Biochemical Pharmacology, Vol. 58, pp. 563–569, 1999.

ELSEVIER

ISSN 0006-2952/99/$–see front matter
PII S0006-2952(99)00115-X

Effects of 1*H*-[1,2,4]Oxadiazolo[4,3-*a*]quinoxalin-1-one (ODQ) and N^{ω}(6)-Nitro-L-arginine Methyl Ester (NAME) on Cyclic GMP Levels during Muscarinic Activation of Tracheal Smooth Muscle

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ABSTRACT. The effects of carbachol on the cyclic GMP (cGMP) content of bovine tracheal smooth muscle in the absence of phosphodiesterase inhibitors were evaluated. Carbachol (1×10^{-5} M) induced two cGMP peaks, at 20 and 60 sec. Both cGMP signals were carbachol concentration-dependent (1×10^{-11} to 1×10^{-5} M), the first being higher than the second. The cGMP signal induction was studied using an inhibitor of the soluble guanylyl cyclase (GC), 1*H*-[1,2,4]oxadiazolo[4,3-*a*]quinoxalin-1-one (ODQ), and a nitric oxide (NO) synthase inhibitor, N^{ω}(6)-nitro-L-arginine methyl ester (NAME). ODQ (1×10^{-7} M) did not affect the second cGMP peak but abolished the first peak, suggesting that a soluble GC may be involved. NAME (1×10^{-4} M) did not affect the cGMP signals, but changed their 2:1 ratio and also induced a time-shift of the first peak to 10 sec and the second to 50 sec. These results indicate that the NO-soluble GC cascade is not responsible for these muscarinic effects on cGMP levels. BIOCHEM PHARMACOL **58**;4:563–569, 1999. © 1999 Elsevier Science Inc.

KEY WORDS. cGMP; muscarinic agonists; guanylyl cyclase; nitric oxide

Muscarinic activation of TSM† and the relationship between cGMP levels and TSM function have been described [1]. Studies of muscarinic activation and cGMP levels in airway smooth muscle have produced controversial results. Thus, both contraction and relaxation responses have been associated with increased levels of cGMP [1–5]. Elevations of cGMP are related to NO generation and subsequent activation of soluble GC in several tissues [2–4, 6, 7], and are reduced by NOS inhibitors [3]. It seems reasonable to postulate that these two opposite cellular responses, operating with the same second messenger cyclic nucleotide, must have different signaling cascades and regulation mechanisms.

The role of cGMP in the muscarinic activation of the TSM contraction remains obscure [1, 5]. The aim of the present study was to evaluate the effect of carbachol on the cGMP levels without PDE inhibitors in isolated TSM strips during short periods of time. Thus, these carbachol-elicited cGMP responses were studied in the presence of NAME, a specific inhibitor of NOS [3], and ODQ, an inhibitor of soluble GC [8].

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† *Abbreviations*: AMG, aminoguanidine; CC, carbachol; cGMP, cyclic GMP; DTT, dithiothreitol; GC, guanylyl cyclase; KRB, Krebs-Ringer-bicarbonate; mAChR, muscarinic acetylcholine receptor; NAME, N^{ω}(6)-nitro-L-arginine methyl ester; NO, nitric oxide; NOS, nitric oxide synthase; ODQ, 1*H*-[1, 2, 4]oxadiazolo[4,3-*a*]quinoxalin-1-one; OPD, *o*-phenylenediamine; PDE, cyclic nucleotide phosphodiesterase; PMSF, phenylmethylsulfonyl fluoride; SNP, sodium nitroprusside; TCA, trichloroacetic acid; and TSM, tracheal smooth muscle.

Received 21 May 1998; accepted 23 November 1998.

MATERIALS AND METHODS

Materials

The following compounds were purchased from the Sigma Chemical Co.: atropine, NAME, $NADPH_2$, FAD, tetrahydro-L-biopterin, L-arginine, DTT, AMG, creatine phosphate, creatine phosphokinase (rabbit muscle, type I), GTP, PMSF, Trizma base, Tween-20, sucrose, carbachol, and OPD. ODQ was purchased from Tocris-Cookson. Kits for cGMP determination (TRK–500) were obtained from the Radiochemical Center, Amersham. Cyclic [8,5-^{3}H] GMP (25–50 Ci/mmol) was purchased from New England Nuclear. BSA (Fraction V) was from Armour. Polyclonal antibodies against NOS-2 and NOS-3 were purchased from Santa Cruz Biotechnology. Other chemical reagents were obtained from E. Merck.

