British Journal of Pharmacology (2003) 139, 1203–1211
www.nature.com/bjp

Acetylcholine and tachykinins involvement in the caffeine-induced biphasic change in intracellular Ca^{2+} in bovine airway smooth muscle

*,[1,2]Luis M. Montaño, [2]Verónica Carbajal, [2]José L. Arreola, [3]Carlos Barajas-López, [2]Edgar Flores-Soto & [2]Mario H. Vargas

[1]Departamento de Farmacología, Facultad de Medicina, UNAM, Ciudad Universitaria, CP 04510, México DF, México; [2]Departamento de Investigación en Asma, Instituto Nacional de Enfermedades Respiratorias, Tlalpan 4502, CP 14080, México DF, México and [3]Department of Anatomy & Cell Biology, Queen's University, Kingston, ON K7L 3N6, Canada

1 Caffeine has been widely used as a pharmacological tool to evaluate Ca^{2+} release from the sarcoplasmic reticulum in isolated smooth muscle cells. However, in nervous tissue this drug also causes neurotransmitters release, which might cause additional effects when smooth muscle strips are evaluated. To assess this last possibility, simultaneous measurements of contraction and cytosolic Ca^{2+} concentration (using Fura–2/AM) were carried out in bovine airway smooth muscle strips during caffeine stimulation.

2 A first stimulation (S1, $n=11$) with caffeine (10 mM) induced a biphasic change in cytosolic Ca^{2+}, which consisted of a transient Ca^{2+} peak (254 ± 40 nM, $\bar{X}\pm$SEM) followed by a plateau (92 ± 13 nM), and a transient contraction (204.72 ± 31.56 mg tension mg tissue^{-1}). A second caffeine stimulation (S2) produced a similar response but these parameters had a different magnitude. The S2/S1 ratios for these parameters were 0.69 ± 0.02, 0.83 ± 0.06 and 1.01 ± 0.03, respectively. Addition of ω-conotoxin GVIA (1 μM) and tetrodotoxin (3.1 μM) before S2 significantly diminished these S2/S1 ratios (0.26 ± 0.05, 0.26 ± 0.09 and 0.64 ± 0.11, respectively, $n=5$, $P<0.05$), implicating the neurotransmitters release involvement in the response to caffeine. A similar effect ($P<0.01$) was observed with atropine (1 μM, $n=4$), the fragment 4–11 of substance P (SP) (an SP receptor antagonist, 10 μM, $n=5$), and with both substances ($n=4$).

3 We discarded a direct effect of ω-conotoxin GVIA (1 μM) plus tetrodotoxin (3.1 μM) or of atropine (1 μM) plus SP fragment 4–11 on smooth muscle cells because they did not modify caffeine responses in isolated tracheal myocytes.

4 We confirmed by HPLC that caffeine increased the release of acetylcholine (from 0.43 ± 0.19 to 2.07 ± 0.56 nM mg tissue^{-1}, $P<0.02$) in bovine airway smooth muscle strips. Detection of substance P by ELISA was not statistically different after caffeine stimulation (geometric means before and after caffeine, 0.69 vs. 1.97 pg ml^{-1} mg tissue^{-1}, respectively, $P=0.053$).

5 We concluded that acetylcholine and tachykinins release are involved in the caffeine-induced biphasic changes in cytosolic Ca^{2+} concentration.
British Journal of Pharmacology (2003) **139,** 1203–1211. doi:10.1038/sj.bjp.0705348

Keywords: Bovine airway smooth muscle; intracellular Ca^{2+}; acetylcholine; substance P; tachykinins; neurotransmitter release; Ca^{2+} oscillations

Abbreviations: $[Ca^{2+}]i$, intracellular Ca^{2+} concentration; S1, first caffeine stimulation; S2, second caffeine stimulation; SR, sarcoplasmic reticulum

Introduction

Caffeine is a xanthine derivative that enhances the sensitivity of ryanodine receptor channels to a low intracellular Ca^{2+} concentration ($[Ca^{2+}]i$). Due to this effect, caffeine makes that basal Ca^{2+} levels become sufficient to induce channel opening and Ca^{2+} release from the sarcoplasmic reticulum (SR) (Pessah *et al*., 1987). Thus, caffeine has been extensively used as a pharmacological tool to evaluate Ca^{2+} release from isolated excitable cells, including airway smooth muscle cells (Taniguchi & Nagai, 1970; Rousseau & Meissner, 1989; Nehlig *et al*., 1992; Usachev *et al*., 1993; Bazán-Perkins *et al*., 2000). Other caffeine-induced effects, such as adenosine receptor antagonism (Evoniuk *et al*., 1987), and phosphodiesterase inhibition (Beavo *et al*., 1970; Pauvert *et al*., 2002) have been described. These effects might be responsible for neurotransmitters release, as has been found in the brain and in a pheochromocytoma cell line (Nehlig *et al*., 1992; Avidor *et al*., 1994). Although in a single cell preparation these effects apparently do not interfere with the SR-Ca^{2+} release induced by caffeine, in multicellular preparations such as airway smooth muscle strips they might be able to modify the final response, but this last possibility has been scantily investigated.

*Author for correspondence; E-mail: lmmr@servidor.unam.mx
Current address: Instituto Nacional de Enfermedades Respiratorias, Tlalpan 4502, CP 14080, México DF, México

Thus, the aim of the present work was to evaluate the role of neurotransmitters release in the caffeine-induced mobilization of intracellular Ca^{2+} and smooth muscle contraction in bovine tracheal strips.

Methods

Study design

In bovine tracheal strips the following experiments were conducted (Figure 1): (a) Tissues were stimulated with 60 mM KCl for 15 min in order to corroborate their viability. (b) After washing the tracheal strips, and when they returned to basal conditions (~10 min), a first stimulation (S1) with 10 mM caffeine was carried out for 10 min. This was considered the basal response, and we only included tissues showing a good first response to caffeine defined by the presence of a Ca^{2+} transient peak followed by a plateau with oscillations. This criterion was established because we corroborated that the lack of oscillations was associated with a low contraction after caffeine. (c) Because caffeine stimulation depletes the SR-Ca^{2+} stores, tissues were stimulated again with 60 mM KCl during 30 min in Krebs solution containing 2 mM Ca^{2+} in order to enhance the SR refilling. It has already been shown that a minimum of 20 min is enough to accomplish ~75% of the active SR-replenishment (Bourreau *et al*., 1991). (d) A second stimulation (S2) with 10 mM caffeine was performed with or without preincubation with ω-conotoxin GVIA (an N-type voltage-dependent Ca^{2+} channel blocker) plus tetrodotoxin (a Na^{+} channel blocker), or with atropine and/or substance P (SP) fragment 4–11. The SP fragment 4–11 is an unspecific drug that blocks both NK1 and NK2 receptors, as it was demonstrated by its ability to block the tracheal contraction elicited by substance P (an NK1 agonist) and by eledoisin and kassinin (two NK2 agonists) (Mizrahi *et al*., 1984; Tucci *et al*., 2001). We corroborated that the concentration of SP fragment 4–11 used in our study (10 μM) significantly displaced to the right the concentration–response curve to SP (data not shown). Finally, with the aim to further investigate the role of the SR in the caffeine response, the same protocol as described above was carried out in a separate group of tracheal strips, but with S2 being accomplished in a Ca^{2+} free medium (ie, Krebs without Ca^{2+} and with 0.1 mM EGTA).

