Involvement of apamin-sensitive K^+ channels in antigen-induced spasm of guinea-pig isolated trachea

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1 In order to examine whether K^+ channels play a role in antigen-induced airway responses, the effect of K^+ channel blockers on antigen-induced airway smooth muscle contraction and mediator release was examined *in vitro* in guinea-pigs actively sensitized with ovalbumin (OA).

2 Tracheal strips from sensitized animals were suspended in organ baths under a resting tension of 1 g and isometric tension was continuously measured. Cumulative concentration-response curves to OA (0.1 – 1000 ng ml⁻¹) or histamine (10 nM– 1 mM) were obtained in the presence and absence of K^+ channel blockers.

3 OA (10 , 100 or 1000 ng ml⁻¹) was incubated with minced lung tissues from the same animals for 15 min in the presence and absence of K^+ channel blockers, and released histamine and leukotriene C₄ (LTC₄) in the incubating medium were measured.

4 Apamin, a small conductance Ca^{2+} -activated K^+ channel ($P_{K,Ca}$) blocker, (0.1 , 0.3 and 1 μ M) significantly inhibited OA-induced smooth muscle contraction, while charybdotoxin (ChTX, 10 nM), an intermediate and large conductance $P_{K,Ca}$ blocker, and iberiotoxin (IbTX, 3 nM), a large conductance $P_{K,Ca}$ blocker, were without effect. Apamin (0.3 μ M) had no effect on exogenously administered histamine-induced airway smooth muscle contraction, suggesting that the inhibition of OA-induced contraction by apamin did not occur at the smooth muscle level.

5 The inhibition of OA-induced contraction by apamin (0.3 μ M) was not significantly affected by pretreatment with a leukotriene antagonist, ONO-1078 (10 μ M), but was abolished by pretreatment with a histamine H₁-receptor blocker, pyrilamine (1 μ M).

6 Apamin by itself (up to 0.1 μ M) had no effect on spontaneous histamine release from minced lung tissues. Histamine release induced by low and intermediate concentrations of OA (10 and 100 ng ml⁻¹) was significantly suppressed by apamin pretreatment ($P < 0.05$ and $P < 0.001$), whereas LTC₄ release was not affected. ChTX (0.1 μ M) and IbTX (10 nM) had no significant effect on either spontaneous or OA (100 ng ml⁻¹)-induced histamine release.

7 These results suggest that apamin partially but substantially inhibits antigen-induced smooth muscle contraction, presumably by inhibiting antigen-induced histamine release from airway mast cells through small conductance $P_{K,Ca}$ closure.

Keywords: Apamin; K^+ channel; antigen; mast cell degranulation; histamine; leukotriene C₄; charybdotoxin; iberiotoxin

Introduction

The role of K^+ channels has been demonstrated in many types of airway cells (Black & Barnes, 1990). In airway smooth muscle cells, K^+ channels play a role not only in regulating membrane potential (Kotlikoff, 1990) but also in the relaxation responses induced by β -adrenoceptor agonists (Jones *et al.*, 1990; Miura *et al.*, 1992). The activation of certain types of K^+ channels is also involved in neural function including repolarization of nerve fibres (Conti & Neher, 1980) and presynaptic modulation of neurotransmission (Ichinose & Barnes, 1990; Stretton *et al.*, 1992). Moreover, airway non-excitabile cells such as airway epithelial cells (McCann & Welsh, 1990) and T-lymphocytes (Leonard *et al.*, 1992) possess K^+ channels on the cell membrane which play a role in the exocytotic process.

The present study was designed to examine whether K^+ channels are involved in antigen-induced airway responses, consisting of mast cell degranulation and subsequent smooth muscle contraction. Whether IgE-mediated mast cell degranulation requires the opening of ion channels has not yet been established. Lindau & Fernandez (1986) reported that mast cells do not use ion channels in stimulation-secretion coupling, whereas Ca^{2+} influx through membrane ion chan-

nels is thought to be necessary to complete or maintain the degranulation of mast cells (Penner & Neher, 1988). Recently, changes in membrane permeability to other ions, including Cl^- , Na^+ and K^+ , have also been recognized as an important element of the signal transduction process in mast cells (Janiszewski *et al.*, 1992).

