Journal of Receptors and Signal Transduction

ISSN: 1079-9893 (Print) 1532-4281 (Online) Journal homepage: http://www.tandfonline.com/loi/irst20

Muscarinic drugs regulate the PKG-II-dependent phosphorylation of M_3 muscarinic acetylcholine receptors at plasma membranes from airway smooth muscle

Marcelo J. Alfonzo, Ramona González de Alfonzo, Marcelo Alfonzo González & Itala Lippo de Becemberg

To cite this article: Marcelo J. Alfonzo, Ramona González de Alfonzo, Marcelo Alfonzo González & Itala Lippo de Becemberg (2015) Muscarinic drugs regulate the PKG-II-dependent phosphorylation of M_3 muscarinic acetylcholine receptors at plasma membranes from airway smooth muscle, Journal of Receptors and Signal Transduction, 35:4, 319-328, DOI: 10.3109/10799893.2014.982826

To link to this article: http://dx.doi.org/10.3109/10799893.2014.982826

Journal of Receptors and Signal Transduction

http://informahealthcare.com/rst
ISSN: 1079-9893 (print), 1532-4281 (electronic)

J Recept Signal Transduct Res, 2015; 35(4): 319–328
 DOI: 10.3109/10799893.2014.982826

informa healthcare

RESEARCH ARTICLE

Muscarinic drugs regulate the PKG-II-dependent phosphorylation of M_3 muscarinic acetylcholine receptors at plasma membranes from airway smooth muscle

Marcelo J. Alfonzo, Ramona González de Alfonzo, Marcelo Alfonzo González, and Itala Lippo de Becemberg

Facultad de Medicina, Sección de Biomembranas, Instituto de Medicina Experimental (IME), Universidad Central de Venezuela, Sabana Grande, Caracas, Venezuela

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 M_2/M_3 muscarinic acetylcholine receptors (mAChRs). Muscarinic receptor activity, expressed as [^{3}H]QNB binding at plasma membranes from bovine tracheal smooth muscle (BTSM), increased with cGMP and was augmented significantly cGMP plus ATP but diminished with the PKG-II inhibitor, Sp-8-pCPT-cGMPS. The [^{3}H]-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-[^{32}P]γATP 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 [^{32}P]-labeling. A selective M_3mAChR antagonist, 4-DAMP produced a dramatic inhibition of the basal and CC-dependent [^{32}P]-labeling. The [^{32}P] labeled membrane sediments were detergent solubilized and immunoprecipitated with specific M_2/M_3mAChR antibodies. The M_3mAChR immuno-precipitates exhibited the highest cGMP-dependent [^{32}P]-labeling, indicating it is a PKG-II substrate. Experiments using synthetic peptides from the C-terminal of the third intracellular loop (i_3) of both M_2mAChR (356–369) and M_3mAChR (480–493) as external PKG-II substrates resulted in the i_3M_3-peptide being heavily phosphorylated. These results indicated that PKG-II phosphorylated the M_3mAChR at the i_3M_3 domain (^{480}MSLIKEKK^{485}), suggesting that Ser^{481} 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 M_3mAChR in the patho-physiology of ASM involved in asthma and COPD.

Keywords

cGMP, carbamylcholine, muscarinic receptors, tracheal smooth muscle

History

Received 15 August 2014
Revised 26 October 2014
Accepted 28 October 2014
Published online 8 June 2015

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 M_2 and M_3 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 M_2/M_3mAChRs 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 M_2mAChR 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 M_3mAChR coupled to a Gq_{16} 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 [^{3}H]QNB binding studies on a plasma membranes fraction from BTSM demonstrate a high activity of M_2/M_3mAChR subtypes (13). Functional studies have shown that the M_2/M_3 mAChR subtypes are present in these BTSM plasma membranes (7,8,11,12). Recently, we showed that cGMP affects the M_3mAChR functionality expressed as [^{3}H]QNB binding activity (14).

Using a plasma membranes fractions from BTSM, we demonstrate that cGMP, via PKG-II, phosphorylated the M_3mAChR affected its functionality, expressed by an increment in the B_{max} for [^{3}H]QNB binding activity and displaying a positive co-operativity. Moreover, okadaic acid

Address for correspondence: Dr. Marcelo J. Alfonzo, Facultad de Medicina, Sección de Biomembranas, Instituto de Medicina Experimental, Universidad Central de Venezuela, Apdo. 50587, Sabana Grande, Caracas, Venezuela. Fax: 58-2-662-88-77. E-mail: hmag5@hotmail.com

(OKA), (a protein phosphatase (PPase inhibitor) induced a faster [^{3}H]QNB binding, suggesting the involvement of a membrane-bound PPase. Furthermore, a cGMP-dependent [^{32}P]-phosphorylation of membrane protein was specific for the M_3mAChR. This [^{32}P]-membrane labeling was affected by muscarinic agonists such as carbamylcholine (CC) displaying agonist-dependent phospho/dephosphorylation reactions. Conversely, 4-DAMP, a selective M_3mAChR antagonist, inhibited both the basal and cGMP-dependent membrane protein [^{32}P]-phosphorylations, supporting the involvement of an unknown PPase.

In this work, a putative cGMP regulatory feedback 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, $MgCl_2$, 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-M_2 and anti-mAChR antibodies were acquired from Santa Cruz Biotechnology, Inc. (Dallas, TX). L-[^{3}H]QNB (45.5 Ci/mmol) from the Radiochemical Centre (Amersham, UK), GE/Healthcare Bio-Sciences (Pittsburgh, PA), Life Sciences. [^{32}P]γATP (3000 Ci/mmol) was purchased from Perkin-Elmer (Alameda, CA). Other chemical reagents were obtained from Merck (Darmstadt, Germany) and Fisher Scientific (Waltham, MA).

Synthetic tetradecapeptides derived from the C-terminal of third intracellular loop (i3) of mAChR subtypes M_2 (amino acid sequence: ^{356}KQNIVARKIVKMTK^{369}) and M_3 (^{480}MSLIKEKKAAQTLS^{493}) 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 (P_1) 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 N_2 and stored at −80 °C until use.

