Muscarinic drugs regulate the PKG-II-dependent phosphorylation of M₃ muscarinic acetylcholine receptors at plasma membranes from airway smooth muscle

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Abstract
Muscarinic agonists induce the activation of the airway smooth muscle (ASM) leading to smooth muscle contraction, important in asthma. This activation is mediated through M₂/M₃ muscarinic acetylcholine receptors (mAChRs). Muscarinic receptor activity, expressed as [³H]QNB binding at plasma membranes from bovine tracheal smooth muscle (BTSM), increased with cGMP and was augmented significantly by cGMP plus ATP but diminished with the PKG-II inhibitor, Sp-8-pCPT-cGMPS. The [³H]-QNB binding was accelerated by okadaic acid, (OKA), a protein phosphatase (PPase) inhibitor. These two results indicated the involvement of a membrane-bound PPase. Moreover, a cGMP-dependent [³²P]-ATP phosphorylation of plasma membranes from BTSM was stimulated at low concentrations of muscarinic agonist carbamylcholine (CC). However, higher amounts of CC produced a significant decrement of [³²P]-labeling. A selective M₃mAChR antagonist, 4-DAMP produced a dramatic inhibition of the basal and CC-dependent [³²P]-labeling. The [³²P]-labeled membrane sediments were detergent solubilized and immunoprecipitated with specific M₂/M₃mAChR antibodies. The M₂mAChR immuno-precipitates exhibited the highest cGMP-dependent [³²P]-labeling, indicating it is a PKG-II substrate. Experiments using synthetic peptides from the C-terminal of the third intracellular loop (i3) of both M₂mAChR (356–369) and M₃mAChR (480–493) as external PKG-II substrates resulted in the i3M₃-peptide being heavily phosphorylated. These results indicated that PKG-II phosphorylated the M₃mAChR at the i3M₃ domain (480MSLIKEKK⁴⁸⁵), suggesting that Ser⁴⁸¹ may be the target. Finally, this phosphorylation site seems to be regulated by a membrane-bound PPase linked to muscarinic receptor. These findings are important to understand the role of M₃mAChR in the pathophysiology of ASM involved in asthma and COPD.

Keywords
cGMP, carbamylcholine, muscarinic receptors, tracheal smooth muscle

History
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Introduction
Muscarinic agonists induce the activation of the airway smooth muscle (ASM) leading to smooth muscle contraction, related to asthma. This activation is mediated through the M₂ and M₃ muscarinic acetylcholine receptors subtypes (mAChRs) (1–3). Muscarinic agonists increase intracellular cGMP levels and contractility in ASM from guinea pigs (4) and bovine tracheal smooth muscle (BTSM) (5). Likewise, muscarinic agonists acting on both M₂/M₃mAChRs in BTSM isolated strips induce the generation of two cGMP signal peaks at 20-s and 60-s (6). The first signal (20-s) produced by the activation of M₂mAChR coupled to a Gi/o protein induces the transient translocation of NO-sensitive soluble guanylyl cyclase (NO-sGC) from cytoplasm to plasma membranes (7,8). The second signal (60-s), produced by the stimulation of the M₃mAChR coupled to a Gq₁₆ protein leads to the activation of a membrane-bound natriuretic peptide receptor-guanylyl cyclase B (NPR-GC-B) (9–12). We have previously shown that [³H]QNB binding studies on a plasma membranes fraction from BTSM demonstrate a high activity of M₂/ M₃mAChR subtypes (13). Functional studies have shown that the M₂/M₃ mAChR subtypes are present in these BTSM plasma membranes (7,8,11,12). Recently, we showed that cGMP affects the M₃mAChR functionality expressed as [³H]QNB binding activity (14).

Using a plasma membranes fractions from BTSM, we demonstrate that cGMP, via PKG-II, phosphorylated the M₃mAChR affected its functionality, expressed by an increment in the Bₘₐₓ for [³H]QNB binding activity and displaying a positive co-operativity. Moreover, okadaic acid
(OKA), (a protein phosphatase (PPase) inhibitor) induced a faster [3H]QNB binding, suggesting the involvement of a membrane-bound PPase. Furthermore, a cGMP-dependent [32P]-phosphorylation of membrane protein was specific for the M₃mAChR. This [32P]-membrane labeling was affected by muscarinic agonists such as carbamylcholine (CC) displaying agonist-dependent phospho/dephosphorylation reactions. Conversely, 4-DAMP, a selective M₃mAChR antagonist, inhibited both the basal and cGMP-dependent membrane protein [32P]-phosphorylations, supporting the involvement of an unknown PPase.

In this work, a putative cGMP regulatory mechanism on the mAChR activity at plasma membranes from ASM was studied.

Methods

The following compounds were purchased from Sigma Chemical Co. (St. Louis, MO): Trizma base, sucrose, DTT, PMSF, MgCl₂, ATP, cGMP, Protein A/G-agarose beads and CC. BSA standard was purchased from Pierce Thermo Scientific (Rockford, IL). 4-DAMP mustard, 4-DAMP, Rp-8-pCPT-cGMPS and a Protein Phosphatase Inhibitor kit (containing cypermethrin, dephostatin, OKA and NIPP-1 Bovine Thymus recombinant), were obtained from Calbiochem (San Diego, CA). Rabbit anti-M₂ and anti-mAChR antibodies were acquired from Santa Cruz Biotechnology, Inc. (Dallas, TX).

