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Anti-proliferating effect of iptakalim, a novel K_{ATP} channel opener, in cultured rabbit pulmonary arterial smooth muscle cells

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Abstract

ATP-sensitive potassium (K_{ATP}) channels of pulmonary arterial smooth muscle cells (SMCs) have been implicated in pulmonary hypertension. Iptakalim, designed and synthesized by ourselves, is a newly selective K_{ATP} channel opener. Here, we explored the effects of iptakalim on the rise of cytoplasmic free Ca^{2+} concentration ($[Ca^{2+}]_{cyt}$) induced by endothelin-1 (ET-1) and on the proliferation of cultured rabbit pulmonary arterial SMCs. The results showed that iptakalim inhibited the $[Ca^{2+}]_{cyt}$ increase. The enhanced [^{3}H]thymidine incorporation was inhibited and the transition of cells from static phase (G_0/G_1) to DNA synthesis (S) and mitotic phase (G_2/M) was held back by iptakalim in a concentration-dependent manner. Glyburide abolished the inhibitory effect of iptakalim. In conclusion, we have shown that iptakalim had an inhibitory effect on $[Ca^{2+}]_{cyt}$ increase and the proliferation of pulmonary arterial SMCs induced by endothelin-1 through activation of K_{ATP} channels. These findings suggest that iptakalim might be a promising candidate for the treatment of pulmonary arterial remodeling in pulmonary hypertension.

Keywords: Proliferation; Iptakalim; ATP-sensitive potassium channel; Fluo-3; Pulmonary arterial smooth muscle cell

1. Introduction

Pulmonary arterial smooth muscle cell (SMC) proliferation is a key feature during the development of hypoxic pulmonary hypertension. K^+ channel dysfunction in the plasma membrane plays a key role in pulmonary arterial SMC proliferation by modulating cytoplasmic free Ca^{2+} concentration ($[Ca^{2+}]_{cyt}$) (Mandegar and Yuan, 2002). The biophysical properties, regulation, and pharmacology of ATP-sensitive potassium (K_{ATP}) channels have received intense scrutiny during the past twenty years (Cole and Clement-Chomienne, 2003). The alterations of K_{ATP} channel activity in vascular SMCs in response to vasoactive agonists such as endothelin-1 (ET-1) contribute to the regulation of arterial diameter (Nelson and Quayle, 1995; Quayle et al., 1997; Brayden, 2002). The ability of K_{ATP} channel activation to regulate pulmonary pressure has prompted a widespread interest in these channels as potential targets for the pharmacologic control of vascular contractility in disease conditions, such as pulmonary hypertension (Cole and Clement-Chomienne, 2003).

Iptakalim, a fatty para-amino compound with low molecular weight, has been confirmed by substantial pharmacological, biochemical, and electrophysiological studies, as well as receptor-combining tests as a newly selective K_{ATP} channel opener (K_{ATP}CO) (Wang, 2003). Our studies have shown that outward potassium currents of freshly isolated SMCs from rat intrapulmonary arteries could be enhanced in the presence of iptakalim and pulmonary artery remodeling could be alleviated by chronic iptakalim treatment (Xie et al., 2004). The present study was undertaken to explore the effects of iptakalim on pulmonary arterial SMC proliferation and the $[Ca^{2+}]_{cyt}$ rise induced by endothelin-1.