Measurement of muscarinic acetylcholine receptor activity

The mAChR activity was evaluated using the [^{3}H]QNB binding studies, which were performed as described previously (13). Briefly, PM (P_1) 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 [^{3}H]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-[^{3}H]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 [^{3}H]QNB. The column effluent containing 97–98% of the bound [^{3}H]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 B_{max}, K_D and Hill coefficient n_H were calculated as described (17).

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

Protein kinase G (PKG) activity and [^{32}P]-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 $MgCl_2$, 20 mM KPi (pH 7.0), 100 μM of the cocktail of phosphatase inhibitors (OKA, NIPP-1) and 0.1–1 mM [^{32}P] γATP (3 μCi/assay). In the assays using synthetic peptides from i_3M_2/M_3AChRs, which were based on mAChRs M_2[Bos Taurus] NCBI reference sequence NP_001074202.1 and mAChRs M_3[Bos Taurus] NCBI reference sequence NP_776695.1. The peptides were synthesized and purified by HPLC and later used as exogenous substrates to ^{32}P-ATP-dependent membrane phosphorylation. To calculate these values, basal ^{32}P-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 [^{32}P]-labeled lipids. Discs were allowed to dry under air stream and ^{32}P was counted using Cêrenkov radiation (20) in a liquid scintillation counter.

mAChR [^{32}P]phosphorylation and selective immunoprecipitation assays

BTSM plasma membranes (P_1 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^{32}P]ATP (Specific activity: 3000 μCi/μmole), 1 mM $MgCl_2$, 20 mM KPi (pH 7.2), 1 mM DTT, 10 μM IBMX, 100 μM protein phosphatase inhibitor kit containing (cypermethrin,

dephostatin, OKA and NIPP-1 Bovine Thymus recombinant) and other compounds as indicated. [^{32}P]-labeled plasma membranes were separated from the excess of [γ^{32}P]ATP by centrifugation at $12\,000 \times g$ for 15 min at 4 °C in an Eppendorf® (Hamburg, Germany) centrifuge. [^{32}P]-labeled 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). [^{32}P]-labeled membranes (5 mg/ml) were solubilized by incubation at 4 °C for 15 min in a mixture containing 5 mM $MgCl_2$, 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 $150\,000 \times g$ 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-M_3 or anti-M_2mAChR 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 [^{32}P]-immunoprecipitates was used to measure [^{32}P] 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 [^{3}H]QNB antagonist binding at plasma membranes from BTSM

The effect of increasing concentrations of cGMP on the [^{3}H]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 [^{3}H]QNB to plasma membranes from BTSM reached a maximum around 50 nM cGMP. It remained constant at higher cyclic nucleotide concentrations, giving an ED_{50} 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 [^{3}H]QNB binding, saturation experiments were performed to estimate the kinetic parameters of this process, such as the maximal binding activity (B_{max}), the dissociation constant (K_D and the Hill coefficient (n_H) associated with the cGMP effect (Table 1). The K_D values for ATP plus cGMP remained unchanged but the B_{max} and n_H parameters were markedly affected. Thus, B_{max} values (pmoles [^{3}H]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, n_H 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 [^{3}H]QNB binding activity

These changes induced by ATP plus cGMP in the [^{3}H]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 [^{3}H]QNB binding activity was evaluated. In these experiments, the [^{3}H]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 [^{3}H]QNB binding is mediated via a PKG-II isoenzyme anchored to the plasma membrane fraction from BTSM.

Effect of OKA on [^{3}H]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 [^{3}H]QNB binding. The inclusion of OKA in the [^{3}H]QNB binding assays increased the velocity of [^{3}H]QNB binding (Figure 3).

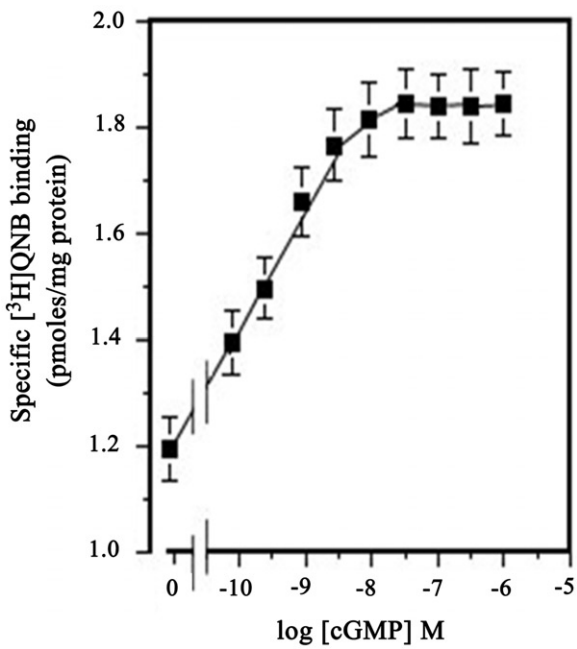

Figure 1. The effect of cGMP on the [3H]QNB binding in the plasma membranes of BTSM. Experiments were performed at 37 °C in the presence of 1,250 nM [^{3}H]QNB, 2–3 μg of membrane proteins, 5 mM ATP and increasing the concentration of cGMP as described in Methods section. Each point represents the mean ± S.E. of four different membranes preparations assayed in triplicate.

J Recept Signal Transduct Res, 2015; 35(4): 319–328

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

Additions	B_{max}	K_D (pM)	n_H
cGMP	1002E20 ± 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 (*)

Tracheal smooth muscle plasma membranes were prepared as described in Methods section. The [^{3}H]QNB binding saturation experiments were carried out at 37 °C in the presence of 10 μM IBMX, 5 mM $MgCl_2$ 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 b triplicate. The values of B_{max} as pmoles [^{3}H]QNB/mg of protein), K_D and n_H were calculated as described (17).

(*)The difference in the B_{max} and n_H for the assays with both ATP and cGMP was statistically significant at $p < 0.01$.

