Volume 253, number 1,2, 16–22 FEB 07399 August 1989

Muscarinic agents modify kinetics properties of membrane-bound guanylyl cyclase activity

I. Lippo de Becemberg, A.E. Peña de Aguilar, I. Camarillo, R. Gonzalez de Alfonzo and M. Alfonzo

Sección de Biomembranas, Instituto de Medicina Experimental, Cátedras de Patología General y Fisiopatología, y Bioquímica, Facultad de Medicina, Universidad Central de Venezuela (UCV), Apartado 50587, Sabana Grande, Caracas, Venezuela

Received 30 May 1989

Plasma membranes from bovine tracheal smooth muscle show guanylyl cyclase activity, which can be stimulated by muscarinic agonists such carbamylcholine and oxotremorine and blocked by atropine. This stimulation was observed in the presence of 150 mM NaCl. In the absence of this salt, guanylyl cyclase activity was considerably higher but was not affected by muscarinic agonists. Carbamylcholine decreased the apparent K_m but did not change the V_{max} of this enzyme. When plasma membrane fractions were extracted with 1% octylglucoside, guanylyl cyclase activity was preserved, however the muscarinic activation was abolished, despite a muscarinic receptor capable of [^{3}H]quinuclidinylbenzilate binding being present in the extract. The detergent extraction changed the affinity of guanylyl cyclase for GTP but the Mn^{2+} kinetics was unaltered. Based on these findings and on current information in the literature, we propose that another component is required to restore the link between the muscarinic receptor and guanylyl cyclase, however the nature of this component remains to be established.

NaCl inhibition; Muscarinic receptor; Guanylyl cyclase; Enzyme-receptor complex; Plasma membrane; (Tracheal smooth muscle)

1. INTRODUCTION

Acetylcholine and other cholinergic agents are known to increase the concentration of cGMP in mammalian tissues, including several types of smooth muscle [1–5]. These agents induce smooth muscle contraction by a process which appears to be associated with elevation in cGMP concentrations, however a direct causal relationship has not been established [6–10] and the biochemical link between the muscarinic receptor and guanylyl cyclase remains unknown [11–17]. We have previously shown that in isolated plasma membrane fractions from rat liver, muscarinic agents stimulate cGMP production, an effect that is inhibited by atropine [18]. Here, we describe isolation of a plasma membrane fraction from tracheal smooth muscle that is greatly enriched in muscarinic receptor and guanylyl cyclase. This guanylyl cyclase was stimulated by muscarinic agonists and by modifications in the apparent K_m for GTP. Of particular significance is the observation that the presence of NaCl was required for stimulation of the cyclase by muscarinic agents.

Correspondence address: M. Alfonzo, Sección de Biomembranas, Instituto de Medicina Experimental, Cátedras de Patología General y Fisiopatología, y Bioquímica, Facultad de Medicina, Universidad Central de Venezuela (UCV), Apartado 50587, Sabana Grande, Caracas, Venezuela

2. MATERIALS AND METHODS

2.1. *Chemicals*

The following compounds were purchased from Sigma (St. Louis, MO). AMP, GTP, creatine phosphate, creatine phosphokinase (rabbit muscle, type I), Trizma base, DTT, Sephadex G-50, carbamylcholine, atropine sulphate, oxotremorine and hexamethonium bromide. Sucrose (Analar grade) was obtained from BDH (England). DL-Quinuclidinylbenzilate (QNB) was a gift from Dr W.E. Scott of Hoffman-La Roche, Inc. (Nutley, NJ). Octylglucoside was purchased from Calbiochem-Behring (La Jolla, CA); L-[^{3}H]QNB

Published by Elsevier Science Publishers B.V. (Biomedical Division)
00145793/89/$3.50

(35.5 Ci/mmol), cGMP radioimmunoassay and ACS from the Radiochemical Centre, Amersham (England); and Aquasol from New England Nuclear (Boston, MA).

