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α-Methyl-5-HT, a 5-HT$_2$ receptor agonist, stimulates β$_2$-adrenoceptors in guinea pig airway smooth muscle

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Abstract

α-Methyl-5-HT is widely used as a high-affinity 5-HT$_2$ receptors agonist, though some studies have postulated that this drug also activates other serotonergic receptors. In the present work, we found that a wide range of concentrations of α-methyl-5-HT induced biphasic responses (contraction followed by relaxation) in guinea pig tracheal rings. The relaxing phase caused by 32 μM α-methyl-5-HT was blocked by 0.1 μM propranolol. Furthermore, during an ongoing histamine-induced contraction, α-methyl-5-HT (0.1–100 μM) produced a concentration-dependent relaxation starting at 10 μM. This relaxation was fully abolished by 0.1 μM propranolol or 1 μM ICI 118,551 (a selective β$_2$-adrenoceptor antagonist). Additionally, in electrophysiological recordings, 32 μM α-methyl-5-HT also enhanced the membrane K$^+$ currents of single tracheal myocytes, an effect reverted by propranolol and ICI 118,551, and mimicked by 0.1 μM salbutamol. Thus, we concluded that α-methyl-5-HT activates β$_2$-adrenoceptors in guinea pig tracheal smooth muscle at concentrations ≥10 μM. This effect must be taken into account when this drug is used in airway smooth muscle and in other tissues expressing β$_2$-adrenoceptors.

Keywords: α-Methyl-5-HT; α-Methyl-serotonin; Airway smooth muscle; β$_2$-Adrenoceptors; K$^+$ currents

1. Introduction

A number of studies have demonstrated that the contractile response induced by 5-hydroxytryptamine (5-HT or serotonin) in airway smooth muscle is mainly mediated by 5-HT$_2$ receptors [1–4]. α-Methyl-5-HT is considered as a high-affinity 5-HT$_2$ receptors agonist, and it is widely used to characterize the 5-HT$_2$ responses in many tissues, including the airway smooth muscle [2,5,6]. Nevertheless, some researchers have reported that α-methyl-5-HT may act as a mixed 5-HT$_1$/5-HT$_2$ agonist [7], and that it may also stimulate 5-HT$_7$ receptors [8]. Thus, it seems that α-methyl-5-HT should not be considered as selective for 5-HT$_2$ receptors as previously thought. In the present work, we provide evidence that α-methyl-5-HT also stimulates β$_2$-adrenoceptors in guinea pig tracheal smooth muscle.

2. Materials and methods

2.1. Animals

Male Hartley guinea pigs (500–600 g) bred in conventional conditions in our institutional animal facilities (filtered conditioned air, 21 ± 1 °C, 50–70% humidity, sterilized bed) and fed with Harlan® pellets and sterilized water were used. The protocol was approved by the Scientific and Bioethics Committees of the Instituto Nacional de Enfermedades Respiratorias. The experiments were conducted in accordance with the published Guiding Principles in the Care and Use of Animals, approved by the American Physiological Society.

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2.2. Organ baths

Animals were deeply anesthetized with pentobarbital sodium (35 mg kg^{-1}, i.p.) and exsanguinated. Major airways were carefully dissected and cleaned of connective tissue, and four rings were obtained from the middle of the trachea (each ring was submitted to different experimental conditions). Each tracheal ring was hung in a 5 ml organ bath between two hooks inserted into the lumen. One of the hooks was attached to an isometric transducer (FT03, Grass Instruments, West Warwick, RI) by a 4–0 silk thread. The other hook acted as an anchor by keeping the ring fixed to a Plexiglas rod. Composition of Krebs solution in the organ baths was (mM): NaCl 120, KCl 4.77, KH_2PO_2 1.2, $MgSO_4$ 1.2, $NaHCO_3$ 25, $CaCl_2$ 2.5 and glucose 11, and maintained at 37 °C and bubbled with 5% CO_2 in oxygen (pH 7.4). Tissues were placed under a resting tension of 1 g during 30 min, and washed with fresh Krebs at 15 min intervals meanwhile. Isometric transducers were connected to a preamplifier (Cyber Amp 380, Axon Instruments Inc., Foster City, CA), signals were digitized with an interface (Digidata 1200, Axon), and tension changes were continuously monitored through a software (AxoScope v7.0, Axon) in a microcomputer.