Preparation of Bovine TSM

Bovine tracheas obtained from a local slaughterhouse were placed in cold KRB solution containing 120 mM NaCl,

5 mM KCl, 1.2 mM $MgSO_4$, 1.2 mM KH_2PO_4, 2.5 mM $CaCl_2$, 25 mM $NaHCO_3$, and 10 mM glucose (pH 7.4). In these studies, tracheal rings posterior to the carina were usually employed. The smooth muscle layer (95–98% smooth muscle cells identified by hematoxylin-eosin staining) was separated carefully from cartilage, serosa, mucosa, and connective tissues. Smooth muscle strips of 10 × 2 mm were prepared while immersed in ice-cold KRB (pH 7.4), gassed with 95% O_2 and 5% CO_2, and used within 3 hr.

Incubation of Smooth Muscle Fragments

The evaluation of smooth muscle contraction and nucleotide concentration was performed by using two procedures.

Procedure 1. Smooth muscle fragments were placed into an organ bath (20 mL) and equilibrated for 1 hr 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 hr of incubation, the different pharmacological agents (less than 20 μL) were added. Later, the bath was drained rapidly, and the strip was frozen in liquid nitrogen. The latter step took around 5 sec.

Procedure 2. 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 16 smooth muscle strips at 37°, at 1 g of tension. After addition of drugs, individual fragments were removed from the bath every 10 sec and placed into liquid nitrogen (within less than 1 sec). Samples were kept in liquid nitrogen until nucleotide extraction was performed. There were no differences in the cyclic nucleotide responses to the agents tested under the two incubation conditions.

Measurement of Cyclic Nucleotides

Frozen samples were thawed and homogenized in 6% TCA as previously described [9]. TCA extractions were performed twice, and the insoluble material was removed by centrifugation at 1500 *g* for 10 min at 4°. 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°, and lyophilized. The acid-soluble nucleotide extract was dissolved in a small volume of 50 mM Tris, 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 described above. For this, 0.4 pmol of [^{3}H]cGMP was added to some samples, and the recovery was between 95 and 98% for this nucleotide. This recovery rate was assumed to be the same for all samples. cGMP was determined using a radioimmunoassay as previously described [10] with a commercial kit (TRK 500) from Amersham. TCA-insoluble material was dissolved in 200 μL of 1 N NaOH and diluted five times to determine total protein content by using a procedure described elsewhere [11]. Cyclic nucleotide values are presented as picomoles per milligram of protein.

Subcellular Fractions from TSM

Subcellular fractionation of TSM was performed as described previously [12]. However, in some experiments, a soluble fraction and a sediment fraction at 150,000 *g* for 1 hr were obtained from extracts of TSM.

GC Assay (EC 4.6.1.2)

GC activity was measured, using a procedure described previously [10], in 125 μL of a reaction mixture containing 50 mM Tris–HCl (pH 7.6), 3 mM $MnCl_2$, 1 mM GTP, and a GTP-regenerating system (5 mM creatine phosphate and 10 IU phosphocreatine kinase/assay in 0.1% defatted BSA). For the assay of the soluble enzyme, 1 mM theophylline was included. Reactions were initiated by addition of the enzyme preparation (15–20 μg membrane-bound and 40–60 μg soluble protein fraction) and incubated for 5 min at 37°. Reactions were terminated by the addition of 10 μL of 167 mM EDTA-Tris (pH 7.5), followed by heating for 3 min in a boiling water bath and cooling on ice. Samples incubated with heat-inactivated enzyme were used as blanks. cGMP was estimated by using a TRK-500 kit from Amersham in 50–100 μL of supernatant of the reaction mixture obtained after centrifugation at 12,000 *g* for 3 min at 4°. Radioactivity was determined by liquid scintillation spectrometry.