Synthetic tetradecapeptides derived from the C-terminal of third intracellular loop (i3) of mAChR subtypes M₂ (amino acid sequence: 356KQNIVARKIVKMTK369) and M₃ (380MSLIKEKAAQTLS493) were prepared in the Synthetic Peptide Unit of Tropical Medicine Institute, of Universidad Central de Venezuela. Both peptides were purified by high-performance liquid chromatography (HPLC) and the amino acid sequence was checked by mass spectrometry.

Plasma membrane preparation

The plasma membrane fraction (P₁) was prepared from BTSM as previously described (15). Aliquots (2–3 mg membrane protein/ml) were suspended in a Buffer containing 0.3 M sucrose, 0.5 mM DTT, 20 mM Tris-HCl (pH 7.2), frozen in liquid N₂ and stored at −80°C until use.

Measurement of muscarinic acetylcholine receptor activity

The mAChR activity was evaluated using the [3H]QNB binding studies, which were performed as described previously (13). Briefly, PM (P₁) fraction was diluted with 80 volumes of hypotonic buffer containing 20 mM Tris-HCl (pH 7.2), 0.5 mM DTT and centrifuged at 150 000 × g for 30 min. Washed and suspended in small volume of incubation buffer (50 mM Tris-HCl, pH 7.8), prior to use. The [3H]QNB binding assay was started by adding membrane protein (2–5 μg) in incubation buffer of 50 mM Tris-HCl (pH 7.8), and L-[3H]QNB (1250 nM) to a final volume of 120 μl. Different compounds to be tested were added to the incubation media. After 1 h at 37°C, the incubation mixture was placed onto a pre-centrifuged Sephadex G-50 column (3 ml) equilibrated with 0.3 M sucrose-50 mM Tris-HCl (pH 7.6) and immediately centrifuged at 700 × g for 1.5 min to remove free [3H]QNB. The column effluent containing 97–98% of the bound [3H]QNB was transferred to vials containing the liquid scintillation cocktail. Radioactivity was measured in a RackBeta liquid scintillation counter LKB, Wallac 1214/1219 and all samples counted with approximately the same efficiency (30%). Specific binding was calculated by subtracting non-specific binding (less than 1% of total binding, measured with 1 μM atropine), from the total binding (16). In all binding experiments, no more than 5% of the fixed radioligand concentration was allowed to bind to the membranes to avoid ligand depletion. Similar amounts of active receptors were employed in these binding assays. The values of Bₘₐₓ, Kᵦ and Hill coefficient nᵢ were calculated as described (17).

Protein kinase G and [32P]-incorporation into membrane protein and synthetic peptides assays

Protein kinase G (PKG) activity and [32P]-incorporation into membranes proteins were measured in plasma membrane fractions using endogenous substrates or synthetic peptides as described (18) and modified (19). Briefly, the incubation medium (50 μl), contained 5 mM MgCl₂, 20 mM KPi (pH 7.0), 100 μM of the cocktail of phosphatase inhibitors (OKA, NIPP-1) and 0.1−1 mM [32P]γATP (3 μCi/assay). In the assays using synthetic peptides from i₃M₂/M₃AChRs, which were based on mAChRs M₂[Bo Taurus] NCBRI reference sequence NP_001074202.1 and mAChRs M₃[Bos Taurus] NCBRI reference sequence NP_001074202.1 and mACHRs M₃[Bo Taurus] NCBRI reference sequence NP_776695.1. The peptides were synthesized and purified by HPLC and later used as exogenous substrates to [32P]-ATP-dependent membrane phosphorylation. To calculate these values, basal 32P-endogenous labeling was subtracted.

The phosphotransfer reaction was allowed to proceed for 10−15 min at 37°C, at which time it was terminated by spotting aliquots onto P81 phosphocellulose papers (GE Healthcare Bio-Sciences, Pittsburgh, PA), and immediately dropped into ice-cold 5% TCA (100 ml). Under gentle agitation at 4°C, discs were washed 4 times (100 ml ice-cold 5% TCA). The paper discs were washed with 100% cold ethanol and some 50 ml of cold ether was used to remove possible [32P]-labeled lipids. Discs were allowed to dry under air stream and 32P was counted using Cerenkov radiation (20) in a liquid scintillation counter.

mAChR [32P]phosphorylation and selective immunoprecipitation assays

BTSM plasma membranes (P₁ fraction) was thawed and diluted 80 times with 20 mM Tris-HCl pH 7.2 buffer containing 5 mM DTT and 1 mM PMSF. Washed plasma membranes (0.5–1.0 mg/ml of protein) was subjected to [32P] phosphorylation reactions at 37°C with 0.1 mM [γ-32P]ATP (Specific activity: 3000 μCi/mumole), 1 mM MgCl₂, 20 mM KPi (pH 7.2), 1 mM DTT, 10 μM IBMX, 100 μM protein phosphatase inhibitor kit containing (cypermethrin, dephostatin, OKA), (a protein phosphatase (PPase) inhibitor) induced a faster [3H]QNB binding, suggesting the involvement of a membrane-bound PPase. Furthermore, a cGMP-dependent [32P]-phosphorylation of membrane protein was specific for the M₃mAChR. This [32P]-membrane labeling was affected by muscarinic agonists such as carbamylcholine (CC) displaying agonist-dependent phospho/dephosphorylation reactions. Conversely, 4-DAMP, a selective M₃mAChR antagonist, inhibited both the basal and cGMP-dependent membrane protein [32P]-phosphorylations, supporting the involvement of an unknown PPase.