Tissues were stimulated three times with KCl (60 mM), and then α-methyl-5-HT was used in two types of experiments. Firstly, temporal evolutions of the responses to α-methyl-5-HT were evaluated by adding single concentrations (0.1, 1, 3.2, 10, 32, and 100 μM) to different tracheal rings. Some of these tissues were preincubated with 0.1 μM propranolol during 15 min before addition of a selected concentration of α-methyl-5-HT (32 μM). Secondly, in order to evaluate the relaxing effect of α-methyl-5-HT, tracheal rings were precontracted with 10 μM histamine, and then a cumulative concentration-response curve to α-methyl-5-HT (1, 3, 10, 32 and 100 μM) was done. These experiments were performed in the continuous presence of ketanserine (3 nM) to block contractile responses induced by 5-HT_{2A} receptors. In some of these tissues, 0.1 μM propranolol or 1 μM ICI 118,551 (a selective β_2-adrenoceptor antagonist) were added 10 min before α-methyl-5-HT administration. We corroborated that at these concentrations propranolol and ICI 118,551 shifted the concentration-response curve to salbutamol to the right, reaching statistical differences ($p < 0.01$) in the $-\log$ IC50 (5.2103 ± 0.1566, mean ± S.E.M., $n = 6$, and 2.6766 ± 0.9817, $n = 4$, respectively), as compared with salbutamol alone (6.1503 ± 0.2005, $n = 6$).

Responses to agonists were expressed as percentage of the third KCl response, while relaxing responses were analyzed as percentage inhibition of the maximum histamine contraction.

2.3. Patch clamp recordings

Isolated myocytes from guinea pig trachea were obtained as follows. Tracheal airway smooth muscle freed from any residual connective tissue was placed in 5 ml Hanks solution containing 2 mg cysteine and 0.05 U ml^{-1} papaine, and incubated for 10 min at 37 °C. The tissue was washed with Leibovitz's solution to remove the enzyme excess, and then placed in Hanks solution containing 1 mg ml^{-1} collagenase type I and 4 mg ml^{-1} dispase II (neutral protease) during ∼20 min at 37 °C. The tissue was gently dispersed by mechanical agitation until detached cells were observed. Enzymatic activity was stopped by adding Leibovitz's solution, the cells were centrifuged at 800 rpm at 20 °C during 5 min and the supernatant was discarded. This last step was repeated once.

For myocytes culture, the cell pellet was resuspended in minimum essential medium containing 5% guinea pig serum, 2 mM L-glutamine, 10 U ml^{-1} penicillin, 10 μg ml^{-1} streptomycin and 15 mM glucose, and plated on rounded coverslips coated with sterile rat tail collagen. Cell culture was performed at 37 °C in a 5% CO_2 in oxygen atmosphere during 24–48 h.

Airway smooth muscle cells were allowed to settle down in the bottom of a 0.7 ml coverglass submerged in a perfusion chamber. The cells were observed with an inverted microscope (IDO3, Zeiss, Jena, Germany). The chamber was perfused by gravity (∼1.5–2.0 ml min^{-1}) with external solution (mM): NaCl 130, KCl 5, $CaCl_2$ 1.8, HEPES 10, glucose 10, $MgCl_2$ 0.5, $NaHCO_3$ 3, KH_2PO_4 1.2, and niflumic acid 0.1 (pH 7.4, adjusted with NaOH). Electrophysiological experiments were performed at room temperature (∼21 °C).