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2. Materials and methods

2.1. Cell preparation and culture

The animal study protocol was in compliance with the guidelines of China for animal care, which was conformed to the internationally accepted principles in the care and use of experimental animals. New Zealand rabbits (1500–2500 g) provided by Jiangsu Province Laboratorial Animal Center (China) were anaesthetized with methoxyflurane. Primary cultures of rabbit pulmonary arterial SMCs were prepared as previously described (Ross, 1971). Briefly, the right and left branches (2nd division) of the main pulmonary artery and intrapulmonary arteries (3rd–4th division) were incubated in physiological balanced saline (PBS, in mM) containing NaCl 138.0, KCl 5.0, Na_2HPO_4 0.3, KH_2PO_4 0.3, $NaHCO_3$ 4.0, penicillin 105 U/l and streptomycin 100 mg/l. A thin layer of adventitia was carefully stripped off with a fine forceps, and endothelium was removed by gently scratching the intimal surface with a surgical blade. The remaining portion of the media was cut into approximately 1-mm squares and placed in culture bottle containing Dulbecco's Modified Essential Medium (DMEM) with 20% fetal bovine serum (FBS). The bottle was placed in a moist tissue culture incubator at 37°C containing an atmosphere of 95% O_2 and 5% CO_2. The purity of pulmonary arterial SMCs in the primary cultures was confirmed by positive staining with smooth muscle α-actin antibody compared with a known positive control of SMCs.

2.2. Measurements of $[Ca^{2+}]_{cyt}$ in pulmonary arterial SMCs

The changes of $[Ca^{2+}]_{cyt}$ were measured using the fluo-3 fluorescence method as described previously (Hardingham et al.,1997) under laser scanning confocal microscope (Zeiss LSM510, Oberkochen, Germany). Cells suspended in DMEM containing 10% fetal bovine serum seeded (10^4 cells/well) in 6-well plates on circular 25-mm glass coverslips. After 24 h, cells were loaded with Fluo-3 working solution (Fluo-3 AM 5 mM and Pluronic F-127 0.03% dissolved in standard buffer) at 37 °C. Changes in the Fluo-3 fluorescence intensity (FI) indicating fluctuations in $[Ca^{2+}]_{cyt}$ were recorded. After stable baseline fluorescence intensity was measured, endothelin-1 was added to extracellular medium to yield a concentration of 10 nM, and the fluorescence intensity was recorded for 300 s.

2.3. Measurements of DNA synthesis

Pulmonary arterial SMCs suspended in DMEM containing 10% fetal bovine serum were seeded (10^4 cells/well) in 96-well plates. After 24 h, the cells were made quiescent by incubation in serum-free DMEM for 24 h. Then, agents to be tested, i.e., endothelin-1 alone or in combination with iptakalim, were added into fresh DMEM. Cells were incubated with test substances for 24 h. In experiments using selective K_{ATP} channel antagonists, cells were preincubated with glyburide at the concentrations of 0.1, 1, and 10 μM for 0.5 h prior to addition of other test substances at the start of the experiment, with the antagonist remaining present throughout the experiment. We used pinacidil as one positive control K_{ATP} opener in that pinacidil can potentiate the outward potassium currents in pulmonary arterial SMCs (Wang, 2003), and glyburide (glibenclamide) as one selective K_{ATP} channel antagonist for glibenclamide (0.1–10 μM) reverses the vasodilator effect of cromakalim, one K_{ATP} opener, in a dose-dependent manner in aortic rings (Ravel et al., 2003). [^{3}H]Thymidine was added to each well at 18th hour to a final concentration of 18.5 kBq/ml. At the end of the incubation period, the incorporated radioactivity (counts per minute) was measured by fixation and solubilisation of cells with a Beckman liquid scintillation counter. Experiments were performed 10 times independently in duplicate.

2.4. Analysis of cell cycle progression

Pulmonary arterial SMCs were cultured in culture bottles and were made quiescent as described above. After addition of drug for 24 h, cells were trypsinized and centrifuged at 1500×*g* for 6 min, and then were fixed with ice-cold 70% ethanol for 30 min. Finally, the cells were stained with a mixture of propidium iodide (50 mg/l) for 30 min. The minimum of 10^6 events was collected and analyzed by software Cell Quest (FACScan, Becton Dickson, USA).

