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Cyclic nucleotide-dependent phosphodiesterases (PDEI) inhibition by muscarinic antagonists in bovine tracheal smooth muscle

Lérida Guerra de González, Ramona González de Alfonzo, Itala Lippo de Bécemberg, Marcelo J. Alfonzo*

Sección de Biomembranas y Cátedra de Patología General y Fisiopatología, Instituto de Medicina Experimental, Facultad de Medicina, Universidad Central de Venezuela, Caracas, Venezuela

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Abstract

In bovine tracheal smooth muscle (TSM) strips, muscarinic antagonists (atropine, 4-DAMP, AFDX-116 and methoctramine) were able to increase simultaneously and a similar fashion the intracellular levels of cyclic nucleotides, with a cAMP/cGMP ratio higher than 2.0. These original pharmacological responses were time-and dose-dependent, exhibiting maximal values at 15 min, with a pEC_{50} of 7.4 ± 0.2 for atropine and 4-DAMP. These effects on cAMP and cGMP levels were similar to the ones obtained with isobutyl-methylxantine (IBMX, 10 μM), a non-selective cyclic nucleotide phosphodiesterase (PDE) inhibitor, suggesting the involvement of PDEs in these muscarinic antagonist responses. Neither, rolipram (10 μM), a specific PDEIV inhibitor, nor zaprinast (10 μM), a PDEV inhibitor, exhibited this ''atropine-like'' responses. Instead, atropine enhanced the increments of cAMP levels induced by rolipram and cGMP levels by zaprinast. However, vinpocetine (20 μM), a non-calmodulin dependent PDEIC inhibitor was able to mimic these muscarinic antagonist responses in intact smooth muscle strips. In addition, in cell free systems, muscarinic antagonists inhibited the membrane-bound PDEIC activity whereas soluble (cytosol) PDEIC activity was not affected by these muscarinic drugs. These results indicate that muscarinic antagonists acting possibly as inverse agonists on M_2/M_3mAChRs anchored to sarcolemma membranes can initiate a new signal transducing cascade leading to the PDEIC inhibition, which produced a simultaneous rise in both cAMP and cGMP intracellular levels in tracheal smooth muscle.

Keywords: PDE; cGMP; cAMP; mAChR; Airway smooth muscle; Muscarinic antagonists

1. Introduction

Airway smooth muscle contraction/relaxation processes are crucial to maintain the airways caliber, which is reduced in bronchial asthma. Both processes seem to exhibit distinctive signal transducing cascades at airway smooth muscle as reviewed [1]. Some signal cascades initiated at the smooth muscle sarcolemma generate cyclic nucleotides as cAMP and cGMP as second messengers. Cyclic AMP has been involved in the smooth muscle relaxation. There are two ways to rise cAMP intracellular levels, by the stimulation of the β_2 adrenergic receptor-Gs protein-adenylyl cyclase transducing machinery [2] and through the inhibition of cyclic nucleotide-dependent phosphodiesterases (PDEs) located in tracheal smooth muscle (TSM) [3]. In TSM, more significant cAMP augmentations have been reported using rolipram, a selective PDEIV inhibitor [4].

The role of cGMP in the airway smooth muscle physiology is controversial. Cyclic GMP-dependent relaxation has been demonstrated by the activation of two distinctive signalling cascades. Firstly, the presence of nitric oxide (NO) activated-soluble guanylyl cyclase signalling (sGC) cascade, which regulates a calcium- activated-K^+ channels linked to tracheal smooth muscle relaxation [5]. The second cascade is related to activation of membrane-bound NP-sensitive GCs [6], involved in tracheal smooth muscle

Abbreviations: AFDX-116, 11[[2-[(diethylamino) methyl]-1-piperidinyl]-5,11-dihydro-6H-pyrido[2,3-b][1,4]benzodiazepine-6-one; 4-DAMP, 4-diphenylacetoxi-*N*-methylpiperidine methobromide; BTSM, bovine tracheal smooth muscle; CC, carbachol; CAM, calmodulin; EGTA, ethyleneglycoltetraacetic acid; IBMX, 3-isobutyl-1-methyl-xantine; GC, guanylyl cyclase; KRB, Krebs–Ringer bicarbonate buffer; mAChR, muscarinic acetylcholine receptor; PMSF, phenyl methyl sulphonyl fluoride; rolipram, (4,3(cyclopentyloxy)-4-methoxyphenyl) pyrrolidin-2-one; zaprinast, 2,2(propyl oxyphenyl)-8-azapurin-6-one

* Corresponding author. Tel.: +58 212 6053654; fax: +58 212 6628877.
E-mail address: hmag5@hotmail.com (M.J. Alfonzo).