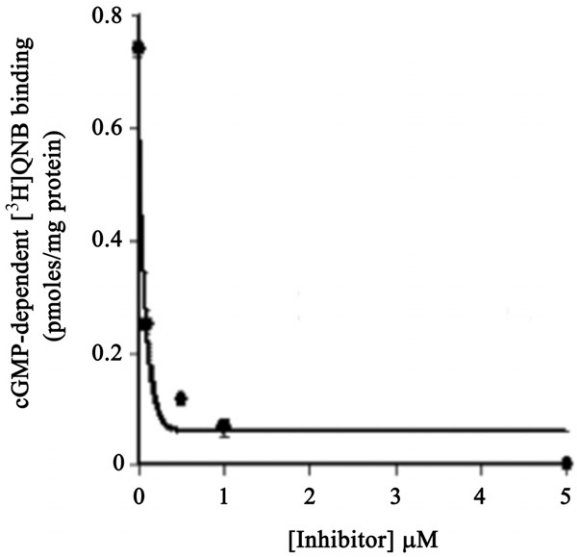

Figure 2. The effect of Rp-8-pCPT-cGMP-inhibitor of PKG on the cGMP-dependent [^{3}H] QNB binding activity at the plasma membranes from BTSM. The [3H]QNB binding activity was assayed at 1250 nM [^{3}H]QNB and 2–3 μg of protein and increasing amounts of Rp-8-pCPT-cGMP at 37 °C as described in Methods section. To evaluate the inhibition effect, cGMP (50 nM) was used as an activator of PKG-II. Each point represents the mean ± S.E. of four different membranes preparations assayed in triplicate.

The time taken to reach maximal [^{3}H]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 [^{3}H]QNB binding was reduced to 10 min. To evaluate more accurately the increase in velocity of [^{3}H]QNB binding, the same data were plotted over a shorter period of 5 min (inset Figure 3). For all experimental conditions tested, the [^{3}H]QNB binding velocity was linear up to 5 min. It can be seen that the [^{3}H]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 [^{3}H]QNB binding.

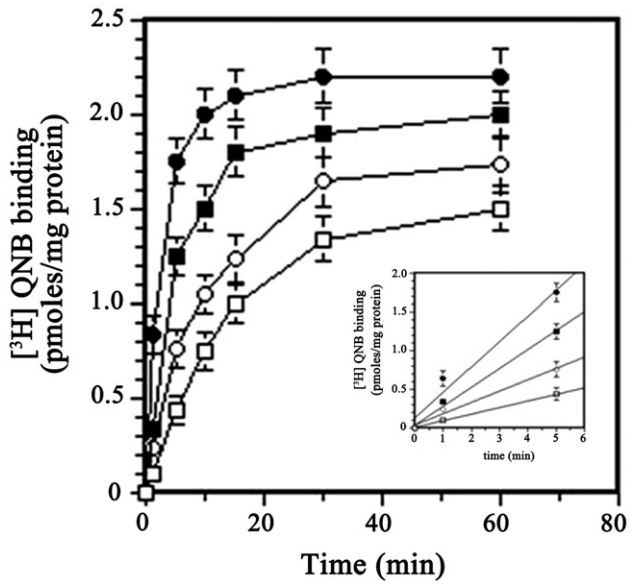

Figure 3. Time course of the effect of okadaic acid (OKA) on the [^{3}H]QNB binding activity to the plasma membranes from BTSM. The [^{3}H]QNB binding in the plasma membranes of the BTSM was assayed at 1250 nM [^{3}H]-QNB and 2–3 μg membrane protein assayed at 37 °C as described in Methods section in the presence of 50 nM cGMP (□), 1 μM OKA (○), 5 mM ATP plus 50 nM cGMP (■), 5 mM ATP, 50 nM cGMP plus 1 μM OKA (●). Insert: [3H]QNB binding activity in a time scale of 5 min. Each point represents the mean ± S.E. of four different membranes preparations assayed in triplicate.

Effect of cGMP on endogenous ^{32}P 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 ^{32}P incorporation from [γ^{32}P]ATP into plasma membrane proteins. A cGMP titration on the ^{32}P-endogenous membrane protein phosphorylation gave maximal ^{32}P incorporation at approximately 5×10^{-7} M of cGMP with a ED_{50} of 1×10^{-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 [^{32}P]-phosphorylation of membrane protein components. Consequently, the [^{32}P]-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 [^{32}P]-labeled material. The difference was statistically significant between the two conditions, which was almost twice in the detergent-solubilized membrane proteins.

To identify specific [^{32}P]-labeled polypeptides, a more detailed [^{32}P]-labeling study was performed. The [^{32}P]-labeling difference between the muscarinic receptors was more significant in the immunoprecipitates using specific anti-M_2 and -anti-M_3mAChR antibodies. Both receptor subtypes were labeled with [γ^{32}P]ATP (Table 2). The M_3mAChR was preferentially and significantly phosphorylated in the presence of 50 nM cGMP, being more than 80% of the ^{32}P solubilized material immunoprecipated by the M_3mAChR antibodies, in comparison only 20% of the phosphorylated M_2mAChR was pull down by the M_2mAChRs antibodies. These data indicate

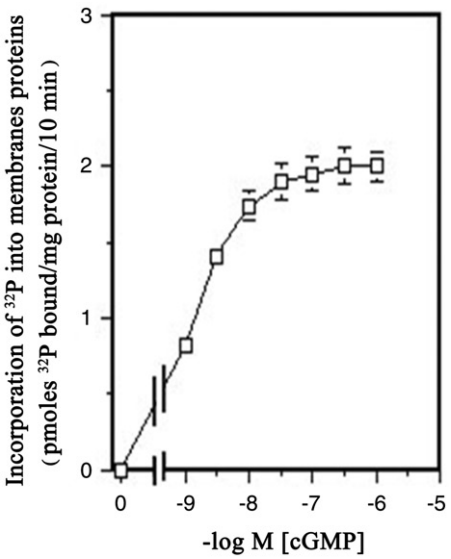

Figure 4. The effect of cGMP on the [^{32}P] labeling into plasma membranes proteins from BTSM. The experiments were performed at 37 °C in the presence of 20–30 μg of membrane proteins, incubated in a medium containing protein phosphatase inhibitors, 5 mM $MgCl_2$, 10 μM IBMX, 0.1 mM [^{32}P]γATP and increasing cGMP concentrations as described in Methods section. After 10 min of incubation, the [^{32}P]-labeled membranes were applied to paper discs and processed as described in Methods section. The disks were counted for [32P] using Cerenkov radiation. 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 phosphor lation in BTSM plasma membranes.