NOS Assay (EC 1.14.13.39)

NOS activity was determined by using the spectrofluorometric determination of nitrite formation as described by Misko *et al.* [13]. Subcellular fractions were dialyzed overnight against 50 mM Tris–HCl, pH 7.6, containing 0.5 mM DTT, 0.1 mM PMSF, and 2 mM EDTA, pH 7.6, and finally for 3–4 hr against 50 mM Tris–HCl, pH 7.6, before the assay. After this procedure, NOS activity was measured at 37° in an incubation medium with the following composition: 50 mM Tris–HCl, pH 7.6, 0.1 mM DTT, 1 μM FAD, 0.03 mM L-arginine, 1 μM tetrahydrobiopterin, 1 mM $NADPH_2$, 3.2% glycerol, and 1 mM $CaCl_2$. NOS activity was only detected in the presence of 1 mM $CaCl_2$. After 20 min, the reaction was stopped by the addition of freshly prepared 2,3-diaminonaphthalene (0.05 mg/mL), and the spectrofluorometric determination of nitrite formation was performed as described by Misko *et al.* [13].

ELISA Measurements of NOS Isoenzymes

Experimental protocols were carried out as described elsewhere [14]. Aliquots of cytosol fractions from tracheal

smooth muscle were adsorbed onto ELISA plates (Immunolon II, Dynatech) with sodium carbonate (0.1 M, pH 9.3) at 4° for 12 hr. Control plates with peptides containing the amino-terminal (amino acids 3–22) sequence from NOS-2 and NOS-3 were run at the same time. After the adsorption step, plates were blocked with 0.1% fetal bovine serum in PBS (pH 7.2) buffer for 1 hr at 37°. Plates were washed five times with PBS-0.5% Tween-20 (Tween buffer). Later, plates were incubated with polyclonal antibodies against NOS-2 and NOS-3 (previously diluted 1:250 in PBS) during 1 hr at 37° under gentle agitation. At the end of this incubation, plates were rinsed four times with the Tween buffer and incubated with horseradish peroxidase coupled to anti-rabbit IgG (1:500 Sigma) for 1 hr at 37°. Finally, plates were rinsed thoroughly with Tween buffer. The colorimetric reaction was started upon the addition of 0.03% OPD and 1% hydrogen peroxide and incubated for 20 min until the color was apparent. The reaction was stopped with 50 μL of 1 M H_2SO_4. The plates were read at 492 nm using an ELISA scanner (Multiscan MCC 340).

Statistical analysis was performed with Student's *t*-test. The estimation of EC_{50} values was performed by computer-assisted nonlinear regression using the InPlot program (Graph Pad® software).

RESULTS

TSM was activated with cumulative concentrations of the muscarinic agonist carbachol, and contractile activity was measured during 3 min after each agonist addition. A concentration-dependent activation was found, as shown in Fig. 1. From these data, an EC_{50} of $1.0 \pm 0.2 \times 10^{-7}$ M was estimated. At carbachol concentrations higher than 1×10^{-5} M, the smooth muscle contraction reached a plateau. This concentration was used to study the muscarinic activation of TSM, employing Procedure 2. Thus, several samples were incubated simultaneously, and kinetic studies were undertaken to evaluate the muscarinic activation of cGMP nucleotide metabolism.

Muscarinic activation (1×10^{-5} M carbachol) of TSM induced two cGMP signal peaks. The first peak was at 20 sec and the second at 60 sec (Fig. 2). It is important to emphasize that both signals were dependent on the muscarinic agonist concentrations (Fig. 3). A 2:1 ratio was observed between peak 1 and peak 2 for all carbachol concentrations (1×10^{-11} to 1×10^{-4} M) assayed. From these data, estimated EC_{50} values were obtained, and similar values, close to 0.5×10^{-9} M carbachol, were estimated for both peaks.