In this work, a putative cGMP regulatory mechanism on the mAChR activity at plasma membranes from ASM was studied.
The effect of cGMP on [3H]QNB antagonist binding

Results

and other compounds as indicated. [32P]-labeled plasma membranes were washed twice with a solution containing, 20 mM EDTA, 1 mM PMSF, 20 mM KPi (pH 7.2) and 100 μM protein phosphatase inhibitor kit (Buffer I). [32P]-labeled membranes (5 mg/ml) were solubilized by incubation at 4°C for 15 min in a mixture containing 5 mM MgCl2, 1 mM DTT, 1 mM EDTA, 0.1 mM PMSF, 20 mM KPi, pH 7.2 (Buffer II). One ninth volume of a detergent mixture containing 0.1% Digitonin-0.02% sodium cholate was added to selected solubilized mAChRs (21). Detergent-solubilized proteins were recovered after centrifugation at 150,000 g for 30 min. The sediment was again re-extracted using the same procedure and both detergent-solubilized supernatants were pooled. The digitonin/cholate-solubilized material was incubated with protein A/G-agarose beads for 1 h at 4°C. The pre-clarified supernatant was incubated overnight with specific anti-M3 or anti-M2mAChR antibodies at 4°C. Immunoprecipitates were collected upon the addition of protein A/G-agarose beads for 6 h at 4°C. The beads were then collected by low speed centrifugation and washed three times using detergent-free buffer II. An aliquot of these [32P]-immunoprecipitates was used to measure [32P] labeling in a RackBeta liquid scintillation counter LKB, Wallac 1214/1219.

Protein measurement

The amount of protein was quantified using bovine serum albumin (BSA) as standard (22).

Data analysis

A computer-assisted non-linear regression program (InPlot, Graph Pad software, La Jolla, CA) was used to analyze binding and competition experiments results (13).

Results

The effect of cGMP on [3H]QNB antagonist binding at plasma membranes from BTSM

The effect of increasing concentrations of cGMP on the [3H]QNB binding activity as an expression of mAChRs functionality was evaluated in the presence of 5 mM ATP and 10 μM IBMX, a non-selective phosphodiesterase (PDE) inhibitor (Figure 1). The binding of [3H]QNB to plasma membranes from BTSM reached a maximum around 50 nM cGMP. It remained constant at higher cyclic nucleotide concentrations, giving an ED50 for cGMP of 0.5 × 10⁻⁹ M. IBMX was included in all assays, otherwise bimodal behavior was observed (data not shown) due to the existence of PDE activity in this plasma membrane fraction. To establish the nature of this cGMP-dependent increase in [3H]QNB binding, saturation experiments were performed to estimate the kinetic parameters of this process, such as the maximal binding activity (Bmax), the dissociation constant (KD) and the Hill coefficient (nH) associated with the cGMP effect (Table 1). The KD values for ATP plus cGMP remained unchanged but the Bmax and nH parameters were markedly affected. Thus, Bmax values (pmoles [3H]QNB/mg of protein) increased significantly from 1.20 ± 0.12, in the presence of cGMP or, 1.34 ± 0.15 in the presence of ATP to 1.98 ± 0.19 for the condition containing ATP plus cGMP. This increment represents more than 65% in comparison to cGMP alone and higher than 40% to only ATP. In addition, nH values shifted significantly from 1.2 ± 0.1 in the assays with either cGMP or ATP to 1.9 ± 0.3 for the condition having ATP plus cGMP. These kinetic parameters indicated that cGMP in the presence of ATP is affecting the mAChR functionality.

Effects of the inhibitor (Rp-8-pCPT-cGMPS) of PKG-II on the [3H]QNB binding activity

These changes induced by ATP plus cGMP in the [3H]QNB binding kinetic parameters may be through the activation of a PKG-II activity, associated with these plasma membranes. In this sense, the effects of a specific inhibitor (Rp-8-pCPT-cGMPS) of PKG on the [3H]QNB binding activity was evaluated. In these experiments, the [3H]QNB binding, induced by cGMP (50 nM), was completely abolished with a dose-dependent titration of this PKG-II inhibitor (Figure 2). These results indicated that the cGMP activator effect on the [3H]QNB binding is mediated via a PKG-II isoenzyme anchored to the plasma membrane fraction from BTSM.

Effect of OKA on [3H]QNB binding

If a phosphorylation of these mAChRs is occurring, a dephosphorylation process must also exist. Thus, protein phosphatase inhibitors such as OKA may affect the [3H]QNB binding. The inclusion of OKA in the [3H]QNB binding assays increased the velocity of [3H]QNB binding (Figure 3).