Additionally, in order to assess whether ω-conotoxin GVIA, tetrodotoxin, atropine, or SP fragment 4–11 have a direct effect on the smooth muscle cell, their effects on the intracellular Ca^{2+} transient peak induced by caffeine were explored in single myocytes isolated from bovine trachea. In these experiments, caffeine stimulations (10 mM) were accomplished approximately 15 min apart in each isolated myocyte. After confirming that two repetitive caffeine stimulations had the same response, a third one was conducted in the presence of ω-conotoxin GVIA plus tetrodotoxin, or of atropine plus SP fragment 4–11.

In another set of experiments we explored the effect of SP on single airway smooth muscle cells and tracheal strips. In these last tissues, 3.2 μM phosphoramidon (a neutral endopeptidase inhibitor) was used in order to avoid degradation of SP.

Simultaneous measurement of [Ca^{2+}]i and contraction in airway strips

Tracheal bovine strips (1–1.5 mm wide, 5 mm long) obtained from a local slaughterhouse were dissected free of cartilage,

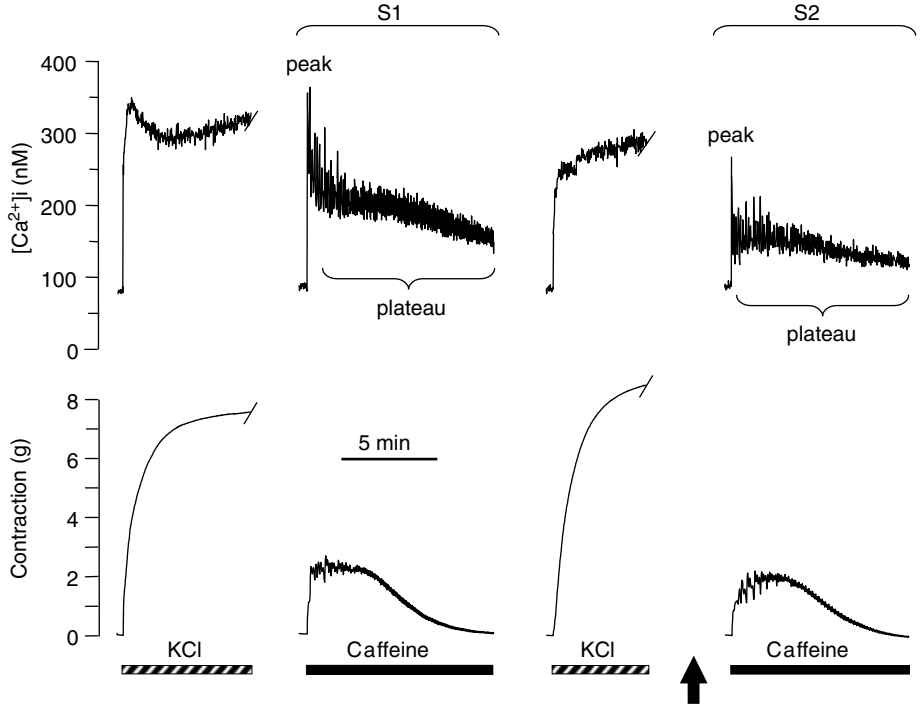

Figure 1 Experimental protocol to evaluate the effect of caffeine (10 mM) in bovine airway smooth muscle strips through simultaneous measurements of the [Ca^{2+}]i and contraction. Arrow indicates when an experimental maneuver was done (drug addition or change to a Ca^{2+}-free medium). S1 and S2 are the first and second caffeine stimulation, respectively. KCl concentration was 60 mM. For details, see the text.

epithelium and connective tissue and immersed in Krebs solution (mM): NaCl 118, $NaHCO_3$ 25, KCl 4.6, KH_2PO_4 1.2, $MgSO_4$ 1.2, glucose 11, $CaCl_2$ 2 (unless otherwise specified). The strips were loaded with the acetoxymethyl ester of fura 2 (Fura-2/AM) by incubating them under protection from light for 3.5 h at 37°C in a shaking bath (54 strokes per min) with 2 ml bubbled (5% CO_2 in O_2) Krebs solution containing 20 μM Fura-2/AM, 1 mM probenecid and 0.01% pluronic F-127. Afterwards, strips were washed for 10–15 min with Krebs solution to remove the dye from the extracellular space. Each preparation was mounted vertically in the bottom of a 4-ml polymethacrylate fluorimeter cuvette using a special adaptor from Photon Technology International (PTI, Princenton, NJ, U.S.A.). This cuvette was placed in a PTI fluorometer and the Krebs solution was continuously bubbled with 5% CO_2 in O_2 and maintained at 37°C. The lower end of the strip was fixed to the adaptor and the upper end was attached to an isometric force transducer (FSG-01, Experimetria Ltd., Budapest, Hungary) connected to an analog-digital interface (PTI) via an EasyGraf recorder (model TA240, Gould Electronics, Cleveland, OH, U.S.A.). Recordings were stored in a microcomputer and analyzed using a data acquisition and analysis software (Felix v1.21, PTI). Preparations were equilibrated for 30 min under a resting tension of 1–1.5 g prior to testing. The special adaptor had two metallic tubes. The first one was used to add solutions (with or without drugs) to tracheal strips through syringes mounted in a serial system of three-way stopcocks. Under these conditions, drugs added to the tissues always had a uniform concentration. The second tube was used to remove the solution.

Fura-2/AM loaded in the smooth muscle strips was excited by alternating pulses of 340 and 380 nm light, and emission was collected at 510 nm using a fluorometer (PTI). The fluorescence acquisition rate ($\sim$0.8 s^{-1}, $[Ca^{2+}]i$) was calculated according to the formula of Grynkiewicz *et al*. (1985). The K_d of Fura-2 was assumed to be 386 nM (Kajita & Yamaguchi, 1993). The mean 340/380 fluorescence ratios R_{max} and R_{min} were obtained by exposing the tissue to 5 mM Ca^{2+} in the presence of 10 μM ionomycin and in Ca^{2+}-free medium with 10 mM EGTA, respectively. R_{max} was 2.49 and R_{min} 1.19. The fluorescence ratio at 380 nm light excitation, in Ca^{2+}-free medium and Ca^{2+} saturated cells (ß), was 1.36. With this procedure, a typical recording of 340/380 in a mirror fashion was obtained, thus corroborating the appropriateness of the Ca^{2+} measurement (Figure 2(a)).