	Total [^{32}P] incorporated (pmol/10 min)	
	A	B
Total endogenous PM [^{32}P]-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)		
anti-m_3AChR	1.43 ± 0.15	2.74 ± 0.18*
anti-m_2AChR	0.89 ± 0.24	1.02 ± 0.21

BTSM plasma membranes (PM) (P_1-fraction, 1 mg protein/assay) were incubated in a medium containing protein phosphatase inhibitors and 5 mM $MgCl_2$, 10 μM IBMX, 0.1 mM [^{32}P-γ]ATP (A) plus 50 nM cGMP (B) as described in Methods section. After 10 min of incubation at 37 °C, [^{32}P]labeled PM was collected b centrifugation and washed twice to remove free [^{32}P]$_i$ or [^{32}P-γ]ATP to obtain the total [^{32}P]labeling endogenous PM. These [^{32}P]-labeled membranes were solubilized with Digitonin-cholate mixture and processed as described in Methods section. This [^{32}P] 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 b duplicate. Statistical differences between A versus B condition: (*) $p < 0.01$.

that cGMP, via a PKG-II, phosphorylates the M_3mAChR subtype. In these experimental conditions, the [^{32}P]-labeled M_3mAChR represents more than the 55% of the total [^{32}P]-labeled plasma membrane proteins from BTSM.

Another biochemical approach was undertaken to identify the M_3mAChR motifs and the putative amino acids involved

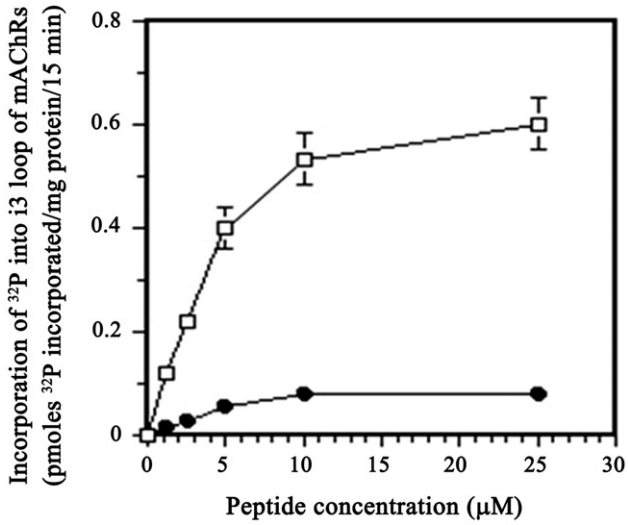

Figure 5. The [^{32}P] labeling of synthetic tetradeca-peptides from i_3 loop of M_2 and M_3 mAChR b endogenous plasma membranes protein kinases. The experiments were performed at 37 °C in the presence of 20–30 μg of membrane proteins, incubated in a medium containing protein phosphatases inhibitors, 5 mM $MgCl_2$, 10 μM IBMX, 50 nM cGMP, 0.1 mM [^{32}P]γATP and increasing concentrations of peptides: M_2 (356KQNIVARKIVKMTK369)(●) and M_3 (480MSLIKEKKAAQTL S493) (□) as described in Methods section. After 15 min of incubation, the [^{32}P]-labeled membranes were applied to P81 discs (anion-exchange paper) and processed as described in Methods section. The disks were counted for [^{32}P] using Cerenkov radiation. The [^{32}P] 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.

in this PKG-II phosphorylation. Thus, an experimental approach using synthetic peptides based on amino acids segments containing the putative consensus phosphorylation sequences for PKG [(R/K)2-3,-X-S*/T*]. These motifs were located close to the C-terminal from the third intracellular loop (i_3) of the M_2 and M_3mAChR subtypes (Figure 5). These peptides have the following amino acid sequences: M_3 (^{480}MSLIKEKKAAQTLS^{493}) and a close related in the M_2 (^{356}KQNIVARKIVKMTK^{369}). These synthetic peptides were phosphorylated in a concentration-dependent manner, by the PKG-II, in the presence of 50 nM cGMP and1 mM [γ^{32}P]ATP and 10 μM IBMX.

The M_3mAChR synthetic peptide was greater phosphorylated than the M_2mAChR peptide by the membrane-bound protein PKG-II. The V_{max} for the M_3mAChR synthetic peptide was 0.75 ± 0.03 pmoles P/min/mg protein, which was 4.6 times higher than for the M_2mAChR synthetic peptide (0.16 ± 0.03 pmoles P/min/mg protein). It is important to emphasize that these kinetic values are relative due to the fact that this ^{32}P-incorporation is the result of a competition between the endogenous mAChRs and the exogenous synthetic peptides. These findings indicate that the intracellular i_3-loop region from the M_3mAChRs is specifically phosphorylated by a PKG-II anchored to the plasma membranes.

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

It was important to establish, whether or not, the cGMP effect on ^{32}P phosphorylations was affected by muscarinic

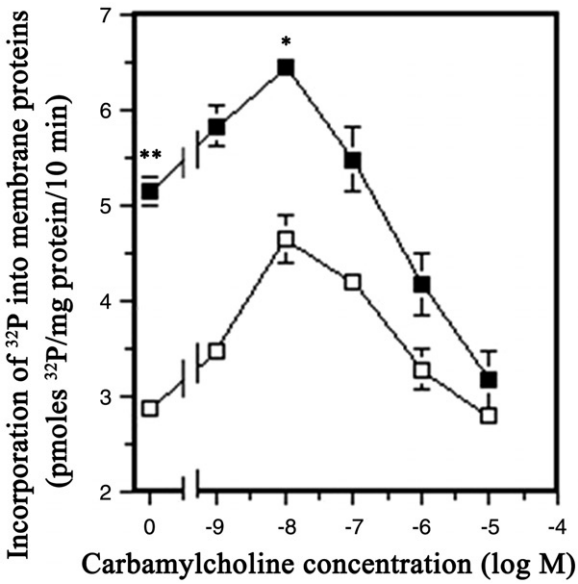

Figure 6. The incorporation of [^{32}P] into the plasma membranes proteins from BTSM in the presence of muscarinic agonist: Carbam lcholine (CC). The experiments were performed at 37 °C in the presence of 20–30 μg of membranes proteins, incubated in a medium containing 5 mM $MgCl_2$, 10 μM IBMX, 0.1 mM [32P]γATP, in the presence (■) or absence (□) of 50 nM cGMP without the protein phosphatase inhibitors and increasing CC concentrations as described in Methods section. After 10 min of incubation, [^{32}P]-labeled membranes was applied to paper discs and processed as described in Methods section. The discs were counted for [32P] using Cerenkov radiation. Each point represents the mean ± S.E. of four different membranes preparations assayed in triplicate. The differences between ATP versus [ATP plus cGMP] assay without CC was statistically significant at $p < 0.001$(**). The difference between ATP versus [ATP plus cGMP] in the presence of CC at a concentration of 1×10^{-8} M was statistically significant at $p < 0.01$ (*).