Several studies on rat basophilic leukaemia (RBL) cells (Mohr & Fewtrell, 1987; Labrecque *et al.*, 1991), which may be regarded as mast cell analogues, suggested the involvement of K^+ channels in mast cell degranulation. However, little is known about the involvement of K^+ channels in mast cell degranulation, especially in airway mast cells. For this reason, we have examined the effects of K^+ channel blockers on antigen-induced mediator release from lung parenchyma and smooth muscle contraction using guinea-pigs actively sensitized with ovalbumin (OA). We used three types of Ca^{2+} -activated K^+ channel ($P_{K,Ca}$) blockers: apamin, a blocker of small conductance $P_{K,Ca}$ (Banks *et al.*, 1979), charybdotoxin (ChTX), an intermediate and large conductance $P_{K,Ca}$ blocker (Miller *et al.*, 1985; Beech *et al.*, 1987) and iberiotoxin (IbTX), a selective blocker of large conductance $P_{K,Ca}$ (Suarez-Kurtz *et al.*, 1991). We chose these blockers since $P_{K,Ca}$ activity is thought to be important in the degranulation of guinea-pig and human basophils (Labrecque *et al.*, 1991; Beauvais *et al.*, 1992).

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Methods

Animal sensitization and preparation

Male Dunkin-Hartley guinea-pigs, weighing 250–300 g, were sensitized by subcutaneous injection of 0.5 ml of saline containing 10 µg of ovalbumin (OA) and 100 mg of aluminum hydroxide on 2 consecutive days. Three weeks after the sensitization procedure, the animals were anaesthetized by intraperitoneal injection of urethane (2 g kg⁻¹) and killed by exsanguination. The trachea and lungs were quickly removed and placed in Krebs-Henseleit (K-H) solution cooled to 4°C. The composition of the K-H solution was as follows (mM): NaCl 118, KCl 5.9, MgSO₄ 1.2, CaCl₂ 2.5, NaH₂PO₄ 1.2, NaHCO₃ 25.5 and glucose 5.6. After being cleaned of connective tissue, the trachea was opened longitudinally by cutting through the cartilage and cut into segments so that each segment contained 3–4 cartilage rings. Each segment of tracheal tissue was mounted in a 10 ml organ bath containing K-H solution maintained at 37°C and gassed with 95% O₂ and 5% CO₂, giving a pH of 7.4. Strips were connected via silk threads to force displacement transducers (UL-10GR, Minebea Co., Ltd., Tokyo, Japan) for the measurement of isometric changes in tension, and the responses were recorded on a polygraph (Rectigraph-8K, San-ei, Tokyo, Japan). The tissues were allowed to equilibrate for 1 h with frequent washing under a resting tension of 1 g which was found to be optimal for measuring the changes in tension. The remaining lungs were dissected free of large vessels and bronchi and cut into small pieces (1–2 mm thick) for the minced lung preparation.

Contraction studies

Indomethacin (10 µM) was present throughout the experiment in order to avoid a possible modulation of contractile responses by prostaglandins derived from epithelium (Holzman, 1992). After the equilibration period, cumulative concentration-response curves to OA (0.1–1000 ng ml⁻¹) or exogenously applied histamine (0.1 µM–1 nM) were obtained in the tracheal strips in the presence and absence of apamin (0.1, 0.3 or 1 µM). Apamin or its vehicle (saline with 0.25% bovine serum albumin (BSA)) was preincubated with tissues for 20 min before the contraction studies. In another set of experiments, the effects of other K⁺ channel blockers, ChTX (10 nM) and IbTX (3 nM), on OA-induced concentration-response curves were also similarly examined. The concentrations of ChTX and IbTX chosen in these experiments were the maximal concentrations which, by themselves, had no effect on baseline tone.