2.2. *Preparation of plasma membrane fractions from tracheal smooth muscle*

Fractionation of bovine tracheal smooth muscle was performed as described [19,20]. The procedure is briefly summarized as follows: Bovine trachea and bronchii from 10 cows were transported on ice from the slaughterhouse to the laboratory. The thin layer of smooth muscle holding the cartilage was dissected on ice after removal of serosal, mucosal and submucosal layers. All subsequent manipulations were performed at 4°C. The smooth muscle (about 250 g) was rinsed with 20 mM Tris-HCl buffer (pH 7.2) containing 0.3 M sucrose, 0.5 mM DTT and 0.1 mM phenylmethylsulphonyl fluoride (PMSF) (ISP buffer), minced and homogenized twice with 3 vols ISP buffer/g wet wt tissue, in a Waring blender operating at full speed for 30 s with a 1 min interval on ice. The dispersed material was centrifuged at 850 × *g* for 10 min and the supernatant fraction retained. The sediment was re-extracted with 2 vols ISP buffer/g wet wt and processed as described above. The supernatant was saved and the sediment again extracted with 2 vols ISP buffer/g wet wt and filtered sequentially through 2, 4, 8 layers of cheesecloth. The filtrate was centrifuged at 1000 × *g* for 10 min to remove nuclei and cell debris, which was discarded, and the supernatant was pooled with two previous supernatant fractions. The combined extract is referred to as fraction E. It was centrifuged at 31000 × *g* for 15 min to sediment mitochondria and the resulting supernatant was centrifuged at 150000 × *g* for 1 h yielding a microsomal fraction (fraction P) and a soluble fraction. Fraction P was dispersed in 50 ml ISP buffer. 15-ml samples were fractionated on a discontinuous sucrose gradient (0.3/0.82/12.8 M) in a Beckman SW 25.2 rotor at 80000 × *g* for 1 h. Three fractions, P-1 (in the interphase between 0.3 and 0.82 M), P-2 (in the interphase between 0.82 and 1.28 M) and P-3 (at the bottom), were thus obtained. P-1 and P-2 (combined from 3 tubes) were each diluted with 80 ml of 20 mM Tris-HCl (pH 7.2), 0.5 mM DTT (I buffer) and centrifuged at 150000 × *g* for 30 min. P-1 and P-2 were separately suspended in about 10 ml of 20 mM Tris-HCl (pH 7.2) buffer containing 0.3 M sucrose and 0.5 mM DTT (IS buffer) divided into small aliquots, frozen in liquid N_2 and stored at −80°C.

Both P-1 and P-2 were enriched in muscarinic receptor and guanylyl cyclase activities. However, P1 was the most active fraction and therefore was used in most experiments.

2.3. *Enzyme assays*

5′-Nucleotidase (EC 3.1.3.5) was estimated via the procedure of Touster et al. [21], the P_i released being determined according to Fiske and Subbarow [22]. Guanylyl cyclase activity (EC 4.6.1.2) was assayed as described by Lippo de Becemberg et al. [18]. Unless indicated otherwise, the reaction mixture contained 50 mM Tris-HCl (pH 7.6), 3 mM $MnCl_2$, 50–1000 μM GTP, 154 mM NaCl, a GTP-regenerating system (5 mM creatine phosphate and 10 IU phosphocreatine kinase in 0.1% defatted bovine serum albumin), 20 μg membrane protein and other components as indicated, in a total volume of 125 μl. Incubations were initiated by addition of the enzyme preparation and continued for 5–10 min at 37°C. Reactions were terminated by addition of 10 μl of 167 mM EDTA-Tris (pH 7.5), followed by heating for 3 min in a boiling water bath, and cooling on ice. Samples incubated without enzyme served as controls. cGMP was determined by radioimmunoassay in 50–100 μl supernatant of the reaction mixture obtained after centrifugation at 12000 × *g* for 3 min at 4°C. Radioactivity was determined by liquid scintillation spectrometry using ACS as scintillation fluid.