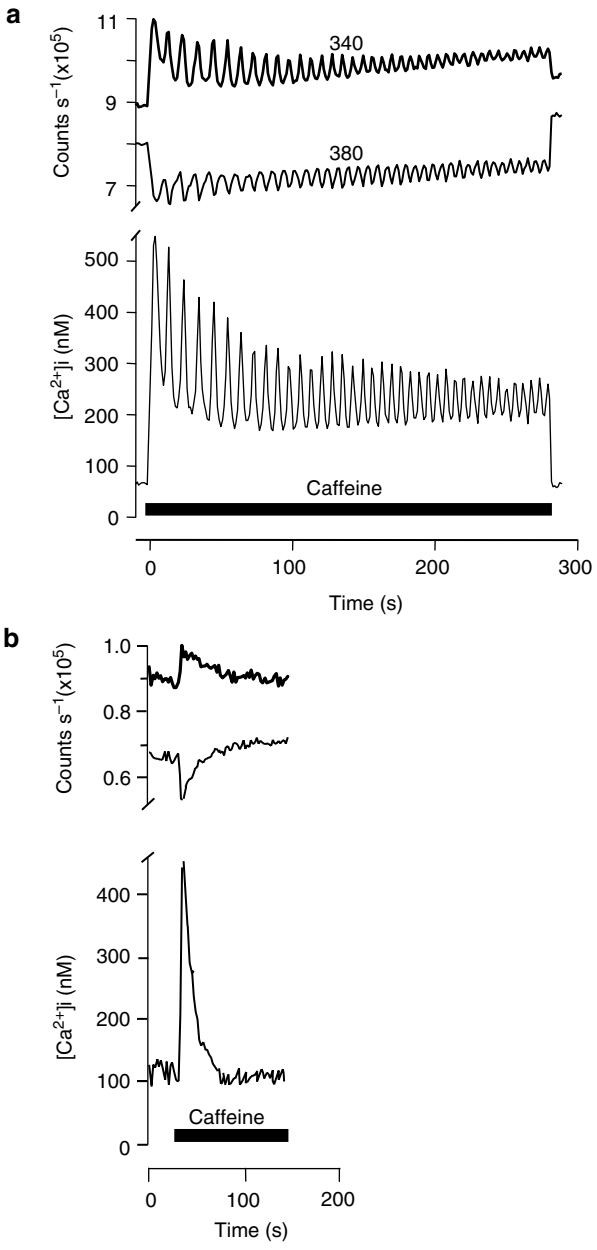

Figure 2 Typical recording of the change in $[Ca^{2+}]i$ induced by caffeine (10 mM) in bovine airway smooth muscle strips (a) and in single myocytes (b). Upper traces display fluorescence recordings at 340 and 380 nm.

Measurement of $[Ca^{2+}]i$ in tracheal myocytes

Airway smooth muscle cells were obtained from bovine trachea as follows. Approximately 200 mg smooth muscle were minced, placed in 5 ml Hanks solution (Gibco BRL, Rockville, MD, U.S.A.) 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 (Gibco) to remove the enzyme excess, and then placed in a Krebs solution containing 0.144 mg ml^{-1} of a highly purified collagenases and neutral proteases mixture (Liberase Blendzyme 2, Roche, Indianapolis, IN, U.S.A.) for $\sim$12 min until dispersed cells were observed. This procedure allowed us to obtain cells with consistent levels of resting $[Ca^{2+}]i$. Cells were then 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 into a 1.5 ml heated perfusion chamber with a glass cover at the bottom. This chamber was mounted on a Nikon inverted microscope (Diaphot 200, Tokyo, Japan) and cells adhered to the glass were continuously perfused at a rate of 2–2.5 ml min^{-1} with Krebs solution (37°C, equilibrated with 5% CO_2 in O_2, pH 7.4) containing 1.5 mM Ca^{2+}.

Cells loaded with Fura-2 were studied as smooth muscle strips (see above) with the following differences: Equipment used was a microphotometer (PTI). Background fluorescence was automatically determined and subtracted by removing the cell from the field before starting the experiments. The fluorescence acquisition rate was 0.5 s^{-1}, $[Ca^{2+}]i$. R_{max} and R_{min} were obtained by exposing the cells to 10 mM Ca^{2+} in the presence of 10 μM ionomycin and in Ca^{2+}-free Krebs solution

with 10 mM EGTA, respectively. R_{max} was 7.16, R_{min} 0.38, and ß 1.8. Data analysis was performed using Felix v1.21 software (PTI).

Drugs previously dissolved at the required concentration in Krebs solution were added to the perfusion chamber by using a 60-ml syringe connected to a four-way stopcock system.

Acetylcholine (ACh) and SP detection

For this purpose, bovine tracheal airway smooth muscle strips (2 mm wide, 5 mm long) were suspended in 5 ml organ baths containing Krebs solution. Preparations were maintained at 37°C, pH 7.4, and bubbled with 5% CO_2 in O_2. Tissues were attached to a isometric force transducer (model FT03, Grass Instruments, West Warwick, RI, U.S.A.) connected to an analog–digital interface (Digidata 1200A, Axon Instruments, Foster City, CA, U.S.A.) *via* a signal conditioner (CyberAmp 380, Axon Instruments). Recordings were stored in a microcomputer and analyzed using a data acquisition and analysis software (AxoScope v7.0, Axon Instruments). Preparations were equilibrated for 30 min under a resting tension of 1–1.5 g prior to testing. Tissues were then stimulated three times with 60 mM KCl (lasting 20 min each) until maximum stable responses were obtained. After washing the tissues, they were incubated for 10 min with 140 KIU ml^{-1} aprotinin (a protease inhibitor) in order to improve the recovery of SP (Scott *et al*., 1996), the bath liquid was recovered, filtered with acrodisc syringe filters with 0.2 μm nylon membrane (Pall Gelman Laboratory, Ann Arbor, MI, U.S.A.), and two aliquots were taken and frozen at −20°C. Subsequently, tissues were incubated again with aprotinin and stimulated with 10 mM caffeine for 10 min and the same protocol described above was followed. Only those tissues in which caffeine induced a transient contraction with oscillations were included.

ACh measurement One of the sample's aliquots was lyophilized with a freeze dry system (model 77520, Labconco Corporation, Kansas City, MI, U.S.A.) and reconstituted with 100 μl of filtered deionized glass-distilled water. ACh and choline concentrations in the sample were measured by cation-exchange HPLC-electrochemical detection method (Potter *et al*., 1983). In this technique, an analytic column for ACh and choline (MF-6150, Bioanalytical Systems, West Lafayette, IN, U.S.A.), an immobilized enzyme reactor (IMER; Bioanalytical Systems) and an electrochemical detector (Coulochem II, ESA Inc., Chelmsford, MA, U.S.A.) were coupled to the HPLC (model 9012, Varian Co., Walnut Creek, CA, U.S.A.). Mobil phase (50 mM Tris/$NaClO_4$ plus 1% ProClin reagent, pH 8.5) was pumped at a rate of 1 ml min^{-1}.

ACh and choline were retained by the analytic column and separated from each other. The IMER contains two enzymes, acetylcholinesterase (AChE) and choline oxidase, which convert the ACh to choline and then to betaine and H_2O_2, respectively. Then, H_2O_2 is oxidized by the platinum electrode (set at 500 mV) of the electrochemical detector producing a current whose magnitude reflects the amount of ACh in the sample. Some minutes later, choline is eluted and transformed by the IMER to H_2O_2, producing a separated peak that reveals the amount of choline in the sample. Chromatography data were recorded and processed with a special software (Star Chromatography Workstation, Varian Inc. version 4.01). Concentration of ACh and choline were calculated by extrapolation of the results with standard curves made just before the HPLC sample measurements. Injection of progressive ACh and choline concentrations showed that the detection limit of our HPLC system was ~1 nM in a 10 μl sample.

Because ACh released by the tissue is quickly hydrolyzed to choline, the ACh measurement should ideally have been done under AChE inhibition in order to avoid its degradation. However, the addition of 1 μM physostigmine to the organ bath induced a sustained tracheal contraction, thus interfering with the transient contraction induced by caffeine. Therefore, ACh production in the samples was expressed as the sum of ACh plus choline (nM mg of $tissue^{-1}$) detected with the HPLC system.