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physiology as previously suggested by Ishii and Murad [7]. In this sense, a CNP-sensitive GC (NP-GC) has been recently identified by Borges et al. [8] and found to participate in muscarinic signalling cascades. However, a muscarinic acetylcholine receptor (mAChR) activation leading to tracheal smooth muscle contraction with a significant increment in cGMP tissue levels have been reported by Katsuki and Murad [9]. Recently, Guerra de Gonzalez et al. [10,11] have established that carbamylcholine (a muscarinic agonist) induces contraction in bovine TSM (BTSM) triggering two cGMP signal events. The first signal (20 s), is the result of activation of soluble guanylyl cyclase (sGC) and the second one (60 s), might be a product of membrane-bound GC as described by Becemberg et al. [12] and Alfonzo et al. [13]. In trying to identify the mAChR subtype involved in these cGMP signal responses, BTSM preparations were pre-incubated during 15 min with a non-selective muscarinic antagonist (atropine) to block agonist actions. Surprisingly, it was found that atropine (0.1–10 μM) increased cAMP and cGMP levels [14], suggesting that this drug is affecting the intracellular cyclic nucleotide content. The aim of this study was to elucidate the molecular mechanisms associated with a simultaneous rise of the cAMP and cGMP intracellular levels induced by the muscarinic antagonists in bovine tracheal smooth muscle cells.

2. Materials and methods

2.1. Materials

The following compounds were purchased from Sigma, Atropine, 4-DAMP, methoctramine, IBMX, rolipram, zaprinast, vinpocetine, carbachol and snake venon 5′ nucleotidase. Kits for cGMP determination (TRK-500) were obtained from Radiochemical Center, Amersham. Cyclic [8,5-^{3}H] GMP (25–50 Ci/mmol) and cyclic [5′,8-^{3}H] AMP (30–60 Ci/mmol), were purchased from Amersham. Kits for cAMP radioassay determination (KAPH2-172) were obtained from Diagnostic Products Corporation (DPC). BSA (Fraction V) was from Armour. Ion-exchange resin (Dowex-1-X-8 chloride form) was purchased from BIORAD. Other chemicals pro-analysis grade reagents were obtained from E. Merck and Fisher.

2.2. Preparation and incubation of Bovine TSM

Bovine tracheas obtained from a local slaughterhouse were placed and transported in cold Krebs ringer bicarbonate (KRB) solution and processed as described previously by Gonzalez et al. [11]. In these studies, tracheal rings posterior to the carina were usually employed. The smooth muscle layer was carefully separated from cartilage, serosa, mucosa and connective tissues. Smooth muscle strips (10 mm × 2 mm) were excised while immersed in ice-cold KRB (pH 7.4), gassed with 95% O_2 and 5% CO_2, and used within 3 h. Evaluation of smooth muscle contraction and cyclic nucleotide concentration was performed by using two procedures.

2.2.1. Procedure 1

Smooth muscle strips were placed into an organ bath (20 ml) and equilibrated for 1 h in KRB with 95% O_2 and 5% CO_2 (pH 7.4) at 37°, with medium replacement every 30 min. Strips were loaded with 1 g of tension, and the contraction was expressed as an increase in tension of these preparations, measured isometrically by using a force displacement transducer (Grass model FT03) attached to a polygraph (Grass model 7-B). After 1 h of incubation, the different pharmacological agents (in less than 20 μl) were added. This assay was routinely used to evaluate the contractile capabilities of each tracheal smooth muscle preparations. Later, the bath was drained rapidly in around 5 s, and the strip was frozen in liquid nitrogen.

2.2.2. Procedure 2

To evaluate the cyclic nucleotide content of several fragments, smooth muscle strips were placed into a specially designed multi-organ chamber with a volume of 400 ml. This chamber has a system of aeration with 95% O_2 and 5% CO_2, and it is able to hold simultaneously sixteen smooth muscle strips at 37°, at 1 g of tension. After addition of drugs, individual fragments were removed from the bath at specific times and placed into liquid nitrogen (within less than 1 s). Samples were kept in liquid nitrogen until cyclic nucleotide extraction was performed. There were no differences in the cyclic nucleotide responses to the agents tested under the two incubation conditions.