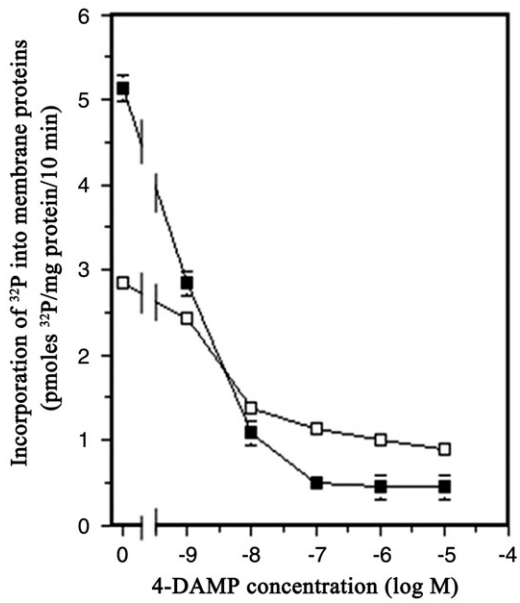

Figure 7. The incorporation of [^{32}P] into the plasma membranes proteins from BTSM in the presence of 4-DAMP (M3 selective muscarinic antagonist). The experiments were performed at 37 °C in the presence of 20–30 μg of membranes proteins, incubated in a medium containing 5 mM $MgCl_2$, 10 μM IBMX, 0.1 mM [32P]γATP, in the presence (■) or absence (□) of 50 nM cGMP and increasing concentrations of 4-DAMP without the protein phosphatase inhibitor kit as described in Methods section. After 10 min of incubation at 37 °C, the [32P]-labeled membranes was applied to paper discs and processed as described in the Methods section. The disks were counted for [^{32}P] using Cerenkov radiation. Each point represents the mean ± S.E. of four different membranes preparations assayed in triplicate.

compounds. In the following experiments, the protein phosphatase inhibitor kit (containing cypermethrin, dephostatin, OKA and NIPP-1 Bovine Thymus recombinant) was omitted for these ^{32}P-phosphorylation assays. A CC titration on ^{32}P protein membranes labeling is shown in Figure 6. It can be seen that this muscarinic agonist induces a ''two opposite responses'' expressed by a significant rise in ^{32}P-phosphorylation of membrane proteins to a concentration of 1×10^{-8} M (Figure 6). However, increasing concentrations of CC produced a dramatic decrease in ^{32}P phosphorylation reaching basal levels at 1×10^{-5} M CC. Similarly, in the presence of 50 nM cGMP, the ^{32}P incorporation into plasma membrane proteins followed a similar ''two opposite responses'' reaching a maximal at a CC concentration of 1×10^{-8} M analogous behavior to the basal condition. However, after this maximal ^{32}P incorporation peak, at higher CC concentrations, muscarinic agonist-dose-dependent dephosphorylations proceed, reaching the same basal ^{32}P labeling at 1×10^{-5} M CC.

To differentiate between the M_2/M_3AChR subtypes being phosphorylated/dephosphorylated, which are present in these plasma membranes fractions, a selective (M_1,M_3,M_5 AChRs) antagonist, such as 4-DAMP was used. A 4-DAMP titration was performed, with both the cGMP-dependent and the basal ^{32}P-phosphorylating activities (Figure 7). Thus, 4-DAMP, in a dose-dependent fashion induced a dramatic inhibition in the basal activity, which was more pronounced for the cGMP-dependent ^{32}P-labeling. As a result, in the presence of cGMP, an IC_{50} for 4-DAMP of 1.0 ± 0.1 nM was estimated. The last results suggest the existence of a putative muscarinic receptor-linked PPase activity.

Discussion

The muscarinic activation of ASM contraction (4–6) involves both muscarinic receptors, M_2/M_3 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 M_3/M_2AChR biological activity as described (11–13). 2. It contains an

active PKG-II isoform, previously identified (14). 3. These membranes 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 M_3mAChR, 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 M_3mAChR.

Cyclic GMP via PKG-II phosphorylation induced the following biological actions on the mAChR binding activity here described: 1. The increase of B_{max} for [^{3}H]QNB binding activity (>60%) indicating that ''new [^{3}H]-QNB binding sites'' are displayed. Nonetheless, this cGMP effect on B_{max}, was obliterated by the 4-DAMP alkylation of M_3mAChR subtype as previously reported (14). 2. A doubling of the Hill coefficients (n_H), which increased from 1.0 to almost 2.0, suggesting a positive co-operativity or homodimer formation of M_3mAChRs as previously postulated (14). 3. The PPase inhibitor OKA induced a faster [^{3}H]QNB binding at these M_3mAChRs indicating a dephosphorylation-linked process is present in these plasma membrane fractions.

These ''new M_3mAChRs'' that exhibited similar affinity constants (K_D), can be excluded as newly synthesized or exposed ''recycled or hidden'' M_3mAChRs from endosomes vesicles since our assays were performed with isolated plasma membrane fragments. The appearance of these ''new M_3mAChR'' 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 M_1mAChR 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 M_3mAChR induced a similar molecular mechanism of homodimer/oligomer formation as previously proposed (14). This rationale is supported by the fact that, the M_3mAChR displays a greater propensity to form a homodimer/oligomer structure at higher density receptor population (31,32). Recent studies demonstrated the formation of M_3mAChR dimers *in vivo* (33,34), which may reflect the situation in these plasma membranes from BTSM, which have higher mAChRs amounts (13,15).