2.4. *[^{3}H]QNB-binding assay*

The [^{3}H]QNB-binding assay [20] was started by adding the protein fraction (2–10 μg protein in I buffer) to 1.5-ml Eppendorf tubes containing 66 mM Tris-HCl (pH 7.8), 154 mM NaCl, L-[^{3}H]QNB (188–625 pM) to a final volume of 60 μl. After 30 min incubation at 37°C, the reaction was terminated by transferring 50 μl incubation mixture to a pre-centrifuged Sephadex G-50 tuberculin syringe equilibrated with 0.25 M sucrose–5 mM Tris-HCl (pH 8.0) to remove unbound [^{3}H]QNB. The syringe and contents were immediately centrifuged at 700 × *g* for 1.5 min [23]. The void volume containing bound [^{3}H]QNB was mixed with deionized water and transferred to vials containing 10 ml Aquasol. Radioactivity was determined by liquid scintillation counting with an efficiency of 40%. Specific binding was determined by subtracting nonspecific binding (measured in the presence of 1 μM atropine sulfate) from total binding (evaluated in the absence of atropine) [20,24].

2.5. *Solubilization of plasma membranes with octylglucoside*

Fraction P-1 containing (3 mg/ml) was diluted with 80 vols I buffer at 4°C. Plasma membranes were collected by centrifugation at 150000 × *g* for 30 min and dispersed into 1/2 vol. IS buffer (washed P-1). Washed P-1 (1–3 mg/ml) was extracted with 1% octylglucoside at 4°C with constant stirring for 30 min as in [20]. Insoluble material was removed by centrifugation at 200000 × *g* for 30 min. Immediately thereafter, the clear supernatant was separated and applied to a pre-packed Sephadex G-50 column previously equilibrated with IS buffer as described above to remove octylglucoside. After centrifugation, the void volume contained very little OG and almost all of the membrane protein and was designated the 1% OG fraction.

Protein concentrations were determined by the method of Bensadoun and Weinstein [25] with bovine serum albumin as standard.

3. RESULTS

3.1. *Characterization of P-1 plasma membranes*

The enzymatic activities of guanylyl cyclase and 5′-nucleotidase (a marker for plasma membranes) of fraction E and P-1 are listed in table 1. The specific activities of 5′-nucleotidase and guanylyl cyclase of P-1 were 7.3- and 4.55-fold greater than in fraction E. Moreover, [^{3}H]QNB-binding activity (a measure of muscarinic receptor) was 7-fold greater in P-1 than in fraction E. Competition experiments on [^{3}H]QNB binding in the presence of muscarinic antagonists and agonists in P-1 have

Table 1

Specific activities of guanylyl cyclase, 5'-nucleotidase and L-[^{3}H]QNB binding in extract (E) and plasma membrane subfraction (P-1) from bovine tracheal smooth muscle

	Extract (E)	P-1	Purification
Guanylyl cyclase	17.05 ± 1.09	77.63 ± 2.17	4.55
5'-Nucleotidase	7.32 ± 1.15	53.60 ± 7.25	7.30
L-[^{3}H]QNB binding	200.86 ± 7.86	1568.57 ± 138.46	7.31

Guanylyl cyclase activity was assayed for 10 min at 37°C in the presence of 1 mM GTP. L-[^{3}H]QNB binding activity was assayed in the presence of 625 pM L-[^{3}H]QNB. Other details as described in the text. The specific activity of guanylyl cyclase is expressed as pmol cGMP/min per mg protein; 5'-nucleotidase as μmol P_i released/h per mg protein; and L-[^{3}H]QNB binding as fmol L-[^{3}H]QNB bound/30 min per mg protein. Data are means ± SE of duplicate determinations from 5 separate experiments

been previously reported [20] and the results were similar to those of other muscarinic receptor preparations [24,26,27].