The standard whole-cell configuration of the patch clamp technique and an Axopatch 200A amplifier (Axon) were used to record the membrane K^+ currents activated by depolarizing voltage steps. Patch pipettes were made with 1B200F-6 glass (Word Precision Instruments, Sarasota, FL) using a horizontal micropipette puller (P-87, Sutter Instruments Co., Novato, CA). Pipette resistance ranged from 2 to 4 MΩ when filled with the internal solution. This solution was composed by (mM): potassium gluconate 140, NaCl 5, HEPES 5, EGTA 10, ATP disodium salt 5, and GTP sodium salt 0.1 (pH 7.3, adjusted with KOH). The series resistance (ranged from 10 to 15 MΩ) and whole-cell capacitance were compensated electronically. Whole-cell currents were filtered at 1–5 KHz using the analog filter of the amplifier, digitized (Digidata 1200, Axon) at 10 KHz, and stored on a microcomputer for later analysis through special software (p-Clamp v8.0, Axon).

Immediately after obtaining the whole-cell configuration, a series of hyperpolarizing and depolarizing square pulses (from −70 to +40 mV) were applied in 10 mV increments from a holding potential of −60 mV. These pulses had a duration of 500 ms and a frequency of 1 Hz. This voltage protocol evoked reproducible and stable outward currents. These currents were abolished by 10 mM tetraethylammonium (TEA), indicating that they are K^+ currents (total potassium-currents, $I_K{}^+$). After the control protocol test, α-methyl-5-HT (32 μM) or salbutamol (1 μM) was added to the perfusion solution and changes in the currents were quantified with the same protocol at 400 ms. In some experiments, 0.1 μM propranolol or 1 μM ICI 118,551 were applied before or during the above mentioned drugs. Cell capacitance was measured in all electrophysiological experiments.

2.4. Intracellular Ca^{2+} measurements in tracheal myocytes

Guinea pig tracheal myocytes were isolated as described above. Cells were loaded with 0.5 μM fura 2/AM in low Ca^{2+}

(0.1 mM) at room temperature (22–25 °C). After 1 h, cells were allowed to settle down into a heated perfusion chamber with a glass cover in the bottom. This chamber was mounted on an inverted microscope (Diaphot 200, Nikon, Tokyo, Japan) and cells adhered to the glass were continuously perfused at a rate of 2–2.5 ml min^{-1} with Krebs solution (composition in mM: NaCl 118, KCl 4.6, $CaCl_2$ 2.0, $MgSO_4$ 1.2, $NaHCO_3$ 25, KH_2PO_4 1.2, glucose 11; 37 °C, equilibrated with 5% CO_2 in oxygen, pH 7.4).

Cells loaded with fura 2 were exposed to alternating pulses of 340 and 380 nm excitation light, and emission light was collected at 510 nm using a microphotometer (Photon Technology International, Princeton, NJ). Background fluorescence was automatically subtracted and determined by removing the cell from the field before starting the experiments. The fluorescence acquisition rate was 0.5 s. Intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) was calculated according to the formula of Grynkiewicz et al. [9]. The K_d of fura 2 was assumed to be 386 nM [10]. The mean 340/380 fluorescence ratios R_{max} and R_{min} were obtained by exposing the cells to 10 mM Ca^{2+} in presence of 10 μM ionomycin and in Ca^{2+}-free Krebs with 1.11 mM EGTA, respectively. R_{max} was 8.99 and R_{min} 0.35. The fluorescence ratio at 380 nm light excitation in Ca^{2+}-free physiological saline solution and Ca^{2+} saturated cells (β) was 3.93.

Single myocytes were stimulated with 10 mM caffeine to evaluate their viability. This drug is known to cause the release of Ca^{2+} from sarcoplasmic reticulum by lowering the ryanodine receptor threshold to intracellular Ca^{2+} [11]. Afterward, cells were stimulated with 100 μM α-methyl-5-HT without or with 10 nM ketanserine. We selected this α-methyl-5-HT concentration, slightly higher than the concentration used in organ baths and patch clamp experiments, because the 32 μM concentration did not cause an optimal $[Ca^{2+}]_i$ response. At the end of the experiment, a second caffeine stimulation was performed in order to corroborate the myocyte responsiveness. In another set of experiments, the effect of 0.1 μM propranolol on the $[Ca^{2+}]_i$ responses to three sequential stimuli with 100 μM α-methyl-5-HT was tested.