2.3. Measurement of cyclic nucleotides

Frozen samples were thawed and homogenized in 6% TCA as previously described by Gonzalez et al. [11]. TCA extractions were performed twice, and the insoluble material was removed by centrifugation at 1500 × *g* for 10 min at 4 °C. The insoluble material was processed for protein determination as described later. The acid supernatants were combined, extracted twice with ether to remove TCA, frozen at −80 °C and lyophilized. The acid-soluble nucleotide extract was dissolved in a small volume of 50 mM Tris–HCl, 4 mM EDTA, pH 7.4, and kept frozen at −80°. In each experiment, some untreated frozen strips were used to evaluate the cyclic nucleotide recovery following the procedure above described. Thus, 0.4 pmol of [^{3}H] cGMP or [^{3}H] cAMP were added to some samples, and the recovery was between 95 and 98% for both nucleotides. This recovery rate was assumed to be the same for all samples. cGMP was determined using a radioimmunoassay as previously described [12] with a commercial kit (TRK500) from Amersham. Cyclic AMP was determined using a radioassay kit (KAPH2-172) from DPC as described previously by Alfonzo et al. [15].

2.4. Preparation of soluble and membranes fractions from tracheal smooth muscle

Bovine tracheal smooth muscle strips were isolated as previously described [11]. A cellular extract from these smooth muscle fragments (20 g) was prepared using a Polytron with a buffer I containing 0.3 M sucrose, 20 mM K-EGTA, 2 mM benzamidine, 0.25 mM PMSF 0.5 mM, DTT, HEPES-KOH (pH 7.0) as described [16]. This homogeneized material was filtered through eight layers of cheesecloth and the filtrate was centrifugated at 10,000 × *g* for 15 min to remove cell debris and nuclei leading to a supernatant called celullar extract. The last fraction was spun down at 150,000 × *g* for 1 h leading to a sediment (membranes fraction) and the soluble (cytosol fraction). The membranes fraction was dispersed into a small volumen of Buffer I and glycerol was added to 30% (v/v) in both cytosol and membranes fractions, frozen in liquid nitrogen and stored for less than a week at −86 °C.

2.5. Cyclic nucleotide phosphodiesterase (PDE) activities

The PDE activities were assayed in duplicate using [^{3}H]cGMP as substrate as described elsewhere [17]. Briefly, 50–100 μg of protein were incubated for 15 min at 30 °C with appropriate concentrations (0.1 μC per assay) 8-[^{3}H] cGMP in a buffer containing 0.3 mM of cGMP, 40 mM Tris–HCl pH 7.5, 10 mM $MgCl_2$ and 1 unit of snake venon 5′ nucleotidase. Reactions were terminated by the addition of 10 mM EDTA and transfer to 4 °C. Later, samples were applied to AG-1-X8 (Chloride form, 200–400 mesh) anion exchange columns (BIO-RAD) and the specific hydrolyzed products ([^{3}H] guanosine) was eluted with 50% ethanol. The [^{3}H] content of the eluate was determined using a liquid scintillation counter.

2.6. Other methods

Total smooth muscle protein content was determined in the TCA-insoluble material, which was dissolved in 200 μL of 1 N NaOH and diluted five times to determine protein according to by Bensaudoun and Weinstein [18], using bovine serum albumin as standard. Cyclic nucleotide values are given as picomoles per milligram of total protein. Statistical analysis was performed with Student's *t* test. Values of $-\log EC_{50}$ (pEC_{50}) were estimated using a computer program Prism.3. (Graph Pad®).

3. Results

3.1. Effects of atropine on cyclic nucleotide levels at BTSM

Bovine tracheal smooth muscle cAMP and cGMP basal levels (in pmoles/mg protein) increased in a time-dependent manner in the presence of 10 μM atropine as shown in Fig. 1. Cyclic AMP content raised from 5.0 ± 0.8 to 25 ± 1.3 in about 10 min ($P < 0.01$) Simultaneously, cGMP basal levels were also increased from 1.0 ± 0.3 to 18 ± 1.2 ($P < 0.01$) during the same incubation period. Both biological responses exhibited similar parallel kinetic pattern.

3.2. Effects of non-selective PDE inhibitors on cyclic nucleotide content in BTSM

These increments in cyclic nucleotide content are similar to the effect produced by the cyclic nucleotide PDE inhibitors described in some biological preparations. In this sense, IBMX, a non-selective inhibitor of mammalian PDEs, was evaluated on the cyclic nucleotide levels in BTSM preparations using the same experimental approach as described for atropine. Thus, IBMX (10 μM) induced a similar response as described for atropine, but the effect was less evident than the atropine-elicited effects as showed in Fig. 1. These data suggest that PDEs may be involved in this atropine effect.