We have previously shown that OA-induced contraction is abolished under similar experimental conditions by pretreatment with a combination of the histamine H₁-receptor blocker, pyrilamine (1 µM), and a leukotriene antagonist, ONO-1078 (10 µM; Obata *et al.*, 1992). Thus, in a separate series of experiments, the effect of apamin (0.3 µM) on the OA-induced contractile response was also examined in the presence and absence of pyrilamine (1 µM) or ONO-1078 (10 µM), respectively.

Mediator release assay

Minced lung fragments (80 mg) were suspended in Eppendorf tubes with 1 ml of Tyrode solution containing CaCl₂ (2 mM) and MgCl₂ (1 mM) prewarmed to 37°C for 15 min. Suspended tissues were preincubated with apamin (0.01, 0.1 and 1 µM), ChTX (0.1 µM), IbTX (0.01 µM) or its vehicle (saline with 0.25% BSA) for 15 min and then challenged with saline and OA (10, 100 or 1000 ng ml⁻¹). After a 30 min incubation at 37°C, tubes were cooled to stop further reactions and centrifuged (10,000 g, 3 min, 4°C).

Histamine was determined fluorimetrically by high performance liquid chromatography (h.p.l.c.; Yamatodani *et al.*,

1985). In brief, 0.2 ml of supernatant was collected from each tube. Then, 20 µl of 60% perchloric acid was added to both the supernatant and tissue fractions. The tissue fraction was homogenized with a Polytron homogenizer (Kinematica, Luzern, Switzerland) at maximal speed for 15 s. The supernatant and homogenate were centrifuged at 10,000 g for 3 min. An aliquot of 50 µl of each supernatant was injected into an h.p.l.c. system, and fluorescence was determined by a fluoromonitor (F-1100, Hitachi, Tokyo, Japan). Net histamine release is expressed as a percentage of the total histamine content of the supernatant and tissue fractions and corrected for the spontaneous release of histamine. Immunoreactive LTC₄ was measured by an enzyme immunoassay system, with a claimed cross-reactivity of <0.15% with other eicosanoids (Amersham International PLC, Amersham). Supernatants of 0.5 ml were collected from each tube, and concentrations of LTC₄ in the supernatants were measured, normalized by tissue weight (mg tissue) and corrected for the spontaneous release of LTC₄. In the mediator release assays, all values were based on means of duplicate or triplicate determinations.

Drugs

The following drugs were used; apamin (Research Biochemicals Inc., Baltimore, U.S.A.), charybdotoxin and iberiotoxin (Peptide Institute Inc., Osaka, Japan), histamine hydrochloride (Wako Chemicals, Sendai, Japan), indomethacin (Sumitomo Pharmaceutical Company, Osaka, Japan), ovalbumin and pyrilamine (Sigma Chemical Co., St Louis, U.S.A.) and ONO-1078 (4-oxo-8-[4-(4-phenylbutoxy)benzoylamino]-2-(tertrazol-5-yl)-4H-1-benzopyran hemihydrate; ONO Pharmaceutical Company, Osaka, Japan).

Statistical analysis

The effect of apamin on OA-induced tracheal contraction was assessed by calculating the EC₄₀, namely the concentration of OA producing 40% of the maximal contraction of the tissue induced by 1 mM of histamine. The geometric mean of EC₄₀ with 95% confidence interval (CI) was calculated in each group. All other data are expressed as the arithmetic means ± s.e.mean. The effect of K⁺ channel blockers on OA or histamine-induced concentration-response studies of airway smooth muscle contraction was statistically analyzed by two-way analysis of variance (two-way ANOVA). Comparisons between paired or unpaired mean data were performed by Wilcoxon's signed rank test or Mann-Whitney U test, respectively. In each case, *P* < 0.05 was considered statistically significant.