2.5. Drugs and reagent

Pluronic F-127, endothelin-1, propidium iodine, glyburide, and Me_2SO (DMSO) were all purchased from Sigma Co. Fluo-3 AM (Molecular Probes, USA) was dissolved in DMSO and stored at −20 °C. Dulbecco's Modified Essential Medium (DMEM), fetal bovine serum (FBS), and trypsin were purchased from GIBCO Co. α-Actin antibody was obtained from Boehringer Mannheim. [^{3}H]Thymidine (specific activity, 1 Ci/l) was obtained from Shanghai Institute of Nuclear Research, China. Iptakalim, with a purity of 99.36%, was synthesized and provided by the Institute of Pharmacology and Toxicology, Academy of Military Medical Sciences, China.

2.6. Statistical analysis

Mean values were calculated based on data obtained in preparations from a number of different cells and were quoted together with their S.D. All parameters compared statistically were first shown to be normally distributed using the Kolmogorov–Smirnov test. Mean values were then analyzed by two-way analysis of variance (ANOVA) with control group and endothelin-1 group as one factor and treatment regime (i.e., no drug, iptakalim at the concentrations of 0.1, 1, and 10 μM) as the other factor. If there was

a significant effect of endothelin-1, a Student's *t*-test (unpaired values) was done to compare between control group and endothelin-1 group. If there was a significant effect of drug treatment, a Student's *t*-test or one-way ANOVA with a Newman–Keul's post hoc test was carried out separately for control group and endothelin-1 group. If there was a significant interaction between the two factors, both of the above post tests were performed regardless of whether the two-way ANOVA indicated a significant effect of endothelin-1 or drug treatment. $P<0.05$ was considered as statistically significant.

3. Results

3.1. Effects of iptakalim on $[Ca^{2+}]_{cyt}$ in pulmonary arterial SMCs

Endothelin-1 at the concentration of 10 nM induced a monophasic increase of Fluo-3 fluorescence intensity (FI); endothelin-1 elicited an initial peak of $[Ca^{2+}]_{cyt}$, in which fluorescence intensity increased to 143.84±28.23 from 73.70±10.12 (100.72±8.16% vs. baseline, n=6) in 60 s, and then fell to a sustained plateau (Fig. 1). Pretreatment of pulmonary arterial SMCs with iptakalim at the concentration of 10 μM for 10 min significantly prevented transient increase of $[Ca^{2+}]_{cyt}$ elicited by endothelin-1. The fluorescence intensity in these cells only increased to 86.03±9.82 from 74.30±10.2 (15.78±2.25% vs. baseline, n=6) in 60 s (Fig. 2).

3.2. Effects of iptakalim on proliferation of pulmonary arterial SMCs

As shown in Fig. 3, endothelin-1 (10 nM) stimulated [^{3}H]thymidine uptake in serum-deprived cells. The value of [^{3}H]thymidine incorporation in the endothelin-1 group was increased 1.46 times higher than that in the control group (n=10). Pinacidil (10 μM) antagonized the effects of endothelin-1 in DNA synthesis of cells. Treatment of pulmonary arterial SMCs with iptakalim resulted in a reduction of endothelin-1-induced [^{3}H]thymidine incorporation in a concentration-dependent manner. Iptakalim at the concentrations of 0.1, 1, and 10 μM lowered [^{3}H]thymidine incorporation by 19.75±4.60% (n=10), 41.20±9.49% (n=10), and 54.74±10.11% (n=10), respec-