Figure 1. The effect of cGMP on the [3H]QNB binding in the plasma membranes of BTSM. Experiments were performed at 37°C in the presence of 1,250 nM [3H]QNB, 2–3 μg of membrane proteins, 5 mM ATP and increasing the concentration of cGMP as described in Methods section. Each point represents the mean ± S.E. of four different membranes preparations assayed in triplicate.
The time taken to reach maximal [3H]QNB binding in the presence of 50 nM cGMP or 1 μM OKA, was approximately 60 min compared to 20 min in the presence of [5 mM ATP plus 50 nM cGMP]. However, in the presence of [5 mM ATP plus 50 nM cGMP and 1 μM OKA], the time to reach maximal [3H]QNB binding was reduced to 10 min. To evaluate more accurately the increase in velocity of [3H]QNB binding, the same data were plotted over a shorter period of 5 min (inset Figure 3). For all experimental conditions tested, the [3H]QNB binding velocity was linear up to 5 min. It can be seen that the [3H]QNB binding in relation to cGMP alone was 1.75 faster for OKA, and 3 times faster for [ATP plus cGMP] and significantly 4.5 times faster in the presence of [ATP plus cGMP and OKA]. These data indicate that a phospho-protein phosphatase is implicated in the regulation of the cGMP-dependent [3H]QNB binding.

Tracheal smooth muscle plasma membranes were prepared as described in Methods section. The [3H]QNB binding saturation experiments were carried out at 37°C in the presence of 10 μM IBMX, 5 mM MgCl2 as described in Methods section. Binding assays were performed with 50 nM cGMP, 5.0 mM ATP and 50 nM cGMP plus 5.0 mM ATP. Results are the mean ± SE of four different membrane preparations assayed in triplicate. The values of Bmax as pmoles [3H]QNB/mg of protein, Kd and nH were calculated as described (17). (*)The difference in the Bmax and nH for the assays with both ATP and cGMP was statistically significant at p < 0.01.

Table 1. The [3H]QNB binding parameters in the presence of ATP and cGMP in plasma membranes from tracheal smooth muscle.

| Additions        | Bmax (pmol/mg protein) | Kd (pM) | nH |
|------------------|------------------------|---------|----|
| cGMP             | 1002 ± 0.12            | 516 ± 79 | 1.1 ± 0.2 |
| ATP              | 1.34 ± 0.15            | 539 ± 47 | 1.2 ± 0.1 |
| [ATP plus cGMP]  | 1.98 ± 0.19 (*)        | 528 ± 57 | 1.9 ± 0.3 (*)|

![Figure 3. Time course of the effect of okadaic acid (OKA) on the [3H]QNB binding activity to the plasma membranes from BTSM.](image)

The time taken to reach maximal [3H]QNB binding in the presence of 50 nM cGMP or 1 μM OKA, was approximately 60 min compared to 20 min in the presence of [5 mM ATP plus 50 nM cGMP]. However, in the presence of [5 mM ATP plus 50 nM cGMP and 1 μM OKA], the time to reach maximal [3H]QNB binding was reduced to 10 min. To evaluate more accurately the increase in velocity of [3H]QNB binding, the same data were plotted over a shorter period of 5 min (inset Figure 3). For all experimental conditions tested, the [3H]QNB binding velocity was linear up to 5 min. It can be seen that the [3H]QNB binding in relation to cGMP alone was 1.75 faster for OKA, and 3 times faster for [ATP plus cGMP] and significantly 4.5 times faster in the presence of [ATP plus cGMP and OKA]. These data indicate that a phospho-protein phosphatase is implicated in the regulation of the cGMP-dependent [3H]QNB binding.

Effect of cGMP on endogenous 32P phosphorylation of plasma membranes proteins

The ATP requirement for this cGMP effect implies that a phospho-transfer reaction is taking place (Figure 4). This was measured as a 32P incorporation from [γ-32P]ATP into plasma membrane proteins. A cGMP titration on the 32P-endogenous membrane protein phosphorylation gave maximal 32P incorporation at approximately 5 x 10^-7 M of cGMP with a ED50 of 1 x 10^-9 M for cGMP. The observations indicated that a PKG-II might be involved. To further examine the character of the PKG-II associated with the BTSM plasma membranes, the protein kinase activity was measured as the [32P]-phosphorylation of membrane protein components. Consequently, the [32P]-labeling was increased significantly from 3.25 ± 0.23 pmoles/10 min for the basal condition to 4.95 ± 0.27 pmoles/10 min in the presence of cGMP (Table 2). The detergent-solubilization procedure extracted more than 66% of the total [32P]-labeled material. The difference was statistically significant between the two conditions, which was almost twice in the detergent-solubilized membrane proteins.

To identify specific [32P]-labeled polypeptides, a more detailed [32P]-labeling study was performed. The [32P]-labeling difference between the muscarinic receptors was more significant in the immunoprecipitates using specific anti-M2 and -anti-M3mAChR antibodies. Both receptor subtypes were labeled with [γ-32P]ATP (Table 2). The M2mAChR was preferentially and significantly phosphorylated in the presence of 50 nM cGMP, being more than 80% of the 32P solubilized material immunoprecipitated by the M2mAChR antibodies, in comparison only 20% of the phosphorylated M2mAChR was pull down by the M3mAChRs antibodies. These data indicate...
that cGMP, via a PKG-II, phosphorylates the M₃mAChR subtype. In these experimental conditions, the [³²P]-labeled M₃mAChR represents more than the 55% of the total [³²P]-labeled plasma membrane proteins from BTSM.