The M_3mAChRs 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 comparison, 4-DAMP, an M_3 selective antagonist, displayed high and low affinity-binding sites. In contrast, a non-selective antagonist atropine and the M_2-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 M_3mAChR 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 [^{3}H]QNB binding and ^{32}P-labeling in the plasma membranes from BTSM. Interestingly, the ED_{50} results for cGMP (1×10^{-9} M) was similar for both the rise of the [^{3}H]QNB binding and the ^{32}P-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 [^{3}H]-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 M_3mAChR 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 M_3mAChRs, induces receptor uncoupling from G-proteins (37), which has been reported in SHSY5Y cell line (38).

Our previous observations using ^{32}P-autoradiographs (14) and the specific immunoprecipitation assays support the argument that M_3mAChR is specifically phosphorylated by PKG-II. Several agonist-dependent phosphorylations of M_3 mAChR have been reported (39). An M_3mAChR hyperphosphorylation occurs following agonist occupation, linked to desensitization of muscarinic receptors. This usually occurs at serine (Ser) and threonine (Thr) residues contained on the i_3-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 i_3-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 (IP_3R), which generates the IP_3Rtide (GRRESLTSFG) and the cAMP response element binding protein (CREB) which contains a CREBtide (KRREILSRRPSYR) (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 i_3-loop of the M_3mAChRs extending from Thr^{450} to Q^{490}. This contains the peptide M_3AChR (^{480}MSLIKEKKAAQTLS^{493}), which

was heavily phosphorylated by PKG-II located in the sarcolemma from BTSM. The known consensus sequence for phosphorylation of PKG is in the domain of i_3M_3 mAChR, specifically ^{480}M**S**LI**K**E**KK**^{485} (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 Ser^{481}.

Whether or not, this PKG-II-dependent M_3mAChR phosphorylation has a relevant biological function was also explored. We found the effect was mediated mainly by M_3mAChRs (Table 2).

Consequently, we evaluated, in the absence of PPase inhibitors, the effect of muscarinic drugs, specifically muscarinic agonist, CC, and the M_3mAChR selective antagonist, 4-DAMP, on these cGMP-dependent ^{32}P-membrane proteins.

In this sense, the ^{32}P labeling of plasma membrane proteins showed a ''bell shaped'' as a dose–response curve for muscarinic agonists. Thus, a rise of the ^{32}P-labeling, induced by CC was maximal at a concentration of 1×10^{-8} M. The agonist-dependent membrane protein phosphorylations may be linked to M_3mAChR 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 ($>1 \times 10^{-8}$ 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 M_3mAChR antagonist, increased this dephosphorylation activity, reducing both the basal and cGMP-dependent phosphorylations. This was shown in the presence of cGMP, with an IC_{50} of 1.0 ± 0.1 nM for 4-DAMP, which was in the expected nM range for a specific inhibition of M_3mAChRs (47). This inhibition of ^{32}P-phosphorylation of membrane proteins was similar to that observed at higher muscarinic agonist doses ($>1 \times 10^{-8}$ M).

Two mechanisms can be proposed to explain the dramatic inhibition of 4-DAMP on ^{32}P-labeling. First, 4-DAMP may be acting as an ''inverse-agonist'' on M_3mAChR, changing the receptor conformation, especially at the i_3-loop of the M_3 mAChRs, which is less susceptible to phosphorylation by PKG-II. Similar ''inverse-agonist'' behavior, reducing the constitutive phosphorylation of the mutant N514Y M_3mAChR, has been reported for some muscarinic antagonists such as atropine, 4-DAMP and pirenzepine (48). Second, 4-DAMP may also be acting as an ''inverse agonist'' on the M_2mAChR. Thus, it is possible that M_2mAChR activates a putative membrane-bound PPase leading to a profound decrease in ^{32}P-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 (B_{max}) of [^{3}H] 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 Ca^{2+}-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 Ca^{2+} 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 M_3mAChR 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 i_3-loop- M_3mAChR (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 M_3mAChR dephosphorylation. Further research will establish the validity of this proposal.

A recent study indicates that the binding site of both SET and PP2A on M_3mAChR occurs at the i_3-loop (^{474}IT**KRKR**MSLI**K**E**KK**AAQ^{490}). SET specifically binds to the site ^{476}**KRKR**^{479} in close vicinity to a domain ^{484}**K**E**KK**AAQ^{490} involved in G protein coupling and activation (55,57,58). Our results based on the synthetic peptide ^{480}M**S**LI**K**E**KK**AAQTLS^{493} led us to propose that the domain ^{480}M**S**LI**K**E**KK**^{485} is the putative phosphorylation site on PKG-II. This domain contains the **S**^{481}, 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 cGMP-dependent M_3mAChR phosphorylation, is a novel mechanism, present in ASM cells to guarantee a feedback control of cGMP on M_3mAChR activation. This post-translational reversible modification at M_3 mAChRs may act as a feedback mechanism to terminate the cGMP-dependent muscarinic signal transduction cascades at the sarcolemma of BTSM.

Finally, the M_3mAChR, 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 M_3mAChRs, 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 M_3mAChR, inducing the desensitization of this M_3mAChR subtype, in an inhibitory feedback

mechanism (14). Furthermore, the presence of a cGMP-PDE in these plasma membranes from BTSM 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 M_3mAChRs, 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 M_3mAChR at the i_3-loop extending from Thr^{450}-Q^{490}, and containing the peptide M_3mAChR (^{480}M**S**LI**KE**K**K**AAQ TLS^{493}). The latter was heavily phosphorylated by PKG-II, *inducing the desensitization of this M_3mAChR subtype, in a feedback mechanism at plasma membrane level* (14). The M_3mAChR, a prototypic class A GPCR, which preferentially couples to the family of G proteins, is involved in numerous important physiological functions in ASM. Interestingly, M_3mAChR 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 M_3mAChR functions at the molecular level is of considerable relevance for designing novel classes of drugs that can modulate M_3mAChR function for therapeutic purposes in pathological conditions such as asthma and COPD.

Acknowledgements

The authors thank Dr. Coral Wynter for revision of the English version of this article.

Declaration of interest

This work was supported by grants from CDCH-UCV # PG -09-7401-2008/2 (R. G. A.); CDCH-UCV # PI-09-7726.2009/ 2 (I. L. B.); CDCH-UCV # PG 09-7772-2009/1 (M. J. A.). The authors declare no conflicts of interests.