To determine the origin of these two cGMP peaks, several experimental approaches were undertaken. First, ODQ, a specific inhibitor of the NO-stimulated soluble GC, was used. Tracheal smooth muscle strips were preincubated with 100 nM ODQ at 37°. After 30 min, carbachol (1×10^{-5} M) was added, and strips were removed at specific times as described in Materials and Methods. Later, all samples were processed to measure the cyclic nucleotide

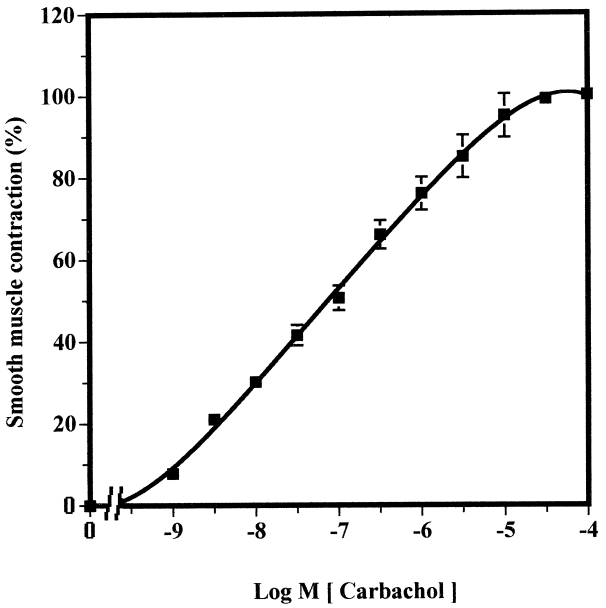

FIG. 1. Carbachol cumulative concentration response from bovine TSM. The contractile activity was measured using Procedure 1 as described in Materials and Methods. Carbachol cumulative concentrations from 1×10^{-9} to 1×10^{-4} M were employed. After each addition, a 3-min recording period was used. The maximal activity was considered as 100% (3.2 ± 0.2 g), and from this value the other responses were calculated. Each value is the mean ± SEM of five different tracheas assayed in duplicate.

levels. Figure 4 shows the effect of ODQ (100 nM) on the cGMP levels. It can be seen that the first cGMP signal peak was abolished by ODQ, while the second peak remained almost unchanged.

It has been reported that there is a close relationship between NO and cGMP. Thus, NAME, an inhibitor of NOS, was tested. TSM strips were preincubated with NAME (100 μM) for 30 min at 37°. Later, carbachol (1×10^{-5} M) was added, and cGMP levels were measured as a function of time. It is important to recognize that the control experiments are the same control data shown in Fig. 4. After NAME treatment, both cGMP signals remained the same, as shown in Fig. 5. However, an interesting time displacement was observed. Thus, the first peak (20 sec in control) shifted to 10 sec and the second peak (60 sec in control) was displaced to 50 sec. The first cGMP peak maintained the same size, but the 2:1 ratio disappeared, showing a similar magnitude for both peaks.

Another group of experiments were performed to confirm the existence of the NO-sensitive soluble GC. Thus, a soluble fraction and a crude particulate fraction from bovine TSM (150,000 *g* for 1 hr) were obtained. In addition, a highly purified plasma membrane fraction (P_2) also was prepared as described [12]. The effect of ODQ on the SNP-activated soluble GC from tracheal smooth muscle can be seen in Table 1. This SNP-NO activation was abolished completely by ODQ. The latter results demonstrate the existence of an ODQ-sensitive NO-activated soluble GC in this type of smooth muscle. Additionally,