Another biochemical approach was undertaken to identify the M₃mAChR motifs and the putative amino acids involved in this PKG-II phosphorylation. Thus, an experimental approach using synthetic peptides based on amino acids sequences for PKG-II consensus phosphorylation. These motifs were located close to the C-terminal from the third intracellular loop (i₃) of the M₂ and M₃mAChR subtypes (Figure 5). These peptides have the following amino acid sequences: M₃ (480MSLIKEKKAAQTL493) and a close related in the M₂ (356KQNIVARKIVMTK369) as described in Methods section. After 15 min of incubation, the [³²P]-labeled membranes were applied to P81 discs (anion-exchange paper) and processed as described in Methods section. The disks were counted for [³²P] using Cerenkov radiation. The [³²P] incorporation of synthetic tetradeca-peptides from i₃ loop of M₂ and M₃ mAChR b endogenous plasma membranes protein kinases. The experiments were performed at 37°C in the presence of 20–30μg of membrane proteins, incubated in a medium containing protein phosphatase inhibitors, 5 mM MgCl₂, 10 μM IBMX, 50 nM cGMP, 0.1 mM [³²P]ATP and increasing concentrations of peptides: M₂ (356KQNIVARKIVMTK369) and M₃ (480MSLIKEKKAAQTL493) described in Methods section. After 15 min of incubation, the ³²P-labeled membranes were applied to P81 discs (anion-exchange paper) and processed as described in Methods section. The disks were counted for [³²P] using Cerenkov radiation. The [³²P] basal incorporation into the plasma membranes was subtracted to estimate the phosphorylation of peptides. Each point represents the mean ± S.E. of four different membranes preparations assayed in triplicate.

Table 2. Effect of cGMP on the endogenous protein and mAChRs phosphorylation in BTSM plasma membranes.

| Total [³²P] incorporated (pmol/10 min) | A         | B       |
|--------------------------------------|-----------|---------|
| Total endogenous PM [³²P]-labeling    | 3.25 ± 0.23 | 4.95 ± 0.27* |
| Digitonin-cholate soluble extract     | 1.66 ± 0.22 | 3.29 ± 0.24* |
| Immunoprecipitates (Protein G Plus-agarose) | 1.43 ± 0.15 | 2.74 ± 0.18* |
| anti-m2AChR                          | 0.89 ± 0.24 | 1.02 ± 0.21 |
| anti-m3AChR                          |           |         |

BTSM plasma membranes (PM) (P₁-fraction, 1 mg protein/assay) were incubated in a medium containing protein phosphatase inhibitors and 5 mM MgCl₂, 10 μM IBMX, 0.1 mM [³²P-γ]ATP (A) plus 50 nM cGMP (B) as described in Methods section. After 10 min of incubation at 37°C, [³²P]-labeled PM was collected by centrifugation and washed twice to remove free [³²P], or [³²P-γ]ATP to obtain the total [³²P]-labeling endogenous PM. These [³²P]-labeled membranes were solubilized with Digitonin-cholate mixture and processed as described in Methods section. This [³²P] detergent extract was separated into two equal portions and each one was immunoprecipitated with specific anti-m3 or anti-m2AChR antibodies and later incubated with Protein G Plus-agarose as described in Methods section. Data represent the mean ± S.E. of four different plasma membranes preparations assayed in duplicate. Statistical differences between A versus B condition: (*) p<0.01.

Figure 4. The effect of cGMP on the [³²P] labeling into plasma membranes proteins from BTSM. The experiments were performed at 37°C in the presence of 20–30 μg of membrane proteins, incubated in a medium containing protein phosphatase inhibitors, 5 mM MgCl₂, 10 μM IBMX, 0.1 μM [³²P]-ATP and increasing cGMP concentrations as described in Methods section. After 10 min of incubation, the [³²P]-labeled membranes were applied to paper discs and processed as described in Methods section. The disks were counted for [³²P] using Cerenkov radiation.

Figure 5. The [³²P] labeling of synthetic tetradeca-peptides from i₃ loop of M₂ and M₃ mAChR b endogenous plasma membranes protein kinases. The experiments were performed at 37°C in the presence of 20–30 μg of membrane proteins, incubated in a medium containing protein phosphatase inhibitors, 5 mM MgCl₂, 10 μM IBMX, 50 nM cGMP, 0.1 mM [³²P]ATP and increasing concentrations of peptides: M₂ (356KQNIVARKIVMTK369) and M₃ (480MSLIKEKKAAQTL493) described in Methods section. After 15 min of incubation, the [³²P]-labeled membranes were applied to P81 discs (anion-exchange paper) and processed as described in Methods section. The disks were counted for [³²P] using Cerenkov radiation. The [³²P] basal incorporation into the plasma membranes was subtracted to estimate the phosphorylation of peptides. Each point represents the mean ± S.E. of four different membranes preparations assayed in triplicate.