Substance P measurement A 1-ml sample was mixed with 1 ml trifluoroacetic acid (1%, TFA), shaken, centrifuged at 14,000 × *g* for 15 min at 4°C (Model Mikro-22-R, Hettich, Tuttlingen, Germany), and the supernatant was applied to a chromatography column of Bio-Gel P-2 Gel Fine, 45–90 μm mesh (Bio-Rad Laboratories, Hercules, CA, U.S.A.). These columns were previously equilibrated with 1 ml acetonitrile, followed by 15 ml TFA. After addition of the supernatant to the columns, they were washed with 20 ml TFA, eluted by applying 3 ml of 60 : 40 solution of acetonitrile:TFA, collected in plastic tubes, and evaporated until dry using a centrifugal concentrator under vacuum (Speed Vac, model SC110, Savant Instruments, Holbrook, NY, U.S.A.). Samples were reconstituted with 100 μl of buffer protein base with preservatives (part R80206, R&D Systems, Minneapolis, MN, U.S.A.). SP concentration was then measured through a competitive ELISA (catalogue #DE1400, R&D Systems). Samples were read at 405 nm using a Labsystems Multiskan MS (V.4.0, Labsystems Oy, Helsinki, Finland). SP concentration was expressed as pg ml^{-1} mg $tissue^{-1}$ after comparing with standard curves made with the same kit. The detection limit of the SP assay was less than 8 pg ml^{-1}.

Drugs

Fura-2 acetoxymethyl ester (Fura-2/AM), probenecid, pluronic F-127, caffeine, ionomycin, tetrodotoxin, [D-Pro^4, D-$Trp^{7,9,10}$, Phe^{11}]-SP (fragment 4–11), methoxyverapamil hydrochloride (D-600), acetylcholine chloride, SP acetate salt, phosphoramidon sodium salt, Trizma® base, and EGTA (ethylene glycol-bis(ß-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid) were obtained from Sigma (St Louis, MO, U.S.A.). ω-Conotoxin GVIA was purchased from Calbiochem (La Joya, CA, U.S.A.). ProClin was purchased from Bioanalytical System. Fura-2/AM and ionomycin were dissolved in dimethylsulfoxide (final concentration 0.025%). In control experiments, dimethylsulfoxide did not have any effect on the 60 mM KCl-induced tracheal strips contraction.

Data analysis

Evaluation of $[Ca^{2+}]i$ was done by measuring the peak response and the averaged plateau (measured from the visually identified beginning of plateau until the end of the 10-min record) using a data acquisition and analysis software (Felix v1.21, PTI), whereas contractile response was evaluated by the maximum contraction.

In order to minimize the biological variability, responses to caffeine under different preincubation conditions were evaluated in S2, as compared with their respective basal responses to caffeine alone (S1). Thus, an S2/S1 ratio was used in all evaluations. Differences in S2/S1 ratios were evaluated through one-way ANOVA followed by Tukey's test. In some experiments we used paired and nonpaired Student's *t*-test. Statistical significance was set at one-tailed (ACh and SP measurements) or two-tailed *P*-value less than 0.05. Data in the text and illustrations are shown as mean ± s.e.m.

Results

The resting $[Ca^{2+}]$i averaged from all strips ($n=32$) was 103 ± 8 nM. In control conditions (ie without preincubation with other drugs), both the first (S1) and second (S2) caffeine addition yielded a biphasic change in $[Ca^{2+}]$i, constituted by a transient Ca^{2+} peak followed by a plateau with oscillations. The plateau reached a $37.6\pm2.4\%$ of the peak value. These $[Ca^{2+}]$i responses were accompanied by a transient contraction (Figure 1). $[Ca^{2+}]$i responses to S2 were always lower than responses to S1, mainly in the peak, while contraction was almost the same in S2 and S1. Therefore, S2/S1 ratios of $[Ca^{2+}]$i peak, $[Ca^{2+}]$i plateau and contraction were 0.69 ± 0.02, 0.83 ± 0.06 and 1.01 ± 0.03, respectively. These responses to caffeine were relatively homogeneous, as can be observed in the remaining control groups. In some experiments, D-600 (methoxyverapamil, 30 μM), a well-known L-type Ca^{2+} channels blocker, greatly decrease the ongoing Ca^{2+} plateau induced by caffeine, with abolishment of the oscillatory pattern (Figure 3). Additionally, combination of 1 μM ACh plus 1 μM SP (in the presence of 3.2 μM phosphoramidon, $n=3$) reproduced the oscillatory pattern observed with caffeine, which was also abolished by D-600 (Figure 3).

Tissues were preincubated with ω-conotoxin GVIA (1 μM) plus tetrodotoxin (3.1 μM), which would be expected to reduce markedly neurotransmitter release (Montaño *et al*., 1988; Johri & Janssen, 1999). This experimental maneuver significantly decreased the S2/S1 ratio of the transient Ca^{2+} peak, Ca^{2+} plateau and muscle contraction (Figure 4). The same effect was

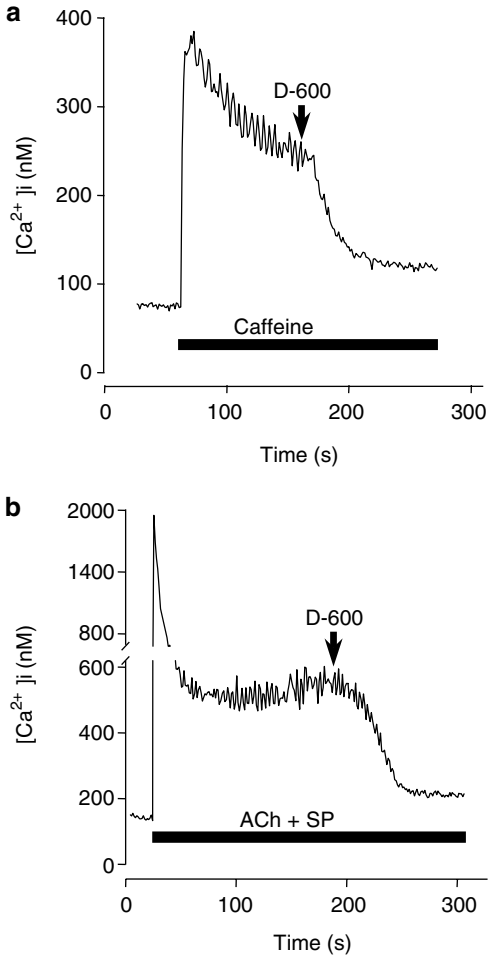

Figure 3 Typical $[Ca^{2+}]$i oscillations observed with 10 mM caffeine in bovine airways smooth muscle strips, and their abolishment with 30 μM D-600 (a). Acetylcholine (ACh, 1 μM) plus substance P (SP, 1 μM) induced a similar pattern of $[Ca^{2+}]$i as caffeine and were also abolished by D-600 (b).