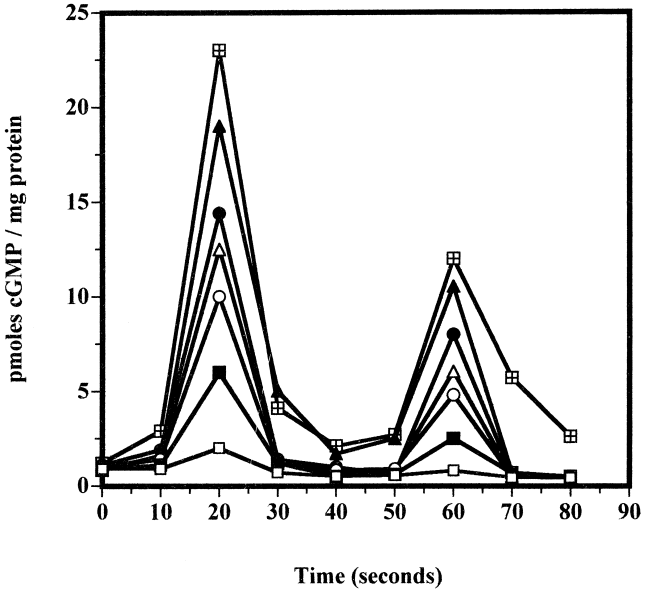

FIG. 2. Time course of muscarinic activation at different carbachol concentrations of the cGMP content of TSM. Experiments were performed using Procedure 2 as described in Materials and Methods. Tracheal smooth muscle strips were preincubated for 30 min at 37°. Carbachol concentrations from 1×10^{-11} (□), 1×10^{-10} (■), 1×10^{-9} (○), 1×10^{-8} (△), 1×10^{-7} (●), 1×10^{-6} (▲), and 1×10^{-5} M (⊞) were added to the multi-organ bath. Every 10 sec, strips were removed and frozen in liquid nitrogen, and cGMP nucleotide content was determined. In each time–course experiment, all smooth muscle strips came from the same trachea. Each value is the mean of two different tracheas, and the cGMP was determined in triplicate.

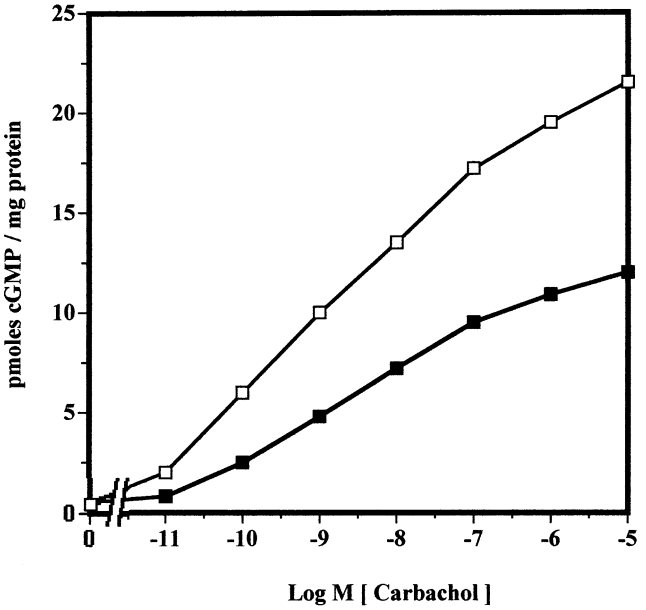

FIG. 3. Carbachol concentration-dependent increments in the two cGMP peaks. Experimental conditions are similar to those described in the legend of Fig. 2. The experimental values of the first (20 sec) (□) and the second (60 sec) (■) cGMP peaks were estimated from the data of Fig. 2 and plotted against the carbachol concentration employed for each experiment.

neither the crude particulate GC (sediment at 150,000 *g* for 1 hr) nor the plasma membrane-bound GC was affected by ODQ, as shown in Table 1.

In addition, it is important to point out that the NO biosynthetic machinery is currently present in this smooth muscle type. Consequently, in the soluble fraction from TSM, the levels of NOS were determined by ELISA [14] with specific antibodies against NOS-2 and NOS-3 isoenzymes. The amount of NOS was determined in the soluble fraction to be 2.5 μg of inducible NOS (NOS-2)/mg protein and 4.8 μg of constitutive NOS-3/mg protein. To strengthen these experimental findings, the effect of NAME on the NOS (Ca^{2+} dependent) activity was determined [13]. It is shown in Table 2 that the activity of NOS is 25.3 ± 3.9 nmol NO released/20 min/mg protein, showing a 21% inhibition by 10 μM NAME and an 82% inhibition at 100 μM NAME. An additional inhibitor, AMG, was employed to corroborate NOS inhibition. It also can be seen in Table 2 that AMG was a weak inhibitor of the NOS activity as predicted [13], reaching 48% inhibition at 100 μM.