Results

Effect of K⁺ channel blockers on OA-induced smooth muscle contraction

OA (0.1–1000 ng ml⁻¹) evoked concentration-dependent smooth muscle contraction in trachea removed from all the experimental animals. Apamin (0.1, 0.3 and 1 µM) pretreatment by itself had no effect on baseline tone. Compared with the concentration-response curves in control tissues (*n* = 10), apamin at 0.3 (*n* = 7) and 1 µM (*n* = 4) significantly suppressed OA-induced contraction concentration-response curves (*P* < 0.01 and *P* < 0.05, respectively, two-way ANOVA, Figure 1), while apamin at 0.1 µM (*n* = 8) failed to affect the curves significantly (*P* > 0.05, two-way ANOVA). The geometric mean of EC₄₀ for ovalbumin in control tissues was 8.2 ng ml⁻¹ (95% CI, 7.1–9.3 ng ml⁻¹), which was significantly increased by pretreatment with each concentration of apamin (0.1, 0.3 and 1 µM) in a concentration-dependent manner; the EC₄₀ values with 95% CI for ovalbumin in the presence of apamin 0.1, 0.3 and 1 µM were 14.2

(12.9 to 15.5) ng ml⁻¹ 29.0 (27.8 to 30.3) ng ml⁻¹ and 31.2 (29.6 to 32.9) ng ml⁻¹ ($P < 0.05$, $P < 0.01$ and $P < 0.05$, respectively, Mann-Whitney *U* test).

Neither ChTX (10 nM, $n = 5$) nor IbTX (3 nM, $n = 5$) had effects on either the spontaneous basal tone or OA-induced contraction concentration-response curves, compared with their control tissues (both $n = 5$).

Effect of apamin on histamine-induced smooth muscle contraction

Pretreatment with apamin (0.3 μ M) did not affect exogenously applied histamine (0.1 μ M–1 mM)-induced contraction concentration-response curves ($n = 6$, $P > 0.05$, two-way ANOVA, Figure 2).

Effect of H_1 blocker and leukotriene antagonist on inhibitory effect of apamin

Neither the H_1 blocker, pyrilamine (1 μ M), nor the leukotriene antagonist, ONO-1078 (10 μ M), had an effect on baseline smooth muscle tone. However, both drugs significantly suppressed OA-induced smooth muscle contraction in our preliminary experiments. After pyrilamine

Figure 1 Effect of apamin on ovalbumin (OA)-induced smooth muscle contraction in actively sensitized guinea-pig trachea. Apamin at 0.3 μ M (■, $n = 7$) and 1 μ M (▲, $n = 4$) but not at 0.1 μ M (●, $n = 8$) significantly suppressed OA (0.1–300 ng ml⁻¹)-induced cumulative concentration-response curves, compared with vehicle-treated tissues (○, $n = 10$). Contractile response is expressed as a percentage of maximal contraction induced by 1 mM of histamine (% contraction) in each tissue. All points represents mean $\pm$ s.e.mean.

Figure 2 Effect of apamin on exogenously administered histamine-induced smooth muscle contraction in actively sensitized guinea-pig trachea. Apamin (0.3 μ M, ●, $n = 6$) had no significant effect on histamine (0.1 μ M–1 mM)-induced cumulative concentration-response curves, compared with vehicle-treated tissues (○, $n = 6$). Contractile response is expressed as a percentage of the maximal contraction induced by 1 mM histamine, which was obtained before the concentration-response study (% contraction) in each tissue. Each value represents mean $\pm$ s.e.mean.

pretreatment, apamin (0.3 μ M) failed to show a significant inhibition of OA-induced smooth muscle contraction ($n = 6$, $P > 0.05$, two-way ANOVA, Figure 3a), while apamin (0.3 μ M) still significantly inhibited OA-induced contraction