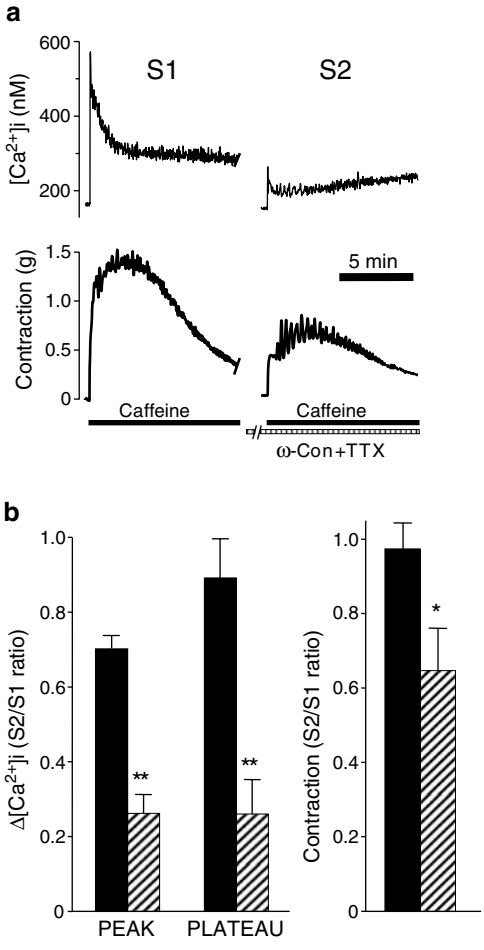

Figure 4 Blockage of neurotransmitter release with ω-conotoxin GVIA (ω-Con, 1 μM) plus tetrodotoxin (TTX, 3.1 μM) significantly inhibited caffeine responses. (a) Typical responses of bovine airway smooth muscle strips to caffeine, before (S1) and after (S2) a 10-min preincubation with ω-Con plus TTX. (b) Averaged observations from five experiments as the one shown in (a). Filled bars represent data obtained in control conditions whereas striped bars were obtained in the presence of the two neurotoxins. Lines on the top of bars are s.e.m. $^*P<0.05$ and $^{**}P<0.01$.

observed when tissues were preincubated with either atropine (1 μM) or SP fragment 4–11 (10 μM), and the inhibition was even more evident when these last two antagonists were simultaneously used (Figures 5 and 6). The removal of Ca^{2+} from the external medium significantly inhibited the actions of caffeine, which only produced a Ca^{2+} transient peak devoid of oscillations and a small transient contraction, as is shown in Figure 7.

In tracheal myocytes the resting $[Ca^{2+}]i$ was 82 ± 13 nM ($n = 6$). As is exemplified in Figure 2(b), stimulation with caffeine produced a Ca^{2+} transient peak (ΔCa^{2+}, 275 ± 7 nM, $n = 6$), which was not modified by ω-conotoxin GVIA plus tetrodotoxin (ΔCa^{2+}, 274 ± 52 nM, $n = 3$), nor by atropine plus SP fragment 4–11 (ΔCa^{2+}, 313 ± 42 nM, $n = 3$).

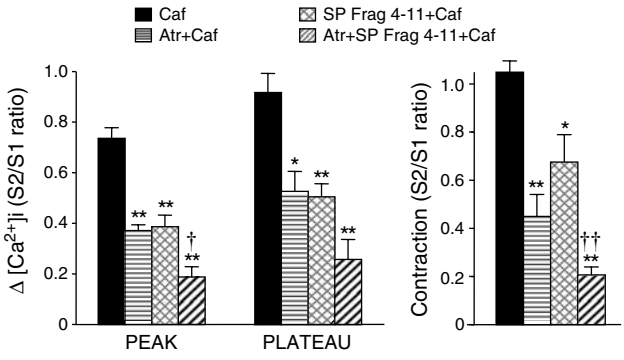

Figure 6 Blockage of muscarinic and SP receptors inhibited caffeine (Caf, 10 mM) responses on bovine airway smooth muscle strips. Bars are averaged data from 4–5 different animals and lines on the top of the bars are s.e.m. $*P<0.05$ and $**P<0.01$ with respect to caffeine group, $\dagger P<0.05$ and $\dagger\dagger P< 0.01$ with respect to caffeine + atropine (Atr, 1 μM) and caffeine + SP fragment 4–11 (SP frag 4–11, 10 μM) groups.

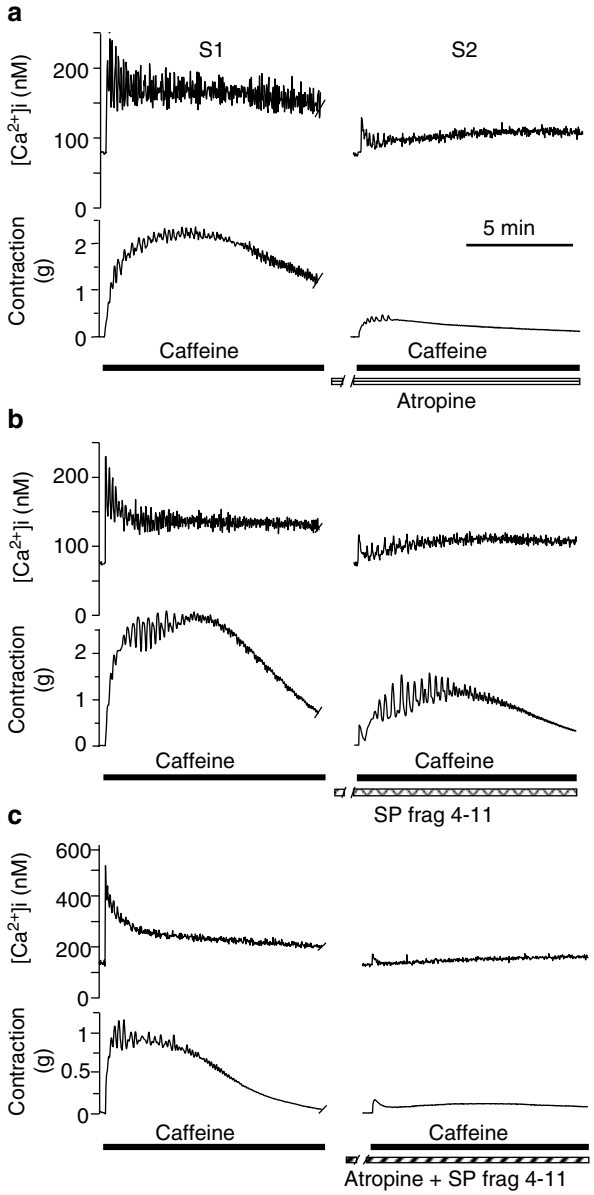

Figure 5 Typical recordings of bovine airway smooth muscle strips responses to caffeine (10 mM) before (S1) and in the presence (S2) of either 1 μM atropine (a), 10 μM SP fragment 4–11 [SP frag 4–11] (b), or both substances together (c).

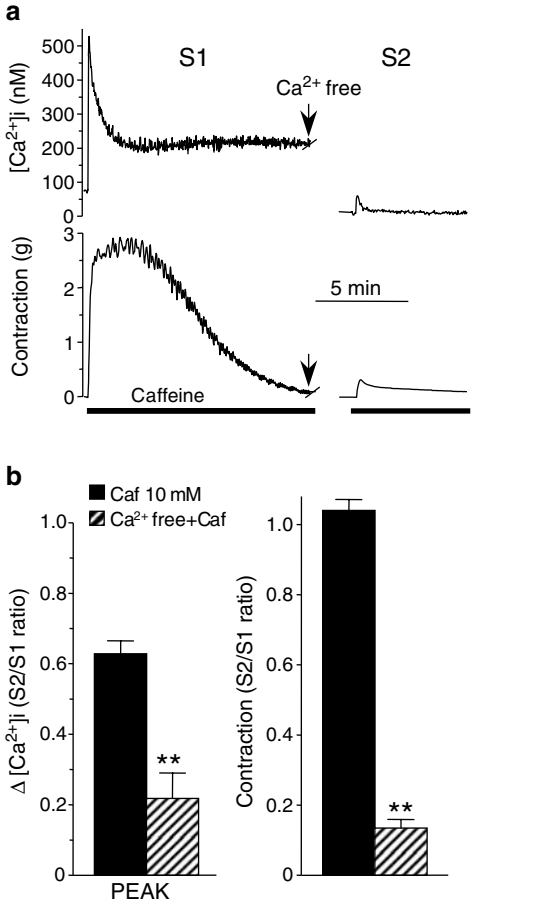

Figure 7 Removal of the extracellular calcium ions inhibits caffeine (Caf, 10 mM) responses. (a) Original recordings of bovine airway smooth muscle strips responses to caffeine. S1 and S2 are the first and second caffeine stimulation, respectively. (b) Effect of Ca^{2+}-free medium on the changes in $[Ca^{2+}]i$ and contraction induced by caffeine in bovine airway smooth muscle strips. Bars represent the average of three animals and lines on the top of the bars are s.e.m. $**P<0.01$.