DISCUSSION

In this study, we determined the initial time course of the cGMP intracellular levels after muscarinic activation of TSM. These studies were undertaken specifically to evaluate the actions of an inhibitor of soluble GC (ODQ) and also an inhibitor of NOS (NAME) on the cGMP levels. In these experiments, the smooth muscle strips were used within 3 hr after arrival of the tracheas. Preparations kept after 3 hr in chilled KRB solution saturated with 95% O_2 and 5% CO_2 showed significantly reduced ATP levels and

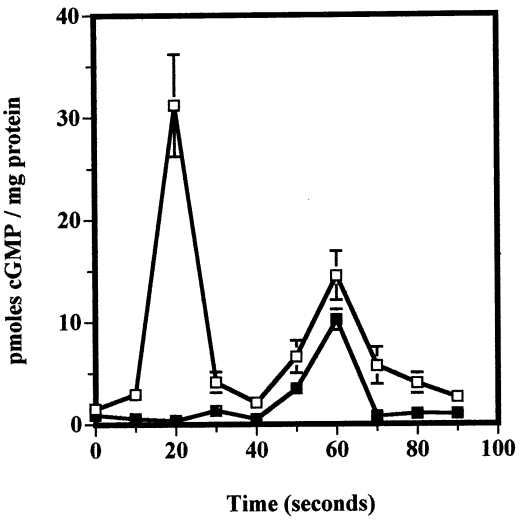

FIG. 4. Effect of ODQ on the time course of cGMP peaks induced by carbachol. TSM strips were preincubated for 30 min with ODQ (■) (100 nM) following Procedure 2. Control experiments (□) without drug were run simultaneously under the same conditions. Each value is the mean ± SEM of four different tracheas, and the cGMP determinations were carried out in triplicate.

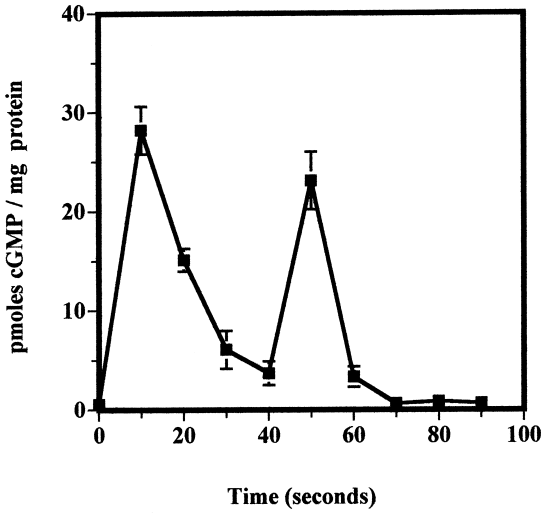

FIG. 5. Effect of NAME on the time course of cGMP signal peaks induced by carbachol. TSM strips were preincubated for 30 min with NAME (100 μM) following Procedure 2. Each value is the mean ± SEM of four different tracheas, and the cGMP determinations were carried out in duplicate.

glycolytic rate (measured as glucose consumption and lactate production at 37° for 30 min) [9]. However, after 3 hr, contractile activity remained, but these responses were delayed and the results became irreproducible.

The cGMP signal peaks must be produced by the most abundant (95–98%) airway smooth muscle cell type. One original finding in this smooth muscle was the presence of two cGMP signal peaks after carbachol activation in the absence of PDE inhibitors. The first peak at 20 sec was followed by a second at 60 sec. cGMP has been implicated in both the contraction [1] and the relaxation [3, 6, 7] of airway smooth muscle. Therefore, it is reasonable to postulate that these two opposite cellular responses using the same second messenger cyclic nucleotide must have different signaling cascades and regulation.

TABLE 1. Effect of ODQ on SNP (NO) stimulated GC activity in soluble (cytosol) fraction and on GC activity in particulate fraction from TSM

Conditions	GC activity (pmol cGMP/min/mg protein)
Soluble fraction*	
Basal	51.2 ± 3.7
+ SNP (NO)	239.4 ± 31.9
+ ODQ	50.7 ± 8.3
+ SNP (NO) + ODQ	70.2 ± 10.5
Particulate fraction†	
−ODQ	35.7 ± 1.9
+ODQ	35.3 ± 1.6
Plasma membranes (P_1 fraction)‡	
−ODQ	69.3 ± 2.8
+ODQ	67.9 ± 8.7

Guanyly cyclase activity was assayed as described in Materials and Methods in the presence of 0.2 mM GTP, 3 mM $MnCl_2$, and a GTP-regenerating system (creatine phosphokinase + creatine phosphate) for 5 min at 37°. For the soluble enzyme assays, 1 mM theophylline was included as described in Materials and Methods. cGMP was determined in the supernatant using radioimmunoassay. Each value is the mean ± SEM of three different preparations assayed in triplicate.