2.5. Drugs

(±)-Propranolol hydrochloride, tetraethylammonium chloride, ketanserine, salbutamol, and the specific β_2-adrenoceptor antagonist ICI 118,551 were all purchased from Sigma Chem. Co. (St. Louis, MO). α-Methyl-5-HT maleate was purchased from Tocris Cookson Inc. (Ellisville, MO). Because α-methyl-5-HT is a light-sensitive chemical compound, all experiments using this drug were performed under darkness conditions.

2.6. Statistical analysis

Differences in the response of tracheal rings and $[Ca^{2+}]_i$ were evaluated through paired or unpaired Student's *t*-test or one-way ANOVA followed by Dunnett's test. These last two analyses were also used to evaluate data from voltage clamp experiments. Statistical significance was set at two-tailed $p < 0.05$. Data are expressed in the text and illustrations as mean ± S.E.M.

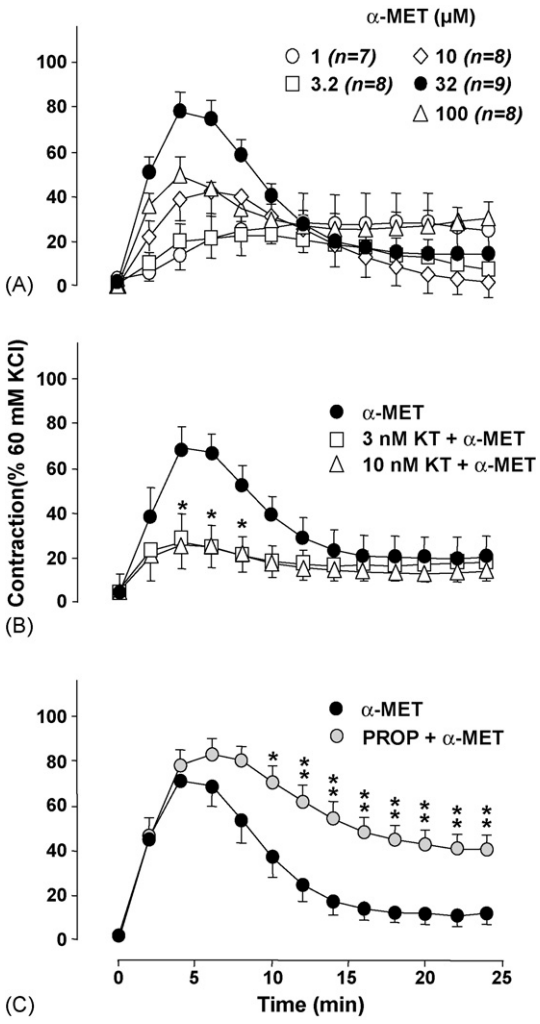

Fig. 1. Responses of guinea pig tracheal rings to α-methyl-5-HT (α-MET), and the effect of ketanserine (KT) and propranolol (PROP). (A) Each non-cumulative single concentration of α-MET produced a biphasic response (contraction followed by relaxation). (B) Both 3 nM ($n = 5$) and 10 nM ($n = 5$) KT greatly reduced the contraction induced by 32 μM α-MET ($n = 5$). (C) PROP ($n = 8$) preincubation notably reduced the relaxation phase induced by 32 μM α-MET ($n = 8$). $^{*}p < 0.05$, and $^{**}p < 0.01$, as compared with their respective α-MET control group. Symbols represent mean ± S.E.M.