3.3. Effects of muscarinic antagonists on BTSM cyclic nucleotide levels

It is important to establish the specificity of this atropine action on cyclic nucleotide content of BTSM. Accordingly, another muscarinic antagonist (4-DAMP) was employed. BTSM strips were incubated during 5 min with increasing concentrations of atropine and 4-DAMP as shown in Fig. 2. In these experiments, atropine and 4-DAMP showed iden-

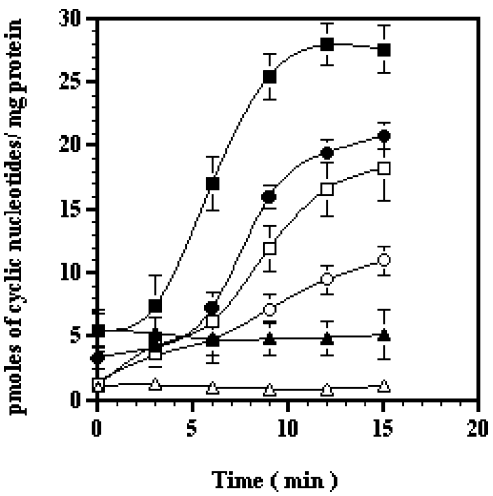

Fig. 1. Time dependence of atropine and IBMX on cAMP and cGMP levels on TSM. Tracheal smooth muscle strips were incubated with atropine (10 μM) and IBMX (10 μM) at different times. The cyclic nucleotides determinations were carried out as described in Section 2. Cyclic AMP tissue levels (dark symbols), on basal condition (▲), in the presence of atropine (■) or IBMX (●). Cyclic GMP levels (empty symbols), on basal condition (△), in the presence of atropine (□) and IBMX (○). Each value is the mean ± S.E.M. of four different TSM preparations, whose nucieotide determinations were performed by triplicate.

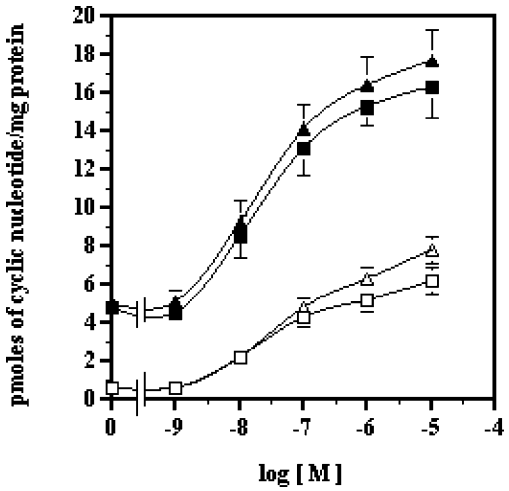

Fig. 2. Dose dependence effect of muscarinic antagonists on cyclic nucieotide levels on TSM. Smooth muscles strips were incubated for 5 min in the presence of different concentrations (1×10^{-9} to 1×10^{-5} M) of atropine (▲, △) or 4-DAMP (□, ■) as described in Section 2. Dark symbols coincide with cAMP tissue levels and empty symbols with cGMP levels. Each value is the mean ± S.E.M. of three different TSM preparations and nucleotide determinations were done by triplicate.

tical pharmacological pattern responses on the cAMP and cGMP levels, being atropine slightly more potent than 4-DAMP. From these data, the pEC_{50} values, for cAMP/cGMP responses for both drugs were calculated. Thus, 4-DAMP (7.88 ± 0.28) and atropine (7.78 ± 0.24) values were estimated for the cAMP elevations, while for the cGMP rise, 4-DAMP (7.32 ± 0.17) and atropine (7.14 ± 0.12) values were obtained.

Tracheal smooth muscle exhibits two pharmacological muscarinic receptors subtypes (M_2 and M_3 AChR) and studies were carried out to identify the mAChR subtype involved in this cyclic nucleotide increments. Further studies were performed with specific M_2 muscarinic antagonists such as AFDX-116 and methoctramine and these data are shown in Fig. 3. Similar pharmacological responses and potencies were found for AFDX-116 and 4-DAMP on the cGMP levels, being methoctramine a less potent drug, since methoctramine induced 50% of cGMP increments in comparison to atropine. In these assays, the estimated pEC_{50} values were 7.4 ± 0.11 (4-DAMP); 7.25 ± 0.12 (AFDX); 7.10 ± 0.11 (methoctramine). These data support a pharmacological profile as follows: Atropine ≥ 4-DAMP ≥ AFDX-116 ≥ methoctramine.