*Guanylyl cyclase was assayed in a cytosol fraction (soluble fraction at 150,000 *g* for 1 hr) prepared as described in Materials and Methods. The soluble guanylyl cyclase activity was assayed in the presence of 100 μM SNP and ODQ (100 nM). After the addition, a preincubation of 10 min was performance, and the enzyme reaction was started with the nucleotide addition.

†Crude particulate fraction was prepared by centrifugation of tracheal smooth muscle extracts at 150,000 *g* for 1 hr to obtain the sediment and the soluble fraction.

‡Plasma membrtanes (P_2 fraction) were prepared as described in Materials and Methods.

TABLE 2. Effects of NAME and AMG on the NOS activity in cytosol fractions from TSM

Conditions	NO synthase activity (nmol NO released/20 min/mg protein)	% of Inhibition
Basal	25.3 ± 3.9	0
NAME		
10 μM	20.1 ± 3.2	21
100 μM	4.5 ± 1.2	82
AMG		
10 μM	20.7 ± 3.5	19
100 μM	13.4 ± 3.3	48

Cytosol fractions from TSM were prepared as described in Materials and Methods. Aliquots were dialyzed (1:1000 with four changes to remove sucrose) against a buffer of 50 mM Tris-HCl, pH 7.6 containing 0.5 mM DTT, 0.5 mM PMSF, 2 mM EDTA. The last change was against 50 mM Tris-HCl, pH 7.6, buffer during 4 hr before the asssay. NOS activity was measured during 20 min at 37° in an incubation medium with the following composition: 50 mM Tris-HCl, pH 7.6, 0.1 mM DTT, 1 μM FAD, 0.03 mM L-arginine, 1 μM tetrahydrobiopterin,. 1 mM $NADPH_2$, 3.2% glycerol and 1 mM $CaCl_2$. After 20 min, the reaction was stopped by the addition of freshly prepared 2,3-diaminonaphthalene (0.05 mg/mL), and the spectrofluorometric determination of nitrite formation was performed as described elewhere [13]. Values are the means ± SEM of four different preparations assayed in triplicate. Inhibition was eastimated using basal activity as 100%.

The rise of the first peak (20 sec) appears to be similar to the cGMP peak induced by acetylcholine in perfused rat heart, as described elsewhere [15]. In TSM, the first peak was ODQ sensitive, whereas the second peak was insensitive to this specific soluble GC inhibitor [8]. Thus, the ability of ODQ to abolish the first peak of cGMP suggests that the mAChR activation at the plasma membrane triggers a cascade leading to the stimulation of a soluble GC. It is important to emphasize the existence of a NO-stimulated GC in cytosol fractions from this TSM. This soluble hemoprotein GC activity is stimulated through the heme moiety by NO-generating compounds such as SNP, and this activation was abolished completely by ODQ.

However, this soluble GC activation is not mediated via NO mechanisms, as suggested by the results obtained with NAME. This powerful NOS inhibitor [3] did not block these peaks in cGMP elicited by muscarinic agonists. Similar results with muscarinic agonists have been reported in cardiomyocytes [16]. In the latter study, carbachol increased cGMP levels by about 200%, but NOS inhibitors did not reduce this response. These authors concluded that the carbachol-mediated increase in cGMP content does not occur through a NO biosynthetic pathway. In addition, they provided evidence of an apparent absence of NOS in cardiomyocytes. In our system, the presence of NO biosynthetic pathways was detected. Thus, the existence of the

inducible NOS-2 and the constitutive NOS-3 enzymes was demonstrated using specific polyclonal antibodies. In addition, the Ca^{2+}-dependent isoform (the most abundant isoform in this smooth muscle type as described here) was very sensitive to NAME inhibition and less sensitive to AMG, as predicted [13]. Thus, the lack of effect of NAME on the cGMP peaks indicates the paucity of involvement of the NO-cGMP transducing cascade in the muscarinic activation described here.