3. Results

Except for the lowest α-methyl-5-HT concentration, all the remaining non-cumulative concentrations induced a biphasic response (contraction followed by relaxation) of tracheal rings (Fig. 1A). The most evident biphasic response was observed with a concentration of 32 μM, therefore, we chose this concentration in the following described experiments.

As it is shown in Fig. 1B, the contraction phase induced by α-methyl-5-HT was largely reduced by the selective 5-HT_{2A} receptor antagonist ketanserine, either at 3 or 10 nM concentrations. However, propranolol (0.1 μM) markedly blocked the relaxing phase (Fig. 1C).

During a cumulative concentration-response curve we found that α-methyl-5-HT caused a relaxation of the ongoing histamine contraction, beginning at 10 μM concentration and

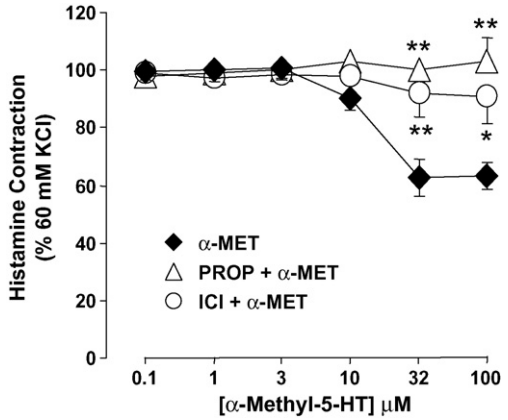

Fig. 2. The relaxing effect of α-methyl-5-HT (α-MET) on the sustained contraction induced by 10 μM histamine in guinea pig tracheal rings is concentration-dependent and mediated by activation of β_2-adrenoceptors. This is supported by the fact that the relaxing effect of α-MET ($n = 11$) was blocked by tissue preincubation with either 0.1 μM propranolol (PROP, $n = 8$) or 1 μM ICI 118,551 (ICI, $n = 7$). To prevent activation of 5-HT_{2A} receptors, all tissues were preincubated with 3 nM ketanserine before adding histamine. $^{*}p < 0.05$, and $^{**}p < 0.01$, as compared with the α-MET group. Symbols represent mean ± S.E.M.

reaching its maximum effect (∼37% relaxation) at 32 μM (Fig. 2). This α-methyl-5-HT-induced relaxation was fully blocked by 0.1 μM propranolol and also by the selective β_2-adrenoceptor antagonist ICI 118,551 (1 μM).

In the electrophysiological experiments of single myocytes, outward potassium-currents (I_K^+) were activated when step depolarisations to −50 mV or more positive potentials were applied, from a holding potential of −60 mV. These currents were significantly ($p < 0.01$) increased by application of 32 μM α-methyl-5-HT (Fig. 3A). At the highest voltage tested (+40 mV), this increment reached ∼4.4-fold the basal response (2.42 ± 0.6 nA versus 0.55 ± 0.08 nA, respectively). The enhanced I_K^+ responses induced by α-methyl-5-HT were completely abolished when 0.1 μM propranolol or 1 μM ICI 118,551 were added *after* this drug (Fig. 3A and D, respectively). Similar results were observed when propranolol or ICI 118,551 was incubated *before* the addition of α-methyl-5-HT (Fig. 3B and E, respectively). Salbutamol (1 μM) mimicked the effect of α-methyl-5-HT on I_K+ and was also inhibited by propranolol or ICI 118,551 (Fig. 3C and F, respectively).

Experiments in isolated tracheal myocytes showed that the resting $[Ca^{2+}]_i$ was 83 ± 18 nM ($n = 5$, Fig. 4). Caffeine (10 mM) addition during 3 min resulted in a transient Ca^{2+} peak (939 ± 26 nM). α-Methyl-5-HT (100 μM) induced an increment of $[Ca^{2+}]_i$ (405 ± 109 nM), which was completely abolished by 10 nM ketanserine. At the end of the experiment, a second stimulation with caffeine produced a response similar to the first one, demonstrating that cells maintained good responsiveness. On the other hand, we found that $[Ca^{2+}]_i$ responses gradu-