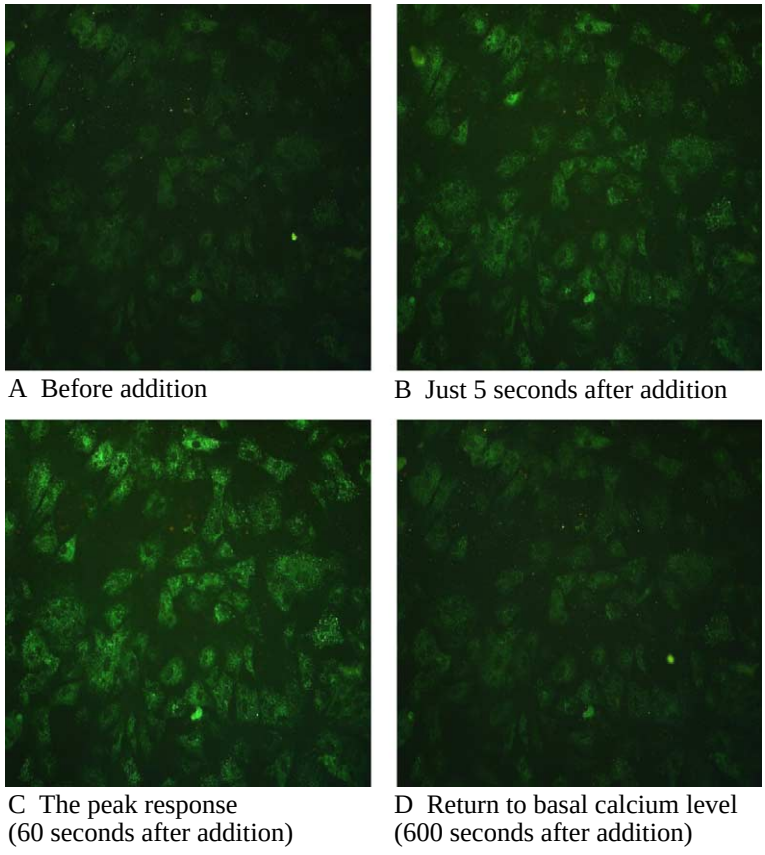

Fig. 1. Increase in $[Ca^{2+}]_{cyt}$ of pulmonary arterial SMCs in response to endothelin-1. The pictures were taken under inverted fluorescence microscope before and after addition of endothelin-1 to pulmonary arterial SMCs. Fluo-3 is used as intracellular calcium indicator. An increase in Fluo-3 fluorescence indicates the increase in intracellular calcium. A. Fluo-3 basal fluorescence. B. Fluo-3 fluorescence 5 s after addition of endothelin-1 (10 nM). C. Fluo-3 fluorescence 60 s after addition of endothelin-1 (10 nM). D. Fluo-3 fluorescence 600 s after addition of endothelin-1 (10 nM).