The time dependence between the smooth muscle contraction and the appearance of these two cGMP signals is an important aspect of this smooth muscle type. It can be speculated that the first cGMP peak (20 sec after agonist addition) is associated with the onset of smooth muscle contraction and may be regulated by the concentration of intracellular calcium [17]. In other biological systems, the formation of cGMP can be accomplished through a Ca^{2+}-linked activation of soluble GC [18]. Thus, the involvement of an ODQ-sensitive soluble GC may be the most feasible mechanism to explain the origin of the first cGMP peak.

Even though the formation of NO is a widespread transduction mechanism for the production of cGMP by the stimulation of soluble GC in many cell types, there is an alternative pathway for elevating cGMP, which is related to the activation of mammalian membrane bound guanylyl cyclases (mmb-GCs) [19]. Taking into account the lack of effect of ODQ on the second cGMP peak and the mmb-GC here described, it seems reasonable to suggest that a mmb-GC is responsible for the generation of the second cGMP peak.

These mmb-GCs do not contain heme as a prosthetic group as does the soluble GC. Thus, in TSM, an atrial natriuretic peptide (ANP)-sensitive GC has been found in a particulate fraction, being involved in the relaxant effect of ANP on the TSM [6, 7]. Furthermore, a mmb-GC activated by muscarinic agonists has been described in a plasma membrane fraction isolated from bovine TSM [10, 20, 21].

It can be postulated that the muscarinic activation begins at the plasmalemma, after agonist binding to mAChRs, which have been described previously in a tracheal smooth muscle plasma membrane fraction [22]. Muscarinic receptor activation leads to the stimulation of several G-proteins associated with specific signal transducing cascades [23]. Even so, a more complex pathway may exist, which can generate G_o/G_i-protein-related messengers such as the α subunits and the $\beta\gamma$ dimers [24]. These G-protein subunits may stimulate the plasma membrane-bound G-protein-sensitive GC through protein–protein interactions. There is evidence to imply that this G-protein-coupled GC activity [20] is the same mAChR-regulated GC activity described in a plasma membrane fraction from TSM [10, 20, 21].

The second cGMP peak (60 sec) appears after the contraction has reached the plateau, and it may be required in the maintenance of the TSM contraction through unknown molecular mechanisms. Likely, this second cGMP signal may be involved in the onset of the relaxation process of the TSM [2–4, 6, 7], which is difficult to rule out at this time.

The apparent time link and the 2:1 ratio between these two cGMP signal peaks deserve some consideration. Consequently, in the presence of NAME, there was an early time shift displacement of these peaks, and the ratio was also changed. Additionally, the ODQ suppressed the first cGMP peak and did not affect the second one. The last statement suggests that the second cGMP signal is not a direct consequence of the first cGMP peak. All these lines of evidence suggest the existence of two distinct but interdependent signal-transducing cascades leading to the production of cGMP.

In summary, muscarinic agonists bind to the mAChR at the plasma membrane in TSM. After agonist binding, several signaling cascades involving G-proteins are initiated. These pathways may start intracellular second messenger cascades that are responsible for these two cGMP peaks. One pathway leads to the stimulation of an ODQ-sensitive GC, presumably a soluble GC, whereas another different pathway seems to activate an ODQ-insensitive GC, most likely an uncharacterized soluble GC or a membrane-bound GC. The cross-talk interactions between these two cGMP signaling cascades are complex, and the molecular events regulating their activities in the muscarinic activation of TSM remain obscure.

This work was supported by Consejo de Desarrollo Científico y Humanístico-Universidad Central de Venezuela (CDCH-UCV) by Grants CDCH-09–33-3683–96 (L.G.G.) and CONICIT S1–2749 (I.L.B.). This work is part of the Ph.D. thesis of Lérida Guerra de González, as a partial fulfillment of Curso de Postgrado en Ciencias Fisiol-gicas. Facultad de Medicina, Universidad Central de Venezuela. The authors thank Dr. J. De Sanctis (I.I.C.-UCV) for the ELISA determination of NOS activity and NO-related products. We also thank Dr. Neil Lynch for correcting the English version of the manuscript.

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