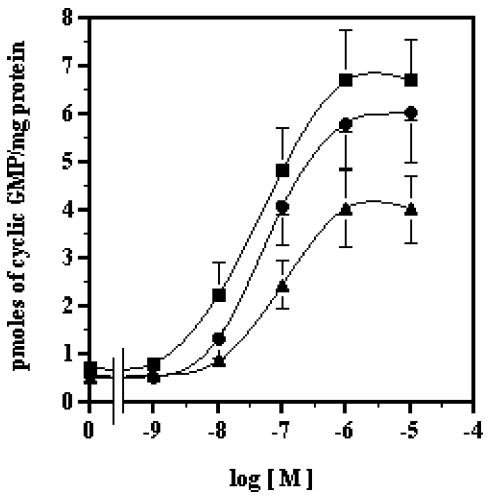

Fig. 3. Time dependence effect of muscarinic antagonists, 4-DAMP, methoctramine and AFDX-116 on the levels of cGMP in TSM. Tracheal smooth muscle strips were incubated as described in Section 2 with concentrations (1×10^{-9} to 1×10^{-5} M) of 4-DAMP (■), AFDX-116 (●) and methoctramine (▲). The incubation time was 5 min, after this time, samples were processed as described in Section 2. Each value is the mean ± S.E.M. of three different preparations and cGMP content was determined by triplicate.

3.4. Effects of rolipram (PDEIV) and zaprinast (PDEV) inhibitors on cyclic nucleotides levels

The most abundant cyclic nucleotide PDE isozymes present in BTSM are potential targets for antagonist muscarinic action, as mentioned above. Hence, the effect of specific PDEIV (rolipram) and PDEV (zaprinast) inhibitors were evaluated on the BTSM cyclic nucleotide levels. As can be seen in Fig. 4, rolipram produced a significant augmentation of cAMP levels. Moreover, the addition of atropine plus rolipram synergistically increased the cAMP levels. Interestingly, zaprinast did not affect the atropine behavior on cAMP content when both drugs were combined, and zaprinast alone was not able to modify the cAMP levels as expected. The cGMP content of BTSM strips was also determined in the same samples of the experiments above described. It can be observed in Fig. 5 that the combination of zaprinast and atropine showed the highest increment in cGMP tissue content being linear as a function of time until 20 min. Also, the mixture of rolipram and atropine showed similar cGMP responses as atropine

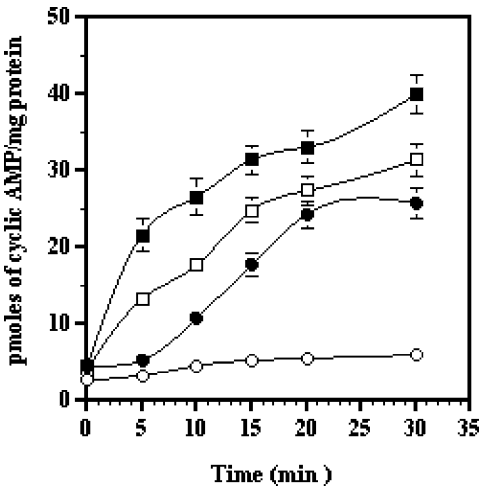

Fig. 4. Time dependence of PDEIV (rolipram) and PDEV (zaprinast) inhibitors on the cAMP levels in TSM. Tracheal smooth muscle strips were incubated as described in Section 2 in the presence of rolipram (10 μM) plus atropine (10 μM) (■), rolipram (10 μM) (□), zaprinast (10 μM) plus atropine (10 μM) (●), zaprinast (10 μM) (○). Each value is the mean + S.E.M. of four experiments performed by triplicate.

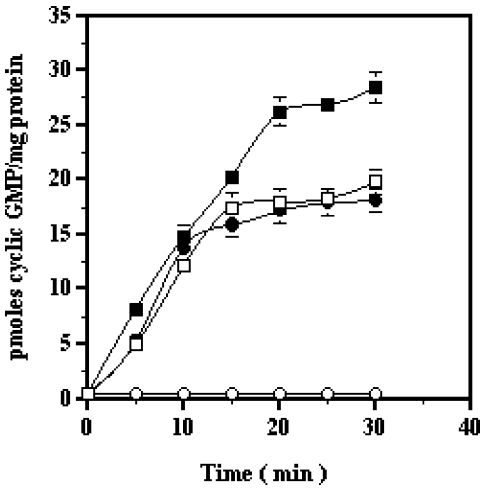

Fig. 5. Effect of PDEIV (rolipram) and V (zaprinast) inhibitors on cGMP levels in TSM in function of time. Tracheal smooth muscle were incubated as described in Section 2 in the presence of zaprinast (10 μM) plus atropine (10 μM) (■), zaprinast (10 μM) (□); rolipram (10 μM) plus atropine (10 μM) (●), rolipram (10 μM) (○). Each value is the mean ± S.E.M. of four different TSM preparations and the cGMP determinations were carried out by triplicate.