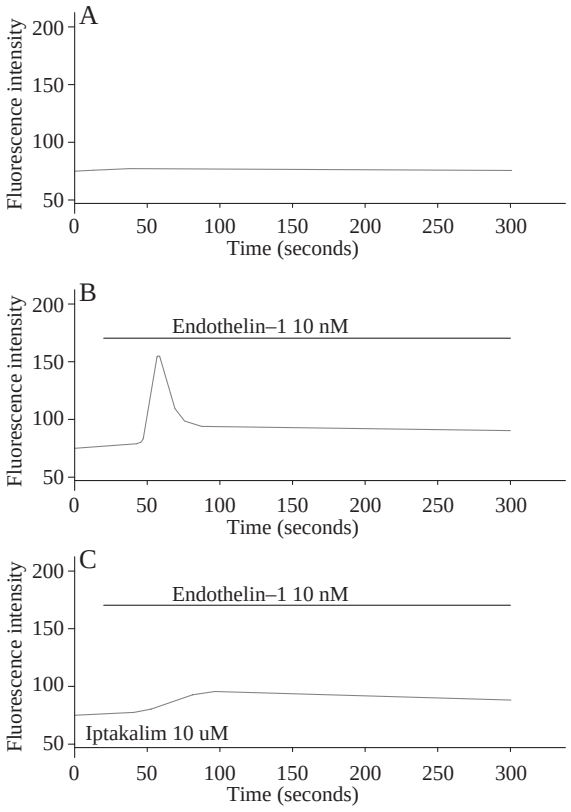

Fig. 2. The effects of iptakalim on changes of $[Ca^{2+}]_{cyt}$ in pulmonary arterial SMCs. Changes in $[Ca^{2+}]_{cyt}$ were estimated by fluorescence measurement using Ca^{2+} indicator Fluo-3 under laser scanning confocal microscope. Changes in the Fluo-3 fluorescence intensity (FI) indicating fluctuations in $[Ca^{2+}]_{cyt}$ were recorded. After stable baseline fluorescence intensity was measured, endothelin-1 was added to extracellular medium to yield a concentration of 10 nM, and the fluorescence intensity was recorded for 300 s. A. Control (6 cells). B. Endothelin-1 induced a monophasic increase in $[Ca^{2+}]_{cyt}$ of pulmonary arterial SMCs (6 cells). C. Pretreatment of pulmonary arterial SMCs with iptakalim significantly prevented $[Ca^{2+}]_{cyt}$ rise induced by endothelin-1 (6 cells).

tively, compared with that of cells treated only with endothelin-1. When cells were preincubated with glyburide at the concentration of 10 μM for 0.5 h prior to addition of pinacidil and endothelin-1, the value of [^{3}H]thymidine uptake was close to the value of cells treated only with endothelin-1. Similar to that, glyburide abolished the inhibitory effect of iptakalim at the concentration of 10 μM on [^{3}H]thymidine uptake in a concentration-dependent manner. The value of [^{3}H]thymidine incorporation was increased by 49.11±8.69% (n=10), 68.63±15.09% (n=10), and 110.05±26.82% (n=10) in the presence of glyburide at the concentrations of 0.1, 1, and 10 μM, respectively, compared with that of cells treated simultaneously with endothelin-1 10 nM and iptakalim 10 μM (Fig. 4). These results suggest that iptakalim antagonized proliferation of pulmonary arterial SMCs induced by endothelin-1 through activation of K_{ATP} channels.

3.3. Effects of iptakalim on cell cycle of pulmonary arterial SMCs

As shown in Table 1, DNA content of nuclei in pulmonary arterial SMCs was measured by flow cytometry analysis after 24 h of exposure to chemical compounds. Endothelin-1 propelled pulmonary arterial SMCs from static phase (G_0/G_1) to DNA synthesis (S) and mitotic phase (G_2/M). The total of S and G_2/M phase ratios in endothelin-1 group was increased by 210.11±37.85% (n=3), while G_0/G_1 phase ratio was decreased by 22.04±1.07% (n=3) compared with control group. When the cells treated simultaneously with endothelin-1 (10 nM) and pinacidil (10 μM), the ratios of G_0/G_1, S, and G_2/M phase were similar to those of the control. However, glyburide (10 μM) antagonized the effects of pinacidil (10 μM). In the groups treated with both endothelin-1 and iptakalim at the concentration of 0.1,