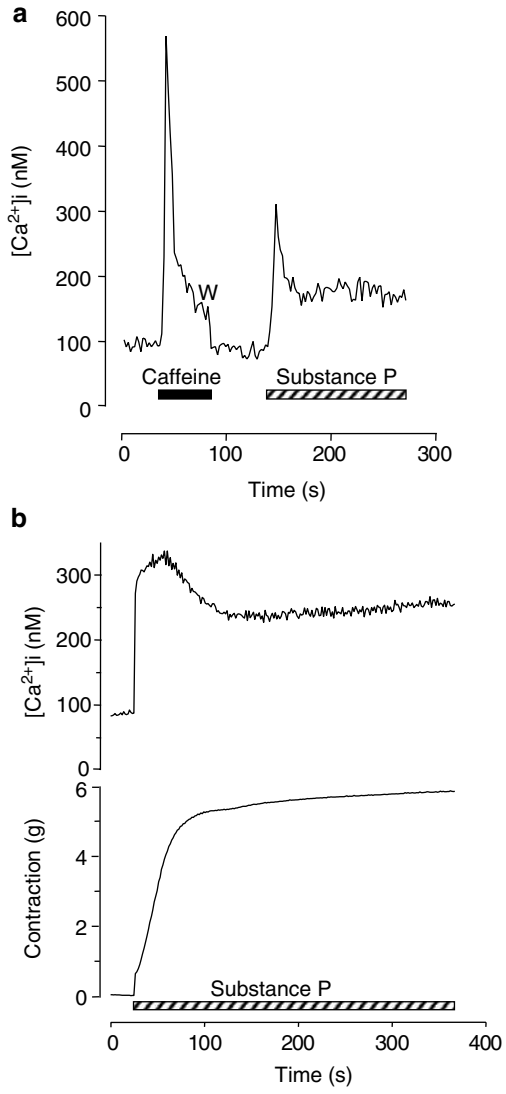

Figure 8 Effect of SP (1 μM) on the $[Ca^{2+}]$i in single airway smooth muscle cells (a) and in the contraction and $[Ca^{2+}]$i in airway smooth muscle strips (b). Tissues were preincubated with 3.2 μM phosphoramidon (inhibitor of neutral endopeptidase).

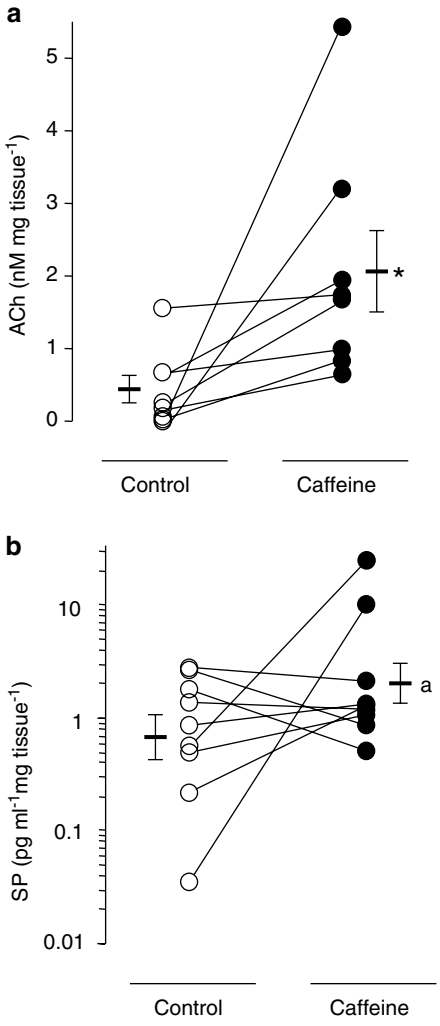

Figure 9 Effect of 10 mM caffeine on the ACh and SP release (in a 10-min period) from bovine airway smooth muscle strips. $^{*}P<0.02$, $^{a}P=0.053$.

Finally, in isolated tracheal myocytes perfusion with 1 μM SP induced a transient Ca^{2+} peak (ΔCa^{2+}, 498 ± 135 nM, $n=5$) followed by a plateau (ΔCa^{2+}, 112 ± 12 nM, Figure 8(a)). Moreover, through simultaneous measurements in tracheal strips we corroborated that 1 μM SP caused smooth muscle contraction during the Ca^{2+} increment in a similar fashion as the pattern reported for other agonists, that is a Ca^{2+} peak (ΔCa^{2+}, 206 ± 23 nM, $n=3$) followed by a plateau (ΔCa^{2+}, 148 ± 27 nM, $n=3$), along with a sustained contraction (0.51 ± 0.09 g tension mg tissue^{-1}, Figure 8(b)). In these last experiments, tissues were preincubated with 3.2 μM phosphoramidon.

The amount of ACh released by bovine trachea strips and accumulated in the organ bath liquid in a 10-min period was higher during caffeine stimulation, as compared with control conditions (0.43 ± 0.19 to 2.07 ± 0.56 nM mg tissue^{-1}, respectively, $P<0.02$, Figure 9(a)). Although the amount of SP released had a tendency to augment after caffeine stimulation, this increment was devoid of statistical significance (geometric means, 0.69 vs 1.97 pg ml^{-1} mg tissue^{-1}, $P=0.053$, Figure 9(b)).

Discussion

Caffeine has been widely used as a pharmacological tool to evaluate Ca^{2+} release from SR stores in single cell experiments (Janssen, 1996; Wang & Kotlikoff, 1997; Madison *et al*., 1998; Bazán-Perkins *et al*., 2000). In these conditions, administration of caffeine produces a $[Ca^{2+}]$i transient peak that rapidly returns to basal levels in less than a minute (Bazán-Perkins *et al*., 2000). This effect of caffeine was corroborated in our study using single tracheal myocytes. However, using a more complex experimental biosystem (tracheal smooth muscle strips), caffeine stimulation induced a biphasic change in $[Ca^{2+}]$i, constituted by a transient Ca^{2+} peak followed by a plateau with oscillations, accompanied by a transient contraction. Similar responses have been previously observed in vascular smooth muscle strips, but up to now the mechanisms involved in the $[Ca^{2+}]$i biphasic changes are unknown (Watanabe *et al*., 1992).