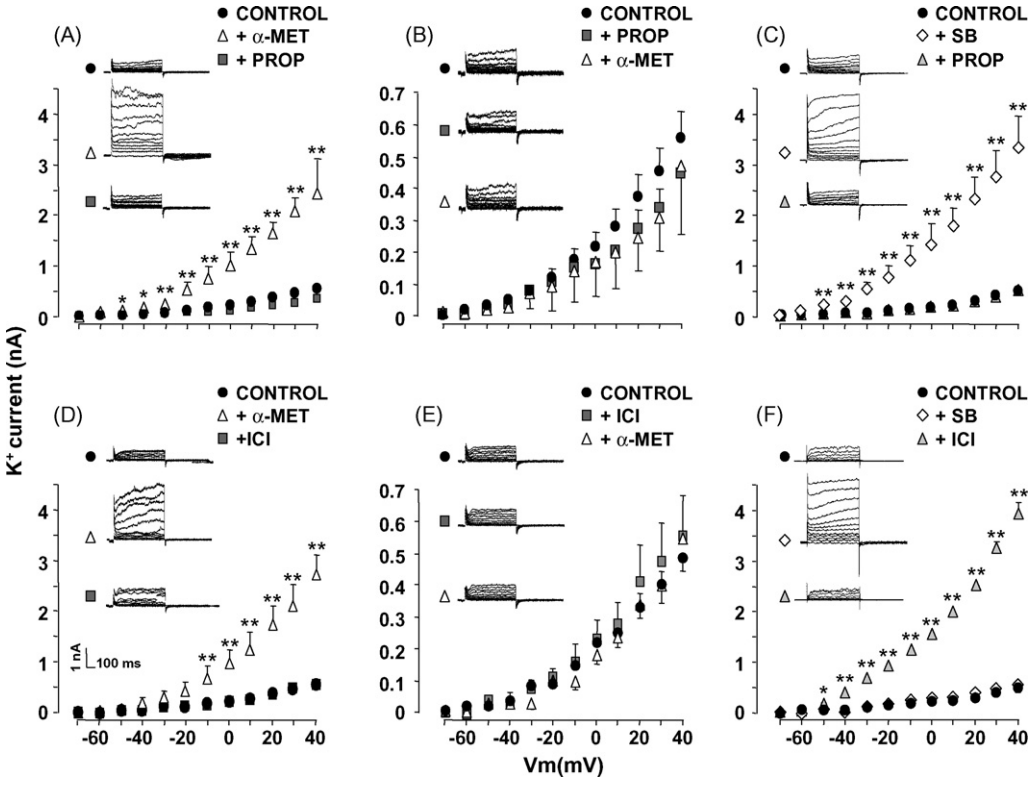

Fig. 3. α-Methyl-5-HT (32 μM, α-MET) increases the membrane K^+ currents of guinea pig airway smooth muscle cells by activation of β_2-adrenoceptors. Voltage steps (10 mV increments, −70 to +40 mV, during 500 ms) from a holding potential of −60 mV, evoked large sustained outward currents. In all cases, representative traces of whole-cell patch clamp recordings are shown in the insets. (A) Perfusion of α-MET induced a significant increase of the control K^+ currents. This effect was blocked by addition of 0.1 μM propranolol (PROP). (B) PROP alone did not modify the current-voltage relationship, as compared with the control group; a subsequent exposure to α-MET did not induce changes in the K^+ currents. (C) 1 μM salbutamol (SB) induced a significant increase in the K^+ currents similar to the one observed with α-MET. This SB effect was reversed by 0.1 μM propranolol. (D, E, F) These PROP effects were mimicked by 1 μM ICI 118,551 (ICI), a more specific β_2-adrenoceptor antagonist. Each curve represents four to six experiments. $^{*}p < 0.05$, $^{**}p < 0.01$. Symbols represent mean ± S.E.M.