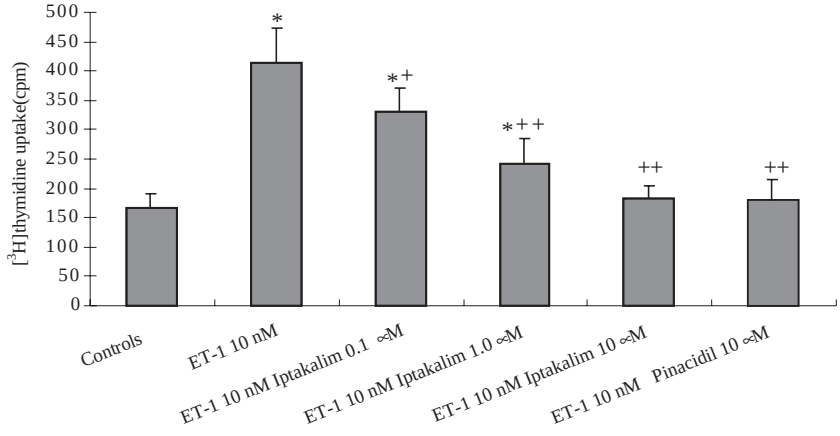

Fig. 3. Effects of iptakalim on [^{3}H]thymidine uptake of pulmonary arterial SMCs. The value of [^{3}H]thymidine incorporation in endothelin-1 group was increased 1.46 times compared with that in control group. Treatment of pulmonary arterial SMCs with iptakalim resulted in a reduction of [^{3}H]thymidine uptake induced by endothelin-1 in a concentration-dependent manner. Experiments were performed 10 times independently in duplicate. *Significantly different from corresponding values in control group (P<0.05). $^+$Significantly different from corresponding values in endothelin-1 group (P<0.05). $^{++}$Significantly different from corresponding values in endothelin-1 group (P<0.01).

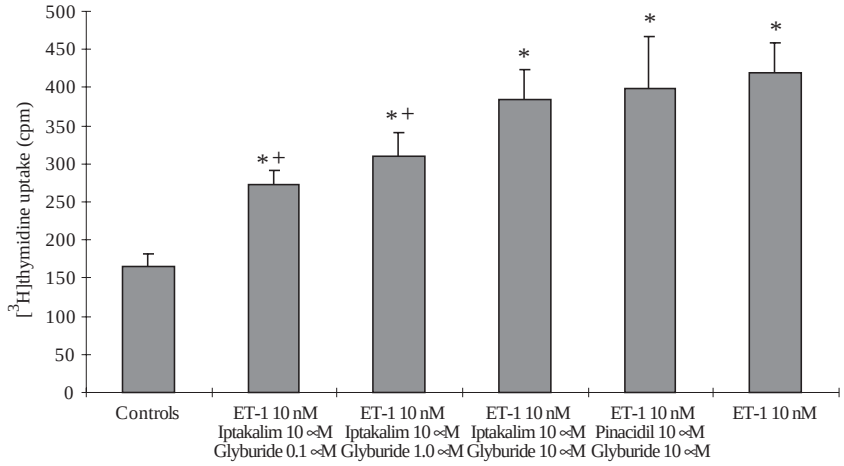

Fig. 4. Glyburide abolished the inhibitory effect of iptakalim on [^{3}H]thymidine uptake of pulmonary arterial SMCs. Cells preincubated with glyburide (0.1, 1.0, and 10 μM) for 0.5 h prior to addition of endothelin-1 (10 nM) in combination with iptakalim (10 μM) for 24 h resulted in a rise of [^{3}H]thymidine incorporation in a concentration-dependent manner compared with that of cells treated simultaneously with endothelin-1 (10 nM) and iptakalim (10 μM). Experiments were performed 10 times independently in duplicate. *Significantly different from corresponding values in control group (P<0.05). $^+$Significantly different from corresponding values in endothelin-1 group (P<0.05).

1, and 10 μM, the total of S and G_2/M phase ratios was decreased by 36.58±5.65% (n=3), 42.59±4.93% (n=3), and 66.27±4.71% (n=3), whereas G_0/G_1 phase ratio was increased by 14.40±2.96% (n=3), 17.90±2.60% (n=3), and 27.85±3.22% (n=3), respectively, compared with the value of the cells treated with endothelin-1 only. Glyburide abolished the effect of iptakalim at the concentration of 10 μM on cell cycle progression of pulmonary arterial SMCs in a concentration-dependent manner (Table 1). Similar to above results from [^{3}H]thymidine incorporation test, these indicated that iptakalim antagonized proliferation of pulmonary arterial SMCs induced also by endothelin-1 through activation of K_{ATP} channels.