Figure 3 Effects of a histamine H_1 -receptor blocker and a leukotriene antagonist on the inhibition of ovalbumin (OA)-induced smooth muscle contraction by apamin in actively-sensitized guinea-pig trachea. In the presence of pyrilamine (1 μ M; a) apamin (0.3 μ M, ●, $n = 6$) failed to affect OA (0.1–1000 ng ml⁻¹)-induced cumulative concentration-response curves, compared with vehicle-treated tissues (○, $n = 6$), while ONO-1078 (10 μ M; b) did not influence the inhibition of OA-induced contraction by apamin (0.3 μ M, $n = 7$) in vehicle-treated tissues (○, $n = 7$). Contractile response is expressed as a percentage of the maximal contraction induced by 1 mM histamine (% contraction) in each tissue. Each value represents mean $\pm$ s.e.mean.

Figure 4 Effect of apamin on ovalbumin (OA)-induced histamine release from minced lung tissues of actively sensitized guinea-pigs. Apamin (0.1 μ M, stippled columns, $n = 8$) significantly inhibited low concentrations of OA (10 and 100 ng ml⁻¹)-induced histamine release, compared with vehicle-treated tissues (open columns, $n = 8$), although 1000 ng ml⁻¹ of OA-induced histamine release was not significantly affected. Histamine release is expressed as a percentage of the total histamine content, corrected for spontaneous release. Each value represents mean $\pm$ s.e.mean. Significant difference from vehicle-treated tissues of * $P < 0.05$ and *** $P < 0.001$, respectively.

in the presence of ONO-1078 pretreatment ($n = 7$, $P < 0.05$, two-way ANOVA, Figure 3b).

Effect of K⁺ channel blockers on OA-induced mediator release

Apamin (0.01 and 0.1 μM , $n = 5$ and $n = 8$, respectively) had no effect on spontaneous histamine release from minced lung tissues. In the presence of apamin (0.1 μM), histamine release induced by 10 and 100 ng ml^{-1} of OA, but not that induced by 1000 ng ml^{-1} , was significantly suppressed (Figure 4). OA-induced LTC₄ release from minced lung tissues ($n = 4$) was not affected by apamin (0.1 μM) pretreatment at any concentration of OA; OA (10, 100 and 1000 ng ml^{-1})-induced LTC₄ release was 6.7 ± 3.5 , 23.4 ± 5.3 and $23.0 \pm 5.1 \text{ pg mg}^{-1}$ tissue without and 6.3 ± 4.2 , 22.7 ± 6.4 and $22.7 \pm 6.6 \text{ pg mg}^{-1}$ tissue with apamin 0.1 μM . Higher concentrations of ChTX (0.1 μM) and IbTX (0.01 μM) than those used in the contraction studies had no significant effect on OA (100 ng ml^{-1})-induced histamine release. Mean histamine release by OA (100 ng ml^{-1}) from control tissues was $12.7 \pm 0.7\%$ ($n = 6$), which was not significantly different from that in the presence of ChTX ($13.6 \pm 1.5\%$) and IbTX ($13.8 \pm 2.9\%$, both $P > 0.05$, Wilcoxon's signed rank test), respectively.

Discussion

The present study shows that apamin, a small conductance $P_{K,Ca}$ blocker, significantly inhibited antigen-induced airway smooth muscle contraction in trachea isolated from actively-sensitized guinea-pigs *in vitro*. This suggests that small conductance $P_{K,Ca}$ play a role in antigen-induced airway contractile responses.

The sensitization procedure used has been demonstrated predominantly to produce IgE antibody (Andersson, 1980), suggesting that OA-induced airway responses were mainly mediated by IgE receptors. Since both pyrilamine and ONO-1078 significantly suppressed OA-induced smooth muscle contraction, histamine and leukotrienes appear to participate in mediating antigen-induced airway smooth muscle contraction. Histamine released by OA challenge is thought to be mainly derived from airway mast cells, because mast cells are a major source of histamine in airways (Riley & West, 1966) and possess high affinity Fc receptors to IgE (Fc ϵ RI; Ishizaka *et al.*, 1970). On the other hand, leukotrienes released by OA could be derived from eosinophils as well as mast cells, as eosinophils have low affinity Fc receptors for IgE (Fc ϵ RII; Capron *et al.*, 1986). Therefore, as far as histamine is concerned, antigen-induced responses are likely to reflect mast cell function through Fc ϵ RI.