While the relaxing phase of the transient response has been attributed to cAMP accumulation produced by the caffeine-induced phosphodiesterase inhibition, the contraction phase has been largely attributed to the Ca^{2+} released from SR stores (Watanabe *et al*., 1992). However, in the present study we found evidences that neurotransmitters, such as ACh and tachykinins, are involved in the contraction and in the $[Ca^{2+}]$i biphasic changes. Firstly, we found indirect data suggesting the involvement of neurotransmitters in the caffeine responses, as follows. (1) The removal of the extracellular Ca^{2+} notably inhibited the caffeine-induced muscle contraction and turned the biphasic Ca^{2+} changes into a small transient peak. This observation would imply that the mechanism of action by which caffeine induced a biphasic increase in the $[Ca^{2+}]$i and muscle contraction is mainly mediated by the external Ca^{2+}, and not solely due to SR Ca^{2+} release. (2) The addition of ACh plus SP to tracheal strips was able to reproduce the oscillatory pattern observed with caffeine, and D-600 abolished it in both experiments. (3) Incubation of tracheas with two toxins able to decrease neurotransmitter release (ω-conotoxin GVIA and tetrodotoxin), notably diminished the biphasic $[Ca^{2+}]$i response to caffeine, with a reduction of the smooth muscle contraction.

Secondly, we were able to identify through pharmacological tools that neurotransmitters released by caffeine corresponded to ACh and tachykinins, since their respective antagonists (atropine and SP fragment 4–11) significantly reduced the caffeine-induced responses. Moreover, combination of both antagonists caused a stronger inhibition of these responses, including the abolishment of $[Ca^{2+}]$i oscillations. Therefore, our results indicate that in tracheal tissue caffeine induced the release of ACh and tachykinins and that these neurotransmitters were responsible for the major part ($>70\%$) of the $[Ca^{2+}]$i increment and muscle contraction. In addition, it was evident that the biphasic nature of the $[Ca^{2+}]$i response to caffeine was fully dependent on these neurotransmitters.

Thirdly, we found a significant increment in the release of ACh in the organ bath liquid after caffeine stimulation of bovine tracheal strips, confirming that this drug induced the release of this neurotransmitter.

Studies in bovine and other mammals have reported the presence of different types of innervation in the trachea (Cameron & Kirkpatrick, 1977). A large network of paratracheal ganglia with positive staining to ACh have been located in the dorsal surface of the membranous portion of the trachea (Baker *et al*., 1986; Baluk & Gabella, 1989). SP-immunoreactive fibers emerging from the vagus nerve, along with sympathetic nerve fibers, are also present within the trachea wall (Baluk & Gabella, 1989). During experiments using tracheal smooth muscle this neuronal component is usually left intact, and its functionality is easily disclosed by electrical field stimulation (Baker *et al*., 1993; Matera *et al*., 1997; Sommer *et al*., 1997). On the other hand, it is now well documented that caffeine induces release of brain neurotransmitters such as serotonin, dopamine and noradrenaline (Nehlig *et al*., 1992). This last effect has been postulated to be mediated *via* the activation of ryanodine Ca^{2+} channel receptors at the SR (Avidor *et al*., 1994), and also by an increment of cAMP after caffeine-induced phosphodiesterase inhibition (Beavo *et al*., 1970). In this last regard, several studies support that ACh and SP are released when cAMP levels are experimentally increased (Blusztajn *et al*., 1992; Kopp *et al*., 2002). Additionally, at least in the hippocampus where ACh release is under tonic inhibitory control by adenosine, caffeine promotes ACh release, although this effect is achieved through its A1 receptor antagonism (Carter *et al*., 1995).

All the above-mentioned studies fully agree with our findings, thus providing further support to our conclusion that caffeine causes ACh and SP tachykinins release from neuronal endings in the bovine airway smooth muscle. As far as we know, this is the first report of such an effect of caffeine in this tissue.

The specific tachykinin involved in the caffeine response is still unclear. In our pharmacological experiments we found that a mixture of ACh and SP added to bovine tissues reproduced the pattern of $[Ca^{2+}]$i response to caffeine (see Figure 3), suggesting that SP could be one of the tachykinins involved. However, we were unable to demonstrate a caffeine-induced increment of this neuropeptide in the organ bath fluids. One possible explanation to these results is that caffeine might be inducing the simultaneous release of tachykinins other than SP, such as neurokinin A. The simultaneous release of SP and neurokinin A has been well documented (Canning & Fischer, 2001), and both neurotransmitters are capable of producing smooth muscle contraction via NK1 (mainly SP) and NK2 (mainly neurokinin A) receptors (Barnes, 2001). Thus, it is likely that neuropeptides such as neurokinin A partially account for the effect of caffeine, although its actual release remains to be demonstrated.

We were able to demonstrate that $[Ca^{2+}]$i oscillations observed in tracheal strips stimulated with caffeine were dependent on extracellular Ca^{2+}, since blockade of L-type Ca^{2+} channels by D-600 abolished them, and they were not observed during incubation in Ca^{2+}-free medium. Moreover, a combination of ACh and SP reproduced such oscillatory pattern, and the blockade of these two neurotransmitter receptors eliminated it. Altogether, these results suggest that ACh and/or tachykinins are responsible for the oscillations induced by caffeine.

We confirmed that SP is able to induce a sustained contraction due to an intracellular Ca^{2+} increment.

Recently, a biphasic $[Ca^{2+}]$i response induced by caffeine was described by Ethier *et al*. (2001) in bovine airway smooth muscle single cells. These authors concluded that SR Ca^{2+} ATPase activity is the main mechanism involved in the sustained plateau phase. However, this Ca^{2+} plateau was a rather small response reaching only 1.9% of the peak response, much lower than the 37.6% observed in our study in smooth muscle strips. Thus, in our study the involvement of the SR Ca^{2+} ATPase activity in the generation of the plateau, if any, seemed to be minimal.

On the other hand, in single tracheal smooth muscle cells we corroborated that none of the drugs used to avoid neurotransmitters release or to block muscarinic or tachykinins receptors had a noticeable effect on the caffeine-induced responses, thus discarding that changes observed in our study could be due to a direct effect on smooth muscle cells.

We concluded that in bovine tracheal strips caffeine releases ACh and tachykinins, thus making this drug an unsuitable pharmacological tool to evaluate Ca^{2+} release from SR in this tissue.

References

AVIDOR, T., CLEMENTI, E., SCHWARTZ, L. & ATLAS, D. (1994). Caffeine-induced transmitter release is mediated via ryanodine-sensitive channel. *Neurosci. Lett.,* **165,** 133–136.

BAKER, D.G., HILLARY, F.D. & BROWN, J.K. (1993). N-type, ω-conotoxin-sensitive Ca^{2+} channels mediate electrically evoked release of ACh in guinea pig trachea. *Am. J. Physiol.,* **264,** L581–L586.

BAKER, D.G., MCDONALD, D.M., BASBAUM, C.B. & MITCHELL, R.A. (1986). The architecture of nerves and ganglia of the ferret trachea as revealed by acetylcholinesterase histochemistry. *J. Comp. Neurol.,* **246,** 513–526.

BALUK, P. & GABELLA, G. (1989). Innervation of the guinea pig trachea: a quantitative morphological study of intrinsic neurons and extrinsic nerves. *J. Comp. Neurol.,* **285,** 117–132.

BARNES, P.J. (2001). Neurogenic inflammation in the airways. *Respir. Physiol.,* **125,** 145–154.

BAZÁN-PERKINS, B., SÁNCHEZ-GUERRERO, E., BARAJAS-LÓPEZ, C. & MONTAÑO, L.M. (2000). Sarcoplasmic reticulum depletion by caffeine and changes of $[Ca^{2+}]$i during refilling in bovine airway smooth muscle cells. *Arch. Med. Res.,* **31,** 558–563.