4. Discussion

In the present study, we have first demonstrated the effects of iptakalim, designed and synthesized by ourselves, on endothelin-1-induced augmentation of cytoplasmic free calcium concentration and on the proliferation of rabbit cultured pulmonary arterial SMCs. Iptakalim inhibited the

Table 1
The effect of iptakalim on cell cycle progression of pulmonary arterial SMCs ($\bar{x}$±S.D., n=3)

Groups	Cell cycle ratios (%)			
	G_0/G_1	S	G_2/M	S+G_2/M
Controls	90.4±1.2	6.1±1.1	3.49±0.91	9.6±1.2
ET-1[a]	70.5±1.0[b]	18.3±1.1[b]	11.28±0.98[b]	29.5±1.0[b]
ET-1+iptakalim 0.1 μM[c]	81.3±1.2[b,d]	12.4±1.7[b,d]	6.36±0.51[b,d]	18.7±1.2[b,d]
ET-1+iptakalim 1.0 μM[e]	83.0±1.3[b,d]	10.5±1.1[b,d]	6.21±0.30[b,d]	17.0±1.3[b,d]
ET-1+iptakalim 10 μM[f]	90.1±1.2[d]	7.20±0.49[d]	2.74±0.76[d]	10.0±1.2[d]
ET-1+pinacidil 10 μM[g]	90.3±1.1[d]	6.6±1.2[d]	3.05±0.12[d]	9.7±1.1[d]
ET-1+iptakalim 10 μM+glyburide 0.1 μM[h]	85.0±1.8[b,d]	9.7±1.8[b,d]	5.90±0.85[b,d]	15.6±1.1[b,d]
ET-1+iptakalim 10 μM+glyburide 1.0 μM[i]	82.2±2.3[b,d]	11.6±2.0[b,d]	6.13±0.36[b,d]	17.8±2.3[b,d]
ET-1+iptakalim 10 μM+glyburide 10 μM[j]	73.4±2.1[b]	17.3±1.6[b]	9.3±1.8[b]	26.6±2.1[b]
ET-1+pinacidil 10 μM+glyburide 10 μM[k]	72.6±3.2[b]	17.6±2.8[b]	9.8±2.9[b]	27.4±1.6[b]

[a] Cells exposed to endothelin-1 10 nM for 24 h.
[b] Significantly different from corresponding values in control group (P<0.05).
[c] Cells exposed to endothelin-1 10 nM in combination with iptakalim 0.1 μM for 24 h.
[d] Significantly different from corresponding values in endothelin-1 group (P<0.05).
[e] Cells exposed to endothelin-1 10 nM in combination with iptakalim 1.0 μM for 24 h.
[f] Cells exposed to endothelin-1 10 nM in combination with iptakalim 10 μM for 24 h.
[g] Cells exposed to endothelin-1 10 nM in combination with pinacidil 10 μM for 24 h.
[h] Cells preincubated with glyburide 0.1 μM for 0.5 h prior to addition of endothelin-1 10 nM in combination with iptakalim 10 μM for 24 h.
[i] Cells preincubated with glyburide 1.0 μM for 0.5 h prior to addition of endothelin-1 10 nM in combination with iptakalim 10 μM for 24 h.
[j] Cells preincubated with glyburide 10 μM for 0.5 h prior to addition of endothelin-1 10 nM in combination with iptakalim 10 μM for 24 h.
[k] Cells preincubated with glyburide 10 μM for 0.5 h prior to addition of endothelin-1 10 nM in combination with pinacidil 10 μM for 24 h.

endothelin-1-induced calcium signal as well as the enhanced [^{3}H]thymidine incorporation, as well as the transition from static phase (G_0/G_1) to DNA synthesis (S) and mitotic phase (G_2/M).