Modulation by muscarinic agonists and antagonists on cGMP-dependent ³²P-ATP phosphorylation

It was important to establish, whether or not, the cGMP effect on ³²P phosphorylations was affected by muscarinic
The muscarinic activation of ASM contraction (4–6) involves both muscarinic receptors, M2/M3 mAChRs, coupled to NPR-GC-B, contributing to the generation of the so-called "membrane-associated cGMP pool" (7,8,11,12). This "membrane-associated cGMP pool" has a different regulation to the "soluble cGMP pool" produced by the NO releasing agents acting on a NO-sGC implicated in the ASM relaxation (23). The role of this "membrane-associated cGMP pool", as a regulatory feedback mechanism, on the mAChRs, anchored to the plasma membranes, was evaluated in this work.

Classically, the effect of cGMP has been studied in intact tissue/cells using cell-permeable cGMP analogs, for example, 8-Bromo-cGMP or the participation of PKG activator, Sp-8-pCPT-cGMPS (24) or PKG inhibitor, Rp-8-pCPT-cGMPS (25). Thus, in intact cells using these tools, it is difficult to discriminate between the cyclic GMP protein kinases (cGKs) substrates under the control of either PKG-I isoforms or membrane-bound PKG-II enzymes (26,27). To overcome this complex experimental task, we used a broken cell system such as a plasma membranes fraction from BTSM, exhibiting the following advantages: 1. It contains a high M3/M2AChR biological activity as described (11–13). 2. It contains an
active PKG-II isoform, previously identified (14). 3. These membrane are depleted of PKG-I isoenzymes and other soluble cGKs substrates and other PKs.

Previously, we have shown that cGMP may regulate mACHR binding via PKG-II activation (14). In the present work, more compelling evidences are given to demonstrate that indeed PKG-II, at plasma membranes from BTSM, phosphorylates the M3mAChR, in a G-protein independent manner, and regulates its receptor activity. Moreover, this phosphorylation was affected by OKA. Interestingly, muscarinic agonists and antagonists regulated this cGMP-dependent phosphorylation, suggesting a relevant role on the M3mAChR.

Cyclic GMP via PKG-II phosphorylation induced the following biological actions on the mACHR binding activity here described: 1. The increase of Bmax for [3H]QNB binding activity (>60%) indicating that ‘new’ [3H]-QNB binding sites’ are displayed. Nonetheless, this cGMP effect on Bmax, was obliterated by the 4-DAMP alkylation of M3mAChR subtype as previously reported (14). 2. A doubling of the Hill coefficients (nH), which increased from 1.0 to almost 2.0, suggesting a positive co-operativity or homodimer formation of M3mAChRs as previously postulated (14). 3. The PPase inhibitor OKA induced a faster [3H]-QNB binding at these M3mAChRs indicating a dephosphorylation-linked process is present in these plasma membrane fractions.

These ‘new M3mAChRs’ that exhibited similar affinity constants (Kd), can be excluded as newly synthesized or exposed ‘recycled or hidden’ M3mAChRs from endosomes vesicles since our assays were performed with isolated plasma membrane fragments. The appearance of these ‘new M3mAChR’ may be related to some complex molecular mechanism, possibly via a two-step isomerization, of the mAChRs induced by antagonist binding (28,29). Molecular biology studies using point mutations and irreversible affinity labeling of the M3mAChR led to the proposal of a tandem two-site model (30). The possibility that the receptor binds two ligand molecules is relevant to the pharmacology and new therapeutic approaches. It is possible that the PKG-II phosphorylation of M3mAChR induced a similar molecular mechanism of homodimer/oligomer formation as previously proposed (14). This rationale is supported by the fact that, the M3mAChR displays a greater propensity to form a homodimer/oligomer structure at higher density receptor population (31,32). Recent studies demonstrated the formation of M3mAChR dimers in vivo (33,34), which may reflect the situation in these plasma membranes from BTSM, which have higher mAChRs amounts (13,15).

The M3mAChRs belong to the class A GPCR regulated by three principal mechanisms: Desensitization, internalization, and down-regulation. Internalization and down-regulation are ruled out in our experiments using isolated plasma membranes fractions. Thus, the receptor desensitization is the unique mechanism to explain these effects induced by cGMP via a PKG-II on mAChR functionality.

In relation to the muscarinic receptor desensitization, it was previously reported that cGMP plus ATP affected the agonist-antagonist muscarinic binding activities (14). Thus, the binding sites for the high affinity-agonist, CC, disappeared as an expression of receptor desensitization. In contrast, a non-selective antagonist atropine and the M2-selective antagonists such as methoctramine and gallamine, revealed only one low affinity binding site, which was not affected by cGMP plus ATP. Moreover, the 4-DAMP-mustard alkylation of the mAChRs blocked the cGMP effect indicating that the M3mAChR is the main receptor target of cGMP (14).