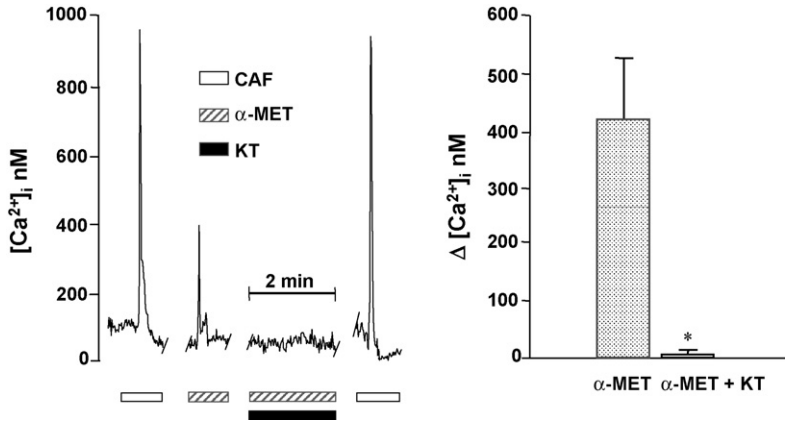

Fig. 4. The increase of the intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) induced by 100 μM α-methyl-5-HT (α-MET) is mediated by activation of 5-HT_{2A} receptors as shown by the fact that this effect is inhibited by 10 nM ketanserine (KT). In left panel, the representative recordings of the $[Ca^{2+}]_i$ changes include an initial and final stimulation by 10 mM caffeine (CAF) to corroborate the cell viability; $^{*}P < 0.01$. Bars represent the mean ± S.E.M. of $n = 5$ experiments.

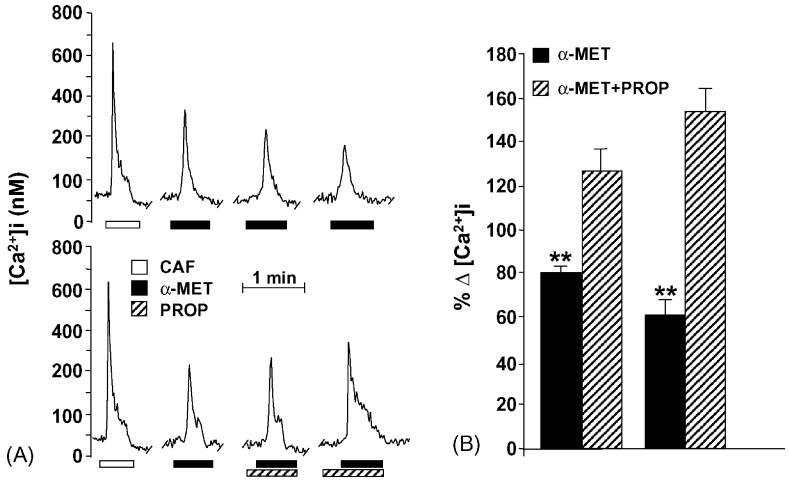

Fig. 5. Effect of propranolol on the increase of the intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) induced by α-methyl-5-HT (α-MET) in guinea pig airway smooth muscle cells. (A) Upper panel: representative recordings of the $[Ca^{2+}]_i$ changes induced by 100 μM α-MET. After stimulation with 10 mM caffeine (CAF) to corroborate the cell viability, responses to three consecutive α-MET stimulation progressively diminished ($n = 5$). Lower panel: propranolol (PROP, 0.1 μM) incubation enhanced the responses induced by α-MET ($n = 4$). (B) Responses of the second and third α-MET stimuli, as percentage of the first one. $^{**}p < 0.01$, as compared with their respective α-MET + PROP group. Bars represent the mean ± S.E.M.

ally diminished during three sequential stimuli with 100 μM α-methyl-5-HT (Fig. 5). By contrast, preincubation with 0.1 μM propranolol caused that such responses to α-methyl-5-HT were progressively higher.