References

1. Mak JC, Barnes PJ. Autoradiographic visualization of muscarinic receptor subtypes in human and guinea pig lung. Am Rev Respir Dis 1990;141:1559–68.
2. Lucchesi PA, Scheid CR, Romano FD, et al. Ligand binding and G protein coupling of muscarinic receptors in airway smooth muscle. Am J Physiol 1990;258:C730–8.
3. Haddad EB, Landry Y, Gies JP. Muscarinic receptor subtypes in guinea pig airways. Am J Physiol 1991;261:L327–33.
4. Murad F, Kimura H. Cyclic nucleotide levels in incubations of guinea pig trachea. Biochim Biophys Acta 1974;343:275–86.
5. Katsuki S, Murad F. Regulation of adenosine cyclic 3′, 5′-monophosphate and guanosine cyclic 3′,5′-monophosphate levels and contractility in bovine tracheal smooth muscle. Mol Pharmacol 1977;13:330–41.
6. Guerra de González L, Misle A, Pacheco G, et al. Effects of 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one (ODQ) and N omega (ω)-nitro-L-arginine methyl ester (NAME) on cyclic GMP levels during muscarinic activation of tracheal smooth muscle. Biochem Pharmacol 1999;58:563–9.
7. Alfonzo MJ, Placeres-Uray F, Hassan-Soto W, et al. Two guanylylcyclases regulated the muscarinic activation of airway smooth muscle. In: Sugi H, ed. Current basic and pathological approaches to the function of muscle cells and tissues-from molecules to humans. Croatia: InTech; 2012:113–32.
8. Uray FP, de Alfonzo RG, de Becemberg IL, Alfonzo MJ. Muscarinic agonists acting through M2 acetylcholine receptors stimulate the migration of an NO-sensitive guanylyl cyclase to the plasma membrane of bovine tracheal smooth muscle. J Recept Signal Transduct Res 2010;30:10–23.
9. Alfonzo MJ, Becemberg IL, Villaroel SS, et al. Two opposite signal transducing mechanisms regulate a G-protein-coupled guanylyl cyclase. Arch Biochem Biophys 1998;350:19–25.
10. Alfonzo MJ, Guerra de González L, Villaroel SS, et al. Signal transduction pathways through mammalian guanylyl cyclases. New Adv Cardiovasc Physiol Pharmacol 1998;1184:147–72.
11. Borges A, Villarroel SS, Winand NJ, et al. Molecular and biochemical characterization of a CNP-sensitive guanylyl cyclase in bovine tracheal smooth muscle. Am J Respir Cell Mol Biol 2001; 25:98–103.
12. Bruges G, Borges A, Villarroel SS, et al. Coupling of M3 acetylcholine receptor to Gq16 activates a natriuretic peptide receptor guanylyl cyclase. J Recept Signal Transduct Res 2007;27: 189–216.
13. Misle AJ, Lippo de Becemberg I, Gonzalez de Alfonzo R, Alfonzo MJ. Methoctramine binding sites sensitive to alkylation on muscarinic receptors from tracheal smooth muscle. Biochem Pharmacol 1994;48:191–5.
14. Alfonzo MJ, González de Alfonzo R, Alfonzo-González MA, Lippo de Bécemberg I. Cyclic GMP regulates M3AChR activity at plasma membranes from airway smooth muscle. Mol Membrane Biol 2013; 30:403–17.
15. González de Alfonzo R, Bécemberg IL, Alfonzo MJ. A Ca^{2+}/Ca M dependent protein kinase associated with Ca^{2+} transport in sarco(endo)plasmic vesicles from tracheal smooth muscle. Life Sci 1996;58:18–24.
16. Fields JZ, Roeske WR, Morkin E, Yamamura HI. Cardiac muscarinic cholinergic receptors. Biochemical identification and characterization. J Biol Chem 1978;253:3251–8.
17. Bennett Jr JP, Yamamura HI. Neurotransmitter, hormone, or drug receptor binding methods. In: Yamamura HI, Enna SJ, Kuhar MJ, eds. Neurotransmitter receptor binding. New York: Raven Press; 1985:61–90.
18. Corbin JD, Reissman EM. Assay of cyclic AMP-dependent protein kinases. Meth Enzymol 1974;38:287–90.
19. Vardanis A. A unique cyclic nucleotide-dependent protein kinase. J Biol Chem 1980;255:7238–43.
20. Clausen T. Measurement of ^{32}P activity in a liquid scintillation counter without the use of scintillator. Anal Biochem 1968;22: 70–3.
21. Peterson GL, Schimerlik MI. Large scale preparation and characterization of membrane-bound and detergent-solubilized muscarinic acetylcholine receptor from pig atria. Prep Biochem 1984;14: 33–74.
22. Bensadoun A, Weinstein D. Assay of proteins in the presence of interfering materials. Anal Biochem 1976;70:241–50.
23. Perez-Zoghbi JF, Bai Y, Sanderson MJ. Nitric oxide induces airway smooth muscle cell relaxation by decreasing the frequency of agonist-induced Ca^{2+} oscillations. J Gen Physiol 2010;135: 247–59.
24. Butt E, Pöhler D, Genieser HG, et al. Inhibition of cyclic GMP-dependent protein kinase-mediated effects by (Rp)-8-bromo-PET-cyclic GMPS. Br J Pharmacol 1995;116:3110–16.
25. Butt E, Nolte C, Schulz S, et al. Analysis of the functional role of cGMP-dependent protein kinase in intact human platelets using a specific activator 8-para-chlorophenylthio-cGMP. Biochem Pharmacol 1992;43:2591–600.
26. Gamm DM, Francis SH, Angelotti TP, et al. The type II isoform of cGMP-dependent protein kinase is dimeric and possesses regulatory and catalytic properties distinct from the type I isoforms. J Biol Chem 1995;270:27380–8.
27. Vaandrager AB, Edixhoven M, Bot AG, et al. Endogenous type II cGMP-dependent protein kinase exists as a dimer in membranes