The mechanisms by which apamin inhibited antigen-induced airway smooth muscle contraction in the present study are still unclear. However, a direct action of apamin on airway smooth muscle is unlikely to explain the inhibitory effect obtained in the present study as the existence of apamin-sensitive K⁺ channels on airway smooth muscle cells has not been reported. It could be suggested rather that apamin inhibits OA-induced smooth muscle contraction by suppressing histamine release from mast cells in guinea-pig airways. This is supported by the following findings in the present study. First, the inhibition of OA-induced contrac-

tion by apamin was abolished by pretreatment with a H₁ receptor blocker, pyrilamine, suggesting that the inhibitory effect of apamin is expressed against a histamine component of the OA-induced contraction. Second, the contraction induced by exogenously administered histamine was not affected by apamin in sensitized tracheal smooth muscle, implying that the inhibition by apamin was not due to the change in responsiveness of airway smooth muscle to histamine. Finally, in lung parenchyma, the histamine release induced by lower concentrations of OA (10 and 100 ng ml^{-1}) was significantly suppressed by apamin.

In studies using rat basophilic leukaemia (RBL)-2H3 cells, depolarization induced by extracellular high K⁺ inhibited not only Ca²⁺ uptake but also exocytosis (Labrecque *et al.*, 1989). In another study from the same group, evidence was obtained to suggest that Ca²⁺-activated K⁺ efflux pathways serve to sustain Ca²⁺ influx which is necessary for IgE receptor-mediated triggering of degranulation (Labrecque *et al.*, 1991). In human basophils, decreasing K⁺ channel conductance using K⁺ channel blockers such as 4-aminopyridine and quinine is also known to inhibit histamine release (Beauvais *et al.*, 1992). These findings from mast cell analogue cells raise the possibility that K⁺ channel activation is involved in the maintenance of Ca²⁺ influx to promote mediator release in mast cells. In this case, apamin-sensitive K⁺ channels appear to play this role in the mast cell degranulation of guinea-pig airways.

OA-induced LTC₄ release was not affected by apamin, whereas lower concentrations of OA-induced histamine release were significantly suppressed. This observation may reflect the possibility that leukotrienes are released by antigen challenge not only from mast cells but also from eosinophils. Alternatively, the difference may be explained by the different releasing mechanisms between preformed mediators such as histamine and non-performed mediators such as leukotrienes, as shown in skin mast cells (Benyon *et al.*, 1989).

In the present study, ChTX and IbTX were without effect on both OA-induced smooth muscle contraction and mediator release. Since airway smooth muscle contains large conductance $P_{K,Ca}$ (McCann & Welsh, 1986), higher concentrations of the blockers for these channels, ChTX (100 nM) and IbTX (10 nM), by themselves increased baseline tone in guinea-pig trachea (Jones *et al.*, 1990; Suarez-Kurtz *et al.*, 1991). Therefore, these concentrations of the blockers could not be used in the contraction studies. However, in the mediator release study, even higher concentrations of the toxins did not show a significant effect on mediator release, suggesting that small conductance $P_{K,Ca}$ but not intermediate or large conductance $P_{K,Ca}$ are involved in airway mast cell degranulation mechanisms.

In summary, apamin, a blocker of small conductance $P_{K,Ca}$, significantly inhibited antigen-induced airway smooth muscle contraction, presumably by suppressing histamine release from airway mast cells in actively-sensitized guinea-pigs. This result provides evidence that the activation of apamin-sensitive K⁺ channels is involved in antigen-induced airway responses.

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