i$ in xanthine and xanthine oxidase-treated rabbit pulmonary artery smooth muscle cell suspension. $n = 6-10$, $\bar{x} \pm s$. $^aP > 0.05$ *vs* xanthine.

	Xanthine	Xanthine + xanthine oxidase
F340/F380 $Ratio_{15\ min}$	0.011 ± 0.028	0.019 ± 0.029[a]
$dratio/dt_{max}$	0.019 ± 0.012	0.020 ± 0.007[a]

DISCUSSION

In this study we measured Ca^{2+} from cell suspension of freshly isolated PASMC. $[Ca^{2+}]_i$ measurement using cell suspension has an advantage for measuring the averaged $[Ca^{2+}]_i$ response, but has disadvantages that cells are detached and continuously stirred throughout the experiment.

ATP induced a rapid increase in the fluorescent ratio (Ca^{2+} transient) in PASMC. Such Ca^{2+} transient was also observed in Ca^{2+} free solution (data not shown), thereby indicating that the Ca^{2+} transient was mainly due to Ca^{2+} release from intracellular store sites. The time course of the decay of increased ratio could not be fitted by a single exponential regression curve, and sustained increase in $[Ca^{2+}]_i$ was observed. These observations taken together indicate that ATP-induced Ca^{2+} influx is present in PASMC as has been reported previously in other smooth muscle cells[6]. Superoxide anion slightly decreased $\Delta ratio_{peak}$ and significantly increased the sustained level of the increase in the ratio, which may be due to the inhibition of Ca^{2+} extrusion or to the increase of ATP-induced Ca^{2+} entry. Extrusion of the elevated cytosolic Ca^{2+} could be obtained through Ca^{2+} pumps[7], mitochondria[8] or intracellular Ca^{2+} buffering system[9], and therefore superoxide anion could impair any of these mechanisms. In the previous report, it was shown that superoxide anion inhibited plasmalemmal Ca^{2+} pump in endothelial cells[2]. Although the precise mechanism has not been clarified in the present experiment, it can be summarized that Ca^{2+} increase in response to ATP becomes sluggish by superoxide anion.

Thapsigargin induced the Ca^{2+} leak from intracellular store sites but not CRAC in PASMC. The presence of CRAC phenomenon has been reported in various smooth muscle cells mainly in cultured cells[10]. However a few reports also have suggested the presence in freshly isolated smooth muscle cells[11]. Previously, it was shown that superoxide anion accelerated thapsigargin induced Ca^{2+} leak and inhibits CRAC in the endothelial cells[2]. Ca^{2+} leak pathway has been reported to play a role in mechanosensitive Ca^{2+} mobilization in A7r5 cultured smooth muscle cell[12]. Superoxide anion did not alter Ca^{2+} leak velocity, thereby indicating that superoxide anion does not affect leak pathway in PASMC.

Superoxide anion is known to be produced at wounded lesion, and accumulation of superoxide anion results in various vascular diseases. Superoxide anion inhibited Ca^{2+} ATPase in IP3-sensitive store sites in cultured smooth muscle cells from pig coronary artery[13]. The present results unveiled that superoxide anion also affected ATP-induced Ca^{2+} mobilization.

Because of the limitation of the experimental device, the effect of superoxide anion on voltage-dependent Ca^{2+} channel could not be examined.

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Further investigation is needed for clarifying the overall effects of superoxide anion on smooth muscle Ca^{2+} homeostasis. In summary, the results indicated that superoxide anion prolonged the ATP-induced Ca^{2+} transient in freshly isolated PASMC.

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超氧阴离子对兔肺动脉平滑肌细胞内钙的影响

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关键词 钙；细胞分离；血管平滑肌；肺动脉；自由基；黄嘌呤；黄嘌呤氧化酶；腺苷三磷酸；thapsigargin

目的：研究超氧阴离子对兔肺动脉平滑肌细胞内钙的影响. **方法**：采用 Fura-2 测定酶分离的兔肺动脉平滑肌细胞内钙. **结果**：ATP 30 $\mu mol \cdot L^{-1}$诱导平滑肌细胞内钙瞬时性增加. Thapsigargin 引起平滑肌细胞内钙缓慢的增加. 超氧阴离子作用于平滑肌细胞后，使 ATP 诱导细胞内钙增加的持续相升高，在 ATP 作用后 5 和 10 min 的比值（$\Delta ratio_{5\ min}$ 和 $\Delta ratio_{10\ min}$）分别由 0.091 ± 0.022 和 0.021 ± 0.020 升高至 0.149 ± 0.048 和 0.117 ± 0.047. 但超氧阴离子对 thapsigargin 诱导的细胞内钙变化没有明显的影响. **结论**：超氧阴离子延迟 ATP 诱导的平滑肌细胞内钙瞬时性增加，而不影响钙的泄漏途径.

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