The involvement of a PKG-II on the desensitization of the muscarinic receptors was further established by the ability of cGMP (activator of PKG) in a dose-dependent manner (35) to increase the [3H]QNB binding and 32P-labeling in the plasma membranes from BTSM. Interestingly, the ED50 results for cGMP (1 × 10−9 M) was similar for both the rise of the [3H]QNB binding and the 32P-labeling in these plasma membranes. Moreover, the effect of the cGMP analogs such as Rp-8-pCPT-cGMPS, a PKG-specific inhibitor (24) suppressed the increase induced by cGMP on the [3H]-QNB binding activity. Similar behavior towards the inhibitor Rp-8-pCPT-cGMPS has been reported for the native (25) and recombinant PKG-II (26). Furthermore, PKG-II has previously been established as a membrane-bound enzyme in plasma membranes from BTSM (14) as well as in other biological systems (27).

The M3mAChR phosphorylation, via PKG-II is a G-protein independent phosphorylation. Other G-protein independent phosphorylations have been involved in the regulation of the muscarinic-antagonist binding to rat cerebral synaptic membranes (36). In the case of the phosphorylation by PKA and PKC of the agonist-unbound M3mAChRs, induces receptor uncoupling from G-proteins (37), which has been reported in SHSY5Y cell line (38).

Our previous observations using 32P-autoradiographs (14) and the specific immunoprecipitation assays support the argument that M3mAChR is specifically phosphorylated by PKG-II. Several agonist-dependent phosphorylations of M3 mAChR have been reported (39). An M3mAChR hyper-phosphorylation occurs following agonist occupation, linked to desensitization of muscarinic receptors. This usually occurs at serine (Ser) and threonine (Thr) residues contained on the i3-loop and C-terminal tail, which has been described for these GPCRs (40–42). Most of these phosphorylations by the kinases GRK, PKA, PKC, and CK1 occur at phosphorylation consensus sites (40,43) located in the i3-loop and C-terminal tail domains of the mAChRs (40,44).

The classic consensus phosphorylation sequences for PKG is [(R/K)2-3,-X-S*/T*], which describes 75% of the sites surveyed (45).

Nevertheless, there are only a few well-characterized proteins preferentially phosphorylated by PKG-II. One is the inositol 1,4,5-trisphosphate receptor (IP3R), which generates the IP3Rtide (GRRES) and the cAMP response element binding protein (CREB) which contains a CREBtide (KRREILS) (26). In both specific peptides, Ser (S) residues are phosphorylated and the cluster of two or more positive cluster charges such as Arg (R) or Lys (K) seems to be the consensus sequences for PKG-II. Taking into account, these observations, a possible PKG-II phosphorylation site may be located in the i3-loop of the M3mAChRs extending from Thr450 to Q490. This contains the peptide M3AChR (480MSLIKEKKAATLS493), which...
was heavily phosphorylated by PKG-II located in the sarclemma from BTSM. The known consensus sequence for phosphorylation of PKG is in the domain of M3mAChR, specifically 488MSLIKEKK485 (45). Based on the data presented here it is proposed that the specific amino acid residue, susceptible to phosphorylation by PKG-II is that of Ser481.

Whether or not, this PKG-II-dependent M3mAChR phosphorylation has a relevant biological function was also explored. We found the effect was mediated mainly by M3mAChRs (Table 2).

Consequently, we evaluated, in the absence of PPase inhibitors, the effect of muscarinic drugs, specifically muscarinic agonist, CC, and the M3mAChR selective antagonist, 4-DAMP, on these cGMP-dependent 32P-membrane proteins.

In this sense, the 32P labeling of plasma membrane proteins showed a “bell shaped” as a dose–response curve for muscarinic agonists. Thus, a rise of the 32P-labeling, induced by CC was maximal at a concentration of 1 × 10^{-5} M. The agonist-dependent membrane protein phosphorylations may be linked to M3mAChR activation, especially as a response to agonist occupation by becoming rapidly hyperphosphorylated at intracellular domains as described (40–43). However, in the plasma membrane fragments, an agonist-dependent dephosphorylation process was observed at higher doses of CC (>1 × 10^{-3} M). This “two opposite responses” phenomenon on these dephospho/phosphorylation processes can be interpreted using a model based on the interactions of a ligand with two different receptors that mediate opposite effects (one stimulatory and one inhibitory) (46). Similar pharmacological muscarinic agonist “two opposite responses” behavior has been previously described to explain the two opposite mAChRs signal transducing mechanisms, regulating a G-protein-coupled NPR-GC-B (11,12).

Surprisingly, 4-DAMP, a selective M3mAChR antagonist, increased this dephosphorylation activity, reducing both the basal and cGMP-dependent phosphorylations. This was shown in the presence of cGMP, with an IC50 of 1.0 ± 0.1 nM for 4-DAMP, which was in the expected nM range for a specific inhibition of M3mAChRs (47). This inhibition of 32P-phosphorylation of membrane proteins was similar to that observed at higher muscarinic agonist doses (>1 × 10^{-3} M).