4. Discussion

α-Methyl-5-HT is a synthetic compound considered as a high affinity agonist for 5-HT_2 receptors. Since early 1980s, this drug has been widely used to characterize the 5-HT_2 responses in many tissues, including the airway smooth muscle [2,5,6]. In the present work, we corroborated that the main receptor activated by α-methyl-5-HT, at least for the contraction, was the 5-HT_{2A} receptor, as such contraction was notably inhibited by ketanserine, and the $[Ca^{2+}]_i$ response was fully abolished by this last drug in isolated myocytes. Nevertheless, in the present study we found that α-methyl-5-HT also stimulates β_2-adrenoceptors, inducing relaxation of the guinea pig airway smooth muscle.

One line of evidence providing indirect support to the activation of β_2-adrenoceptors by α-methyl-5-HT is that the relaxation induced by this drug was notably blocked by either a non-selective β (propranolol) or a selective β_2 (ICI 118,551) adrenoceptor antagonist. Thus, we found that α-methyl-5-HT caused a concentration-dependent relaxation of the histamine-induced contraction, and that this relaxing effect was fully abolished by both β-adrenoceptor antagonists (Fig. 2). Although the relaxing effect of α-methyl-5-HT was also observed during an ongoing contraction induced by carbachol (data not shown), the effect of β-adrenoceptor antagonists was unexplored. In the case of biphasic responses to single concentrations of α-methyl-5-HT, the relaxing phase was partially diminished by preincubation with propranolol (Fig. 1C). In 1994, Ben-Harari et al. [12] observed a similar biphasic response produced by 10 μM 5-HT, and they proposed that the relaxing event was due to a desensitization of the 5-HT_2 receptor via activation of a cAMP-dependent protein kinase A (PKA). Although in our experiments with sin-

gle concentrations of α-methyl-5-HT we cannot discard that this last mechanism of relaxation was present, it could not be operating in the concentration-response curve, since in these last experiments the contraction was induced by histamine and they were performed in the continuous presence of ketanserine, a 5-HT_{2A} receptor antagonist (Fig. 2).

A second line of evidence supporting a major role of β_2-adrenoceptor stimulation in the relaxation process was that α-methyl-5-HT caused an increase of the voltage-activated I_K^+ currents in isolated myocytes, and that these currents were fully prevented or abolished by propranolol and ICI 118,551. This effect of α-methyl-5-HT was mimicked by salbutamol, an effect that was also blocked by propranolol and ICI 118,551. These last findings are in agreement with previous reports which described that activation of β_2-adrenoceptors increases the open probability of K^+ channels, causes cell hyperpolarization and therefore, relaxation of the smooth muscle [13–15].

In agreement with the concept that α-methyl-5-HT activates β_2-adrenoceptors, we found that propranolol notably increased the $[Ca^{2+}]_i$ responses to α-methyl-5-HT, probably by antagonizing its effect on the β_2-adrenoceptor. It has been postulated that the smooth muscle relaxing effect induced by the β_2-adrenoceptor stimulation is due to several mechanisms. Traditionally, PKA-mediated phosphorylation of either phospholamban (a protein that inhibits the sarcoplasmic reticulum ATPase Ca^{2+} pump), phospholipase Cβ (enzyme responsible of the IP_3 production) or IP_3 receptor have been claimed as putative mechanisms of action of β_2-adrenoceptor agonists [16–18]. Recently, it has been proposed that cAMP has an inhibitory effect on the IP_3 receptor, reducing its open probability [19]. All these mechanisms lead to a reduction in the $[Ca^{2+}]_i$, and thus their blockade by propranolol might explain the increased $[Ca^{2+}]_i$ response.

In conclusion, we have demonstrated that α-methyl-5-HT, starting at a concentration of 10 μM, stimulates β_2-adrenoceptors in the guinea pig airway smooth muscle. This effect must be taken into account when this drug is used in airway smooth muscle or in cells expressing β_2-adrenoceptors.

Acknowledgments

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