and can be functionally distinguished from the type I isoforms. J Biol Chem 1997;272:11816–23.
28. Caulfield MP, Birdsall NJ. International Union of Pharmacology. XVII. Classification of muscarinic acetylcholine receptors. Pharmacol Rev 1998;50:279–90.
29. Eller M, Jarv J. Two-step isomerization of quinuclidinyl benzilate-muscarinic receptor complex. Neurochem Int 1998;12:285–9.
30. Jakubik J, El-Fakahany EE, Tucek S. Evidence for a tandem two-site model of ligand binding to muscarinic acetylcholine receptors. J Biol Chem 2000;275:18836–44.
31. Zeng Y, Wess J. Identification and molecular characterization of m3 muscarinic receptor dimmers. J Biol Chem 1999;274: 19487–97.
32. Palczewski K. Oligomeric forms of G-protein coupled receptors (GPCRs). Trends Biochem Sci 2010;35:595–600.
33. Alvarez-Curto E, Ward RJ, Pediani JD, Milligan G. Ligand regulation of the quaternary organization of cell surface M3 muscarinic acetylcholine receptors analyzed by fluorescence resonance energy transfer (FRET) imaging and homogeneous time-resolved FRET. J Biol Chem 2010;285:23318–30.
34. McMillin, SM, Heusel, M, Liu T, et al. Structural basis of M3 muscarinic receptor dimer/oligomer formation. J Biol Chem 2011; 286:28584–98.
35. Francis SH, Busch JL, Jackie D, Corbin JD. cGMP-dependent protein kinases and cGMP phosphodiesterases in nitric oxide and cGMP action. Pharmacol Rev 2010;62:525–63.
36. Burgoyne RD. The loss of muscarinic acetylcholine receptors in synaptic membranes under phosphorylating conditions is dependent on calmodulin. FEBS Lett 1981;127:144–8.
37. Sibley DR, Strasser RH, Benovic JL, et al. Phosphorylation/dephosphorylation of the beta-adrenergic receptor regulates its functional coupling to adenylate cyclase and subcellular distribution. Proc Natl Acad Sci USA 1986;83:9408–12.
38. Bundey RA, Nahorski SR. Homologous and heterologous uncoupling of muscarinic M3 and 1B adrenoceptors to G q/11 in SH-SY5Y human neuroblastoma cells. Br J Pharmacol 2001;134: 257–64.
39. Butcher AJ, Kong KC, Prihandoko R, Tobin AB. Physiological role of G-protein coupled receptor phosphorylation. Handb Exp Pharmacol 2012;208:79–94.
40. Tobin AB. G-protein-coupled receptor phosphorylation: where, when and by whom. Br J Pharmacol 2008;153:S167–76.
41. Hosey MM, Benovic JL, DebBurman SK, Richardson RM. Multiple mechanisms involving protein phosphorylation are linked to desensitization of muscarinic receptors. Life Sci 1995; 56:951–5.
42. Jiansong L, Busillo JM, Benovic JL. M3 muscarinic acetylcholine receptor-mediated signaling is regulated by distinct mechanisms. Mol Pharmacol 2008;74:338–47.
43. Hosey MM. Diversity of structure, signaling and regulation within the family of muscarinic cholinergic receptors. FASEB J 1992;6: 845–52.
44. Kennelly PJ, Krebs EG. Consensus sequences as substrate specificity determinants for protein kinases and protein phosphatases. J Biol Chem 1991;266:15555–8.
45. Kemp BE, Pearson RB. Protein kinase recognition sequence motifs. Trends Biochem Sci 1990;16:342–6.
46. Rovati GE, Nicosia S. Lower efficacy: interaction with an inhibitory or partial agonism? TIPS 1994;15:140–4.
47. Michel AD, Stefanich E, Whiting RL. Direct labeling of rat M3-muscarinic receptors by [^{3}H]-4DAMP. Eur J Pharmacol 1989;166: 459–66.
48. Dowling MR, Willets JM, Budd DC, et al. A single point mutation (N514Y) in the human M3 muscarinic acetylcholine receptor reveals differences in the properties of antagonists: evidence for differential inverse agonism. JPET 2006;317:1134–42.
49. Cohen P. The structure and regulation of protein phosphatases. Annu Rev Biochem 1989;58:453–508.
50. Bialojan C, Takai A. Inhibitory effect of a marine-sponge toxin, okadaic acid, on protein phosphatases: specificity and kinetics. Biochem J 1988;256:283–90.
51. Krause M, Pedarzani P. A protein phosphatase is involved in the cholinergic suppression of the Ca^{2+}-activated K^+ current sIAHP in hippocampal pyramidal neurons. Neuropharmacology 2000;39: 1274–83.
52. Herzig S, Meier A, Pfeiffer M, Neumann J. Stimulation of protein phosphatases as a mechanism of the muscarinic-receptor-mediated inhibition of cardiac L-type Ca2+ channels. Plugers Arch 1995; 429:531–8.
53. Wu G, Bogatkevich GS, Mukhin YV, et al. Identification of G binding sites in the third intracellular loop of the M3-muscarinic receptor and their role in receptor regulation. J Biol Chem 2000; 275:9026–34.
54. Simon V, Guidry J, Gettys TW, et al. The proto-oncogene SET interacts with muscarinic receptors and attenuates receptor signaling. J Biol Chem 2006;281:40310–20.
55. Borroto-Escuela DO, Correia PA, Romero-Fernandez W, et al. Muscarinic receptor family interacting proteins: role in receptor function. J Neurosci Methods 2011;195:161–9.
56. Pitcher JA, Payne ES, Csortos C, et al. The G-protein-coupled receptor phosphatase: a protein phosphatase type 2A with a distinct subcellular distribution and substrate specificity. Proc Natl Acad Sci USA 1995;92:8343–7.
57. Simon V, Oner SS, Cohen-Tannoudji J, et al. Influence of the accessory protein SET on M3 muscarinic receptor phosphorylation and G protein coupling. Mol Pharmacol 2012;82:17–26.
58. Schmidt C, Li B, Bloodworth L, et al. Random mutagenesis of the M3 muscarinic acetylcholine receptor expressed in yeast: identification of point mutations that ''silence'' a constitutively active mutant M3 receptor and greatly impair receptor/G protein coupling. J Biol Chem 2003;278:30248–60.
59. Kistemaker LE, Oenema TA, Meurs H, Gosens R. Regulation of airway inflammation and remodeling by muscarinic receptors: perspectives on anticholinergic therapy in asthma and COPD. Life Sci 2012;91:1126–33.

Supplementary information available online