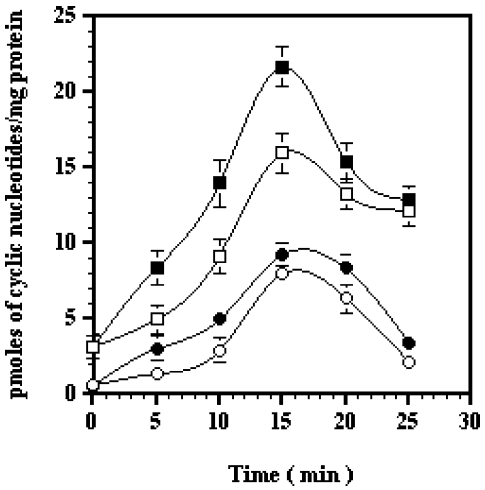

Fig. 6. Time dependence of vinpocetine and atropine on the cyclic nucleotides levels at TSM. Tracheal smooth strips were incubated at different time in the presence of 20 μM vinpocetine (■, ●) and 10 μM atropine (□, ○) as described in Section 2. The squares symbols denote the cAMP and the circles symbols are the cGMP tissue levels. Each value is the mean ± S.E.M. of three different TSM preparations and the cyclic nucleotides concentrations were determined by triplicates.

alone. Moreover, the cGMP levels were not changed in the presence of rolipram, as expected. These data confirm the presence of PDEIV and PDEV isoenzymes in our BTSM preparations.

3.5. Effect of PDEIC inhibitor (vinpocetine) on cyclic nucleotides amounts

Neither rolipram nor zaprinast were able to inhibit the atropine effect on cyclic nucleotide content in BTSM. Instead, atropine enhanced the effects of these selective PDE inhibitors, suggesting that atropine may be affecting other PDE that regulate simultaneously the cAMP and cGMP levels. The PDEI subfamily members hydrolyze both cAMP and cGMP, specially PDEIC, which seems to be the most likely candidate, since this enzyme hydrolyzes cAMP and cGMP with the same affinity. To test this potential target, the effect of vinpocetine, a non-CaM-dependent PDEIc inhibitor, was evaluated in BTSM preparations. As seen in Fig. 6, vinpocetine (20 μM) and atropine (10 μM) displayed identical biological responses on cyclic nucleotide levels, in these experiments both compounds were tested side to side, using similar BTSM preparations. In addition, vinpocetine was more potent than atropine in increasing the cyclic nucleotide levels. Both drug effects were time dependent, showing the maximal levels at 15 min for both nucleotides. However, cGMP levels decreased faster in comparison with cAMP, whose levels remained higher after 25 min of incubation. If muscarinic antagonist-mediated increase of cAMP and cGMP is due to inhibition of PDEIC, it is expected that in the presence of the PDEIC inhibitor vinpocetine, muscarinic antagonists would no longer be able to increase cyclic nucleotides levels. As shown in Fig. 7, pretreatment of BTSM with vinpocetine for 15 min, (followed by 25 min of incubation) after the addition of 10 μM atropine, obliterated the muscarinic antagonist-mediated rise in BTSM cGMP content. This important evidence further supported the notion that muscarinic antagonists are able to inhibit PDEIC activity. Moreover, these data indicate that muscarinic antagonists, such as atropine and vinpocetine are acting on the same molecular entity (PDEIC) in BTSM preparations.

3.6. PDE activities in free-cell systems from BTSM

PDEIC seems to be the intracellular target of vinpocetine and atropine actions as discussed above. To evaluate

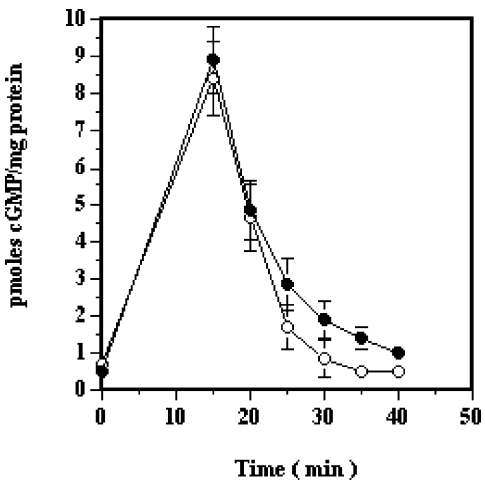

Fig. 7. The effect of vinpocetine pre-treatment on the atropine effect on the cGMP levels at BTSM. Tracheal smooth strips were pre-incubated for 15 min with 20 μM vinpocetine (○). After this time; 10 μM atropine was added in the presence of 20 μM vinpocetine (●) as described in Section 2. Each value is the mean ± S.E.M. of three different TSM preparations and the cyclic nucleotides concentrations were determined by triplicates.