BEAVO, J.A., ROGERS, N.L., CROFFORD, O.B., HARDMAN, J.G., SUTHERLAND, E.W. & NEWMAN, E.V. (1970). Effects of xanthine derivatives on lipolysis and on adenosine 3′,5′-monophosphate phosphodiesterase activity. *Mol. Pharmacol.,* **6,** 597–603.

BLUSZTAJN, J.K., VENTURINI, A., JACKSON, D.A., LEE, H.J. & WAINER, B.H. (1992). Acetylcholine synthesis and release is enhaced by dibutyryl cyclic AMP in a neuronal cell line derived from mouse septum. *J. Neurosci.,* **12,** 793–799.

BOURREAU, J.P., ABELA, A.P., KWAN, C.Y. & DANIEL, E.E. (1991). Acetylcholine Ca^{2+} stores refilling directly involves a dihydropyridine-sensitive channel in dog trachea. *Am. J. Physiol.,* **261,** C497–C505.

CAMERON, A.R. & KIRKPATRICK, C.T. (1977). A study of excitatory neuromuscular transmission in the bovine trachea. *J. Physiol.,* **270,** 733–745.

CANNING, B.J. & FISCHER, A. (2001). Neural regulation of airway smooth muscle tone. *Respir. Physiol.,* **125,** 113–127.

CARTER, A.J., O'CONNOR, W.T., CARTER, M.J. & UNGERSTEDT, U. (1995). Caffeine enhances acetylcholine release in the hippocampus *in vivo* by a selective interaction with adenosine A1 receptors. *J. Pharmacol. Exp. Ther.,* **273,** 637–642.

ETHIER, M.F., YAMAGUCHI, H. & MADISON, J.M. (2001). Effect of cyclopiazonic acid on cytosolic calcium in bovine airway smooth muscle cells. *Am. J. Physiol.,* **281,** L126–L133.

EVONIUK, G., VON BORSTEL, R.W. & WURTMAN, R.J. (1987). Antagonism of the cardiovascular effects of adenosine by caffeine or 8-(*p*-sulfophenyl)theophylline. *J. Pharmacol. Exp. Ther.,* **240,** 428–432.

GRYNKIEWICZ, G., POENIE, M. & TSIEN, R.Y. (1985). A new generation of Ca^{2+} indicators with greatly improved fluorescence properties. *J. Biol. Chem.,* **260,** 3440–3450.

JANSSEN, L.J. (1996). Acetylcholine and caffeine activate Cl^- and suppress K^+ conductances in human bronchial smooth muscle. *Am. J. Physiol.,* **270,** L772–L781.

JOHRI, A.M. & JANSSEN, L.J. (1999). N-Type Ca^{2+} channels trigger release of excitatory and inhibitory neurotransmitter from nerve endings in canine bronchi. *J. Pharmacol. Exp. Ther.,* **290,** 847–853.

KAJITA, J. & YAMAGUCHI, H. (1993). Calcium mobilization by muscarinic cholinergic stimulation in bovine single airway smooth muscle. *Am. J. Physiol.,* **264,** L496–L503.

KOPP, U.C., CICHA, M.Z. & SMITH, L.A. (2002). PGE2 increases release of sustance P from renal sensory nerves by activating the cAMP-PKA transduction cascade. *Am. J. Physiol. Regul. Integr. Comp. Physiol.,* **282,** 1618–1627.

MADISON, J.M., ETHIER, M.F. & YAMAGUCHI, H. (1998). Refilling of caffeine-sensitive intracellular calcium stores in bovine airway smooth muscle cells. *Am. J. Physiol.,* **275,** L852–L860.

MATERA, M.G., AMORENA, M., MARABESE, I., LOFFREDA, A., D'AGOSTINO, B., LUCISANO, A. & ROSSI, F. (1997). Evidence for non-adrenergic non-cholinergic contractile responses in bovine and swine trachea. *Pulm. Pharmacol. Ther.,* **10,** 105–110.

MIZRAHI, J., ESCHER, E., D'ORLEANS-JUSTE, P. & REGOLI, D. (1984). Undeca- and octa-peptide antagonists for substance P, a study on the guinea pig trachea. *Eur. J. Pharmacol.,* **99,** 193–202.

MONTAÑO, L.M., HONG, E. & SELMAN, M. (1988). Difference in beta-blockade on the inhibitory response of cervical and thoracic tracheas to electrical stimulation. *Med. Sci. Res.,* **16,** 193–194.

NEHLIG, A., DAVAL, J.L. & DEBRY, G. (1992). Caffeine and the central nervous system: mechanisms of action, biochemical, metabolic and psychostimulant effect. *Brain Res. Rev.,* **17,** 139–170.

PAUVERT, O., SALVAIL, D., ROUSSEAU, E., LUGNIER, C., MARTHAN, R. & SAVINEAU, J.P. (2002). Characterisation of cyclic nucleotid phosphodiesterase isoforms in the media layer of the main pulmonary artery. *Biochem. Pharmacol.,* **63,** 1763–1772.

PESSAH, I.N., STAMBUK, R.A. & CASIDA, J.E. (1987). Ca^{2+}-activated ryanodine binding: mechanisms of sensitivity and intensity modulation by Mg^{2+}, caffeine and adenine nucleotides. *Mol. Pharmacol.,* **31,** 232–238.

POTTER, P.E., MEEK, J.L. & NEFF, N.H. (1983). Acetylcholine and choline in neuronal tissue measured by HPLC with electrochemical detection. *J. Neurochem.,* **41,** 188–194.

ROUSSEAU, E. & MEISSNER, G. (1989). Single cardiac sarcoplasmic reticulum Ca^{2+}-release channel: activation by caffeine. *Am. J. Physiol.,* **256,** H328–H333.

SCOTT, C., PERRY, M.J.M., KEEN, P.M. & LISNEY, S.J.W. (1996). Use of protease inhibitors increases the amounts of substance P extracted from small specimens of nerve tissue. *Anal. Biochem.,* **233,** 156–159.

SOMMER, B., VARGAS, M.H., SEGURA, P., BAZÁN-PERKINS, B., CARBAJAL, V., CHÁVEZ, J., GUSTIN, P. & MONTAÑO, L.M. (1997). Effect of different ozone concentrations on the neurogenic contraction and relaxation of guinea pig airways. *Fundam. Clin. Pharmacol.,* **11,** 501–511.

TANIGUCHI, M. & NAGAI, T. (1970). Effect of temperature on caffeine-induced calcium release from isolated reticulum in frog skeletal muscle. *Jpn. J. Physiol.,* **20,** 61–71.

TUCCI, P., BOLLE, P. & SEVERINI, C. (2001). Effect of natural tachykinins on ovine lower urinary tract smooth muscle. *J. Auton. Pharmacol.,* **21,** 79–84.

USACHEV, Y., SHMIGOL, A., PRONCHUK, N., KOSTYUK, P. & VERKHRATSKY, A. (1993). Caffeine-induced calcium release from internal stores in cultured rat sensory neurons. *Neuroscience,* **57,** 845–859.

WANG, Y-X. & KOTLIKOFF, M.I. (1997). Muscarinic signaling pathway for calcium release and calcium-activated chloride current in smooth muscle. *Am. J. Physiol.,* **273,** C509–C519.

WATANABE, C., YAMAMOTO, H., HIRANO, K., KOBAYASHI, S. & KANAIDE, H. (1992). Mechanims of caffeine-induced contraction and relaxation of rat aortic smooth muscle. *J. Physiol.,* **456,** 193–213.

(Received November 25, 2002
Revised January 17, 2003
Accepted April 30, 2003)