Two mechanisms can be proposed to explain the dramatic inhibition of 4-DAMP on 32P-labeling. First, 4-DAMP may be acting as an “inverse-agonist” on M3mAChR, changing the receptor conformation, especially at the i3-loop of the M3 mACHRs, which is less susceptible to phosphorylation by PKG-II. Similar “inverse-agonist” behavior, reducing the constitutive phosphorylation of the mutant N514Y M3mAChR, has been reported for some muscarinic antagonists such as atropine, 4-DAMP and pirenzipine (48). Second, 4-DAMP may also be acting as an “inverse agonist” on the M3mAChR. Thus, it is possible that M3mAChR activates a putative membrane-bound PPase leading to a profound decrease in 32P-labeling in plasma membranes. The existence of a membrane-bound PPase activity, involved in these muscarinic actions, is supported by the effect of OKA, a classic inhibitor of PPase activity, which reduced the time required to achieve maximal saturation (Bmax) of [3H] QNB binding from 60 min to 10 min.

The PPase family has been described, as four-major serine/threonine-specific PPases present in animal cells. Moreover, OKA provides important clues to the physiological roles of these PP-1, PP-2A and PP-2B enzymes (49,50). PPases have been suggested to be involved in the modulation of ionic currents by muscarinic agents in several neuronal and non-neuronal cells. In hippocampal pyramidal neurons, a PPase linked to mACHRs is involved in the cholinergic suppression of the Ca2+-activated K+ current (sIAHP) (51). Another non-neuronal system, under muscarinic action linked to PPase, is the ability of acetylcholine to decrease the cAMP-dependent currents through cardiac L-type Ca2+ channels at guinea-pig ventricular myocytes (52). These authors neither identify the muscarinic receptor subtype nor firmly establish the biochemical nature of the PPase involved in these muscarinic actions.

It has been claimed that M3mAChR dephosphorylation regulates the receptor interactions with G proteins (53). The muscarinic receptor signaling regulator named SET is a PPase 2A inhibitor, which binds to the C-terminal of the i3-loop- M3mAChR (54,55) decreasing receptor engagement with G proteins.

All of the above evidences suggest that PPase 2A, which is also a membrane-bound enzyme (56) may be the PPase involved in M3mAChR dephosphorylation. Further research will establish the validity of this proposal.

A recent study indicates that the binding site of both SET and PP2A on M3mAChR occurs at the i3-loop (474ITKRKRMSLIKEKKAQQ489). SET specifically binds to the site 477KRKR479 in close vicinity to a domain KEKKAAQTLS493 located in G protein coupling and activation (55,57,58). Our results based on the synthetic peptide MSLIKEKKAATLS493 led us to propose that the domain MSLIKEK485 is the putative phosphorylation site on PKG-II. This domain contains the S481, which is located between these two relevant regulatory binding sites, suggesting an important biological function for the PKG-II response.

The involvement of PKG-II as a CAMP-dependent M3mAChR phosphorylation, is a novel mechanism, present in ASM cells to guarantee a feedback control of cGMP on M3mAChR activation. This post-translational reversible modification at M3 mAChRs may act as a feedback mechanism to terminate the cGMP-dependent muscarinic signal transduction cascades at the sarclemma of BTSM.

Finally, the M3mAChR, a prototype of class A GPCR that preferentially couples to the family of G proteins, is involved in numerous important physiological functions in ASM. These are the cholinergic tone that contributes to airflow obstruction and chronic airway inflammation in asthma and COPD, where anti-cholinergics are effective bronchodilators by blocking this muscarinic receptor subtype (59). This work supports the existence of “cGMP linked muscarinic signal transducing signalosome” machinery comprised of M3mAChRs, NPR-GC-B, PKG-II and cGMP-PDE located in the plasma membrane of ASM. This signalosome involves a “membrane-associated cGMP pool” as a product of NPR-GC-B (6–12), as a second messenger which streams down to activate a membrane-bound PKG-II, which then phosphorylates the M3mAChR, inducing the desensitization of this M3mAChR subtype, in an inhibitory feedback.
mechanism (14). Furthermore, the presence of a cGMP-PDE in these plasma membranes from BSTM can shut down this ‘‘cGMP linked muscarinic signal transducing signalosome’’ (unpublished results).

Conclusions
This work supports the existence of a ‘‘muscarinic signal transducing signalosome’’ machinery comprised of M3mAChRs, NPR-GC-B, PKG-II and a putative PPase located in the plasma membrane of ASM. This signalosome involves a ‘‘membrane-associated cGMP pool’’ (6–12) as second messenger, which streams down to activate a membrane-bound PKG-II, which then phosphorylates the M3mAChR at the i 3-loop extending from Thr450–Q490, and containing the peptide M3mAChR (480MSLIKEKKAQTLS493). The latter was heavily phosphorylated by PKG-II, inducing the desensitization of this M3mAChR subtype, in a feedback mechanism at plasma membrane level (14). The M3mAChR, a prototypic class A GPCR, which preferentially couples to the family of G proteins, is involved in numerous important physiological functions in ASM. Interestingly, M3mAChR in ASM, the main subject of this work, is involved in the cholinergic tone, which contributes to airflow obstruction and chronic airway inflammation in asthma and COPD. It is known that anti-cholinergics are effective bronchodilators by blocking this muscarinic receptor subtype (59). Thus, understanding how the M3mAChR functions at the molecular level is of considerable relevance for designing novel classes of drugs that can modulate M3mAChR function for therapeutic purposes in pathological conditions such as asthma and COPD.

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