Table 1
Percentage inhibition of cGMP-PDE activity in soluble and membranes fractions from bovine tracheal smooth muscle by muscarinic antagonists and PDEIC inhibitors

Drugs	Subcellular fractions	
	Cytosol	Membranes
Atropine (10 μM)	5	49
Vinpocetine (20 μM)	20	50
IBMX (10 μM)	49	80

PDE activity (expressed as pmoles cGMP hydrolyzed/min/mg protein) was determined using [^{3}H]cGMP as substrate in the presence of 10 μM zaprinast (selective inhibitor of PDEV) as described in Section 2. Cytosol (soluble) and membranes fractions were prepared as described in Methods. The 100% PDE activity at cytosol was 1.094 ± 50 and membranes fraction was 971 ± 40. Each value is the mean of three different subcellular preparations assayed by duplicate. The standard error was less than 5% of the mean.

this hypothesis, PDEIC activity was assayed measuring the cGMP-PDE in cell-free systems, in the presence of a specific inhibitor of cGMP-PDEV (zaprinast) (10 μM). Thus, a zaprinast-insensitive cGMP-PDE activities was assayed in membranes and soluble BTSM subcellular fractions. Table 1 summarizes the effects of selective and non-selective PDE inhibitors on the zaprinast-insensitive cGMP-PDE. Vinpocetine (20 μM) inhibited 20% of this cGMP-PDE activity in the cytosolic fraction whereas this drug inhibited a 50% of the membrane bound enzyme activity suggesting that vinpocetine sensitive PDEIC is mainly located in the membranes fraction. Muscarinic antagonists such as atropine (10 μM) did not affect the PDEs activities in soluble (cytosol) fraction but inhibited about 50% of the membrane bound cGMP PDE activity. It is important to point out that IBMX (10 μM), a non-selective PDE inhibitor was able to inhibit about 50% of the cGMP PDE activity in the cytosolic fraction and inhibited more than 80% the membrane bound cGMP-PDE activities.

4. Discussion

Muscarinic antagonists (atropine, 4-DAMP, AFDX-116 and methoctramine) are charged and bulky molecules unable to cross biological membranes. These drugs act on muscarinic receptors (MAChR) located at the BTSM sarcolema eliciting the increments on cAMP and cGMP content in BTSM described here. Definitive identification of mAChR subtype involved in these biological effects on cyclic nucleotides levels remains uncertain, since BTSM exhibit two pharmacological MAChR subtypes (M_2, M_3) as described elsewhere [19,20]. Nevertheless, the involvement of a M_2 AChR subtype is supported by the pharmacological profile of the pEC_{50} estimated in this work, which was: Atropine ≥ 4-DAMP ≥ AFDX ≥ methoctramine, in agreement with the pK_B profile described by Caulfield and Birdsall [21] for the mammalian M_2 AChR has been characterized displaying this: Atropine (9.0–9.3) ≥ AFDX (8.2–9.0) ≥ 4-DAMP (7.8–8.4) ≥ methoctramine (7.8–8.3). Moreover, these pharmacological responses are similar to the ones reported by Me Intosh and Blazynski [22] for some M_2AChR-mediated actions. Another evidence supporting the involvement of M_2AchR in the above effects is the high density or abundance (70–80%) of this M_2AChR subtype in tracheal smooth muscle [20,23].

Muscarinic receptors may be coupled to signal transducing cascades that can rise these cyclic nucleotide levels due to the inhibition of PDE isozymes. Currently, in TSM, five PDEs families have been identified with different primary structure, kinetic properties, regulatory mechanisms and sensitivity to pharmacological inhibitors [16,24,25]. These families are PDEI (calmodulin [CAM]-dependent PDE), PDEII (cGMP-stimulated PDE), PDEIII (cGMP-inhibited PDE), PDEIV (cAMP-specific PDE) and PDEV (cGMP-specific PDE). In our case, however the role of PDEII and PDEIII isozymes is overlooked, in view of their biochemical properties described by Shahid et al. [16] and Juilfs et al. [26].

The selection of specific PDE inhibitors was based on the type of PDE isoenzymes present in BTSM, as described above, and the pharmacological effects of the drugs selected on airway smooth muscle. It is well known that some PDE inhibitors can display distinctive pharmacological responses upon different smooth muscle types. Thus, rolipram was preferred as a selective PDEIV inhibitor, which has been extensively used in different TSM preparations [16,27,28]. Likewise, zaprinast was chosen as a selective PDEV inhibitor in TSM based on previously published reports [16,27–29].