K^+ channel dysfunction has been demonstrated to play a pivotal role in the development of pulmonary hypertension (Mandegar and Yuan, 2002). Transmembrane K^+ current, which is generated by K^+ efflux through plasmalemmal K^+ channels, is a key determinant factor of the resting membrane potential (Em) in pulmonary arterial SMCs (Yuan, 1995; Evans et al., 1996). Decreased K^+ channel activity, due either to decreased expression of potassium channels and/or to inhibition of the channel open-state probability, reduces transmembrane K^+ current and causes cellular membrane depolarization (Michelakis and Weir, 2001). The membrane potential in pulmonary arterial SMCs governs the activity of voltage-dependent, long-lasting type (L-type) Ca^{2+} channels, which are opened by membrane depolarization and closed by membrane hyperpolarization (Nelson et al., 1990; Fleischmann et al., 1994). Membrane depolarization, therefore, activates these channels and allows for an influx of Ca^{2+} into the cell down a 10,000-fold gradient, thereby increasing $[Ca^{2+}]_{cyt}$. Endothelin-1 inhibits K^+ channels (Betts and Kozlowski, 2000; Li et al., 1999; Peng et al., 1998; Sato et al., 2000), which induce the membrane depolarization and $[Ca^{2+}]_{cyt}$ rise in pulmonary arterial SMCs (Fig. 1). In addition to the control of Ca^{2+} influx into the cell, membrane potential controls other mechanisms and second messenger systems involved in the regulation of tone or the cell's proliferation status, such as inositol-1,4,5-triphosphate (Ganitkevich and Isenberg, 1993; Berridge, 1993).

The cytoplasmic ionized Ca^{2+} acts as a signal transduction element, which is critical in cellular proliferation. Indeed, removal or chelation of extracellular Ca^{2+} significantly inhibits the growth of human pulmonary arterial SMCs in media containing serum and growth factors (Platoshyn et al., 2000). A rise in $[Ca^{2+}]_{cyt}$ increases nuclear Ca^{2+} concentration rapidly (Allbritton et al., 1994), propelling the quiescent cells into cell cycle and through mitosis, and thereby promoting cellular proliferation (Means, 1994; Hardingham et al., 1997).

In contrast, iptakalim induces a hyperpolarization of membrane by activation of K_{ATP} channels (Wang, 2003). This effect lowers the opening probability of L-type Ca^{2+} channels, restrains agonist-induced Ca^{2+} release from intracellular sources through inhibition of inositol-1,4,5-triphosphate formation, decreases the sensitivity of intracellular contractile elements to Ca^{2+}, and accelerates the clearance of intracellular calcium via the Na^+/Ca^{2+} exchanger. As a result, there is a decrease of the $[Ca^{2+}]_{cyt}$ and an attenuation of cell proliferation. Our another study has shown that chronic oral administration of iptakalim alleviated chronic hypoxic pulmonary arterial remodeling and pulmonary hypertension in rats (Xie et al., 2004). The results from the present investigation demonstrated that calcium responses were virtually abolished when cells were incubated in the presence of iptakalim. The inhibition in pulmonary arterial SMC proliferation in the present study may be attributed to activation of K_{ATP} channels for glyburide, a selective K_{ATP} antagonist, and could prevent the inhibitory effect of iptakalim on cell proliferation induced by endothelin-1. Together, these data highlight the potential clinical benefit of K_{ATP} channels in pulmonary hypertension by modulating pulmonary arterial SMC proliferation.

In summary, the novel K_{ATP} channel opener, iptakalim, added to the cells for the initial incubations prior to addition of endothelin-1, substantially attenuated elevation of $[Ca^{2+}]_{cyt}$ and then inhibited DNA synthesis, increased G_0/G_1 phase ratio, and decreased S and G_2/M phase ratio of pulmonary arterial SMCs, respectively. Iptakalim antagonized proliferation of pulmonary arterial SMCs induced by endothelin-1 through activation of K_{ATP} channels for glyburide abolished the inhibitory effect of iptakalim. To the best of our knowledge, iptakalim is the first compound shown to antagonize pulmonary arterial SMC proliferation. These results provided new insights into the molecular mechanism for controlling the pulmonary arterial SMC proliferation and suggest that iptakalim may be a promising therapy for the treatment of pulmonary hypertension (remodeling).

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