Our results showed that rolipram was able to increase the cAMP content without changing the cGMP levels, confirming the presence of PDEIV in BTSM [16,24]. This rolipram-sensitive PDEIV is a molecular target for anti-asthmatic agents [30,31] associated with airway smooth muscle relaxation. Also, we found that zaprinast (a selective PDEV inhibitor) increased the cGMP tissue levels without affecting the cAMP levels, demonstrating the existence of PDEV at BTSM as described in guinea pig TSM [32] and BTSM [16].

Atropine significantly increased, the effects of PDEIV/V selective inhibitors, suggesting that the inhibition of other PDE activity might be responsible for the rise in cyclic nucleotides levels. Tracheal smooth muscle exhibits a set of [CAM]-dependent PDEI subfamily [33,34] that hydrolyzes cAMP and cGMP with equal affinity (low Km = 1–3 μM), specifically PDEIC [16,24], which might be the intracellular target of atropine action. This latter suggestion is strengthened by our results using vinpocetine, a selective non-CAM related PDEIC inhibitor in vascular smooth muscle [35,36]. This drug was able to ‘‘mimic’’ the atropine actions in both the increments on the cyclic nucleotide tissue levels and the inhibition of the membrane bound cGMP-PDE activity. An additional experiment was

performed to evaluate the effect of pre-treatment of PDEIC inhibitor (vinpocetine) on muscarinic antagonists. Under these experimental conditions, muscarinic antagonists were not longer able to increase the cyclic nucleotide levels.

In order to explain these biological activities of the MAChR in the absence of muscarinic agonists (ACh), the MAChR may portray some unknown ''endogenous signaling cascade'' activity modulated by these muscarinic antagonists. Such ''endogenous signaling cascade'' activity has been described by us [13] in a plasma membrane fraction from BTSM, where a M_3AChR coupled to a membrane-bound GC exhibited a ''basal'' activity inhibited by the muscarinic antagonist 4-DAMP. This ''endogenous muscarinic signaling cascade'' activity may be explained by an inverse agonist activity. Muscarinic AChRs have been predicted to exist as a mixture of two functional conformation entities [37] being the so-called conformation (R), induced or stabilized by inverse agonists, while the second conformation state (R*) is the agonist dependent conformation [38]. A classic antagonist has no biological activity. These results further support the notion that muscarinic antagonists may act as inverse agonists of muscarinic receptors and stimulate an unknown signaling cascade that leads to inhibition of PDEIC. If this is the case, this ''basal'' state of MAChR may be linked to the PDEIC activation, therefore implying that muscarinic antagonists, therefore decrease PDEIC activity by inhibiting the ''basal'' muscarinic receptor activation.

In the basal state of TSM, PDEIC activation results in low levels of both cAMP and cGMP, as would be expected based on the enzymatic characteristics of PDE1C [39]. Moreover, such activation is guaranteed at low intracellular [Ca^{2+}] levels since CaM-PDEs contain two high affinity Ca^{2+}/calmodulin binding domains, which are required for activation of these PDEs. Additional questions may be risen. Do muscarinic agonists affect PDEIC? It is important to point out that agonist actions on cyclic nucleotide signals in TSM occur in less than 60 s [9,11] and that these muscarinic antagonist effects are maximal at 15 min of drug exposure.

The lack of effect of atropine on the [^{3}H] cGMP-PDE activities present in the soluble fraction contrasts with the significant inhibition of the membrane-bound enzyme activity, which argues against that atropine is acting directly on the soluble PDE enzymes. However, PDEIC isoforms associated with membranes and cytosol might be structurally different. Thus, the direct inhibition of the membrane-bound PDEIC by muscarinic antagonists is also unlikely given the restrictive active site of membrane-bound cGMP-PDE and the diverse chemical structures of these drugs.

Muscarinic antagonists can affect indirectly this membrane bound-PDE through an unknown signal transducing cascade located in airway smooth muscle membranes. A model implying a MAChR coupled to a novel heterotrimeric G-protein and linked to PDEIC can be postulated by analogy to the light signal transduction cascade in retina, which is composed by Rhodpsin (seven transmembrane receptor)-transducin (heterotrimeric G-protein)–cGMP–PDE [40]. Airway smooth muscle relaxants have been associated with cAMP elevations [2,4,31] and cGMP rise in this smooth muscle type [5,8]. Our findings offer some insight towards the understanding of muscarinic antagonists acting as TSM relaxing drugs since these simultaneous augmentations of cAMP and cGMP can activate specific cyclic nucleotide dependent signal cascades which could act synergistically to produce a substantial relaxation. Moreover, this work defines PDEIC as a new molecular target for novel pharmacological agents able to generate concerted increments in the cyclic nucleotide levels and in order to alleviate the bronchoconstriction imposed in patients suffering from bronchial asthma.

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