3.2. *Cholinergic activation of plasma membrane guanylyl cyclase*

Guanylyl cyclase activity of P-1 was measured in the presence of increasing concentrations of GTP (fig.1). The activity curve was sigmoidal, reaching V_{max} values at about 1000 μM GTP. In the presence of carbamylcholine (CC), activation of the guanylyl cyclase was observed, which was apparent within the range 100–400 μM GTP. These data were linearized using the curve-fitting procedure for a Lineweaver-Burk plot for allosteric enzymes [28] as shown in inset A. Hill plots of these data are shown in inset B. The apparent K_m, V_{max} and n_H values were estimated by these curve-fitting procedures. Basal activity showed an apparent K_m of 574 ± 43 μM; when carbamylcholine was increased from 1×10^{-9} to 1×10^{-8} M, there was a decline in the apparent K_m values for GTP to 465 ± 32 and 365 ± 25 μM, respectively, indicating increased affinity of the enzyme for its substrate. These modifications in the apparent K_m were not accompanied by any significant changes in V_{max}, which had the following values [(in pmol cGMP/min per mg protein) basal, 171 ± 27; 1×10^{-9} M CC, 145 ± 15: 1×10^{-8} M CC, 156 ± 18]. Moreover, no change in cooperativity was found

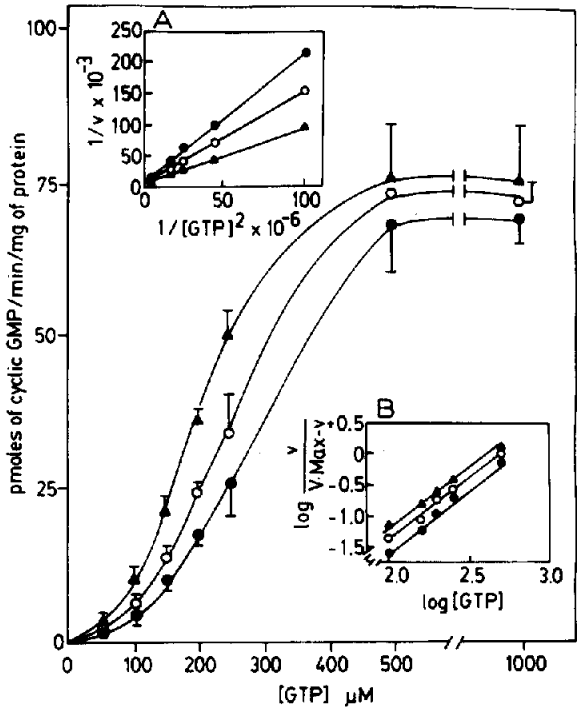

Fig.1. Effects of 1×10^{-9} M (○—○) and 1×10^{-8} M carbamylcholine (▲—▲), on basal (●—●) guanylyl cyclase from plasma membrane fraction P-1. Guanylyl cyclase activity was assayed at 37°C for 5 min in the presence of different GTP concentrations (50–1000 μM). Other details as in section 2. (Inset A) Data linearized using a Lineweaver-Burk curve-fitting procedure for allosteric enzymes [28] to calculate the apparent K_m and V_{max}. (Inset B) Hill plots of data in this figure and n_H calculated as described in section 2. Data are means ± SE of duplicate determinations from six separate experiments.

because the n_H values under all experimental conditions described here remain around 2.0 ± 0.1. In this sense, this particulate guanylyl cyclase behaved as a K-type allosteric enzyme [28]. It is important to emphasize the fact that plotting of $1/v$ vs $1/[GTP]^2$ was the best-fitting procedure for linearization of our experimental data and calculation of apparent V_{max} values, required for further determination of the values of n_H and apparent K_m. The effect of carbamylcholine at concentrations varying over a wide range (1×10^{-10}–1×10^{-5} M) was studied at fixed GTP concentration (200 μM). At low agonist concentration, dose-dependent activation was observed with maximal stimulation at 1×10^{-8}–1×10^{-7} M (fig.2). Another cholinergic agonist, oxotremorine, reacted similarly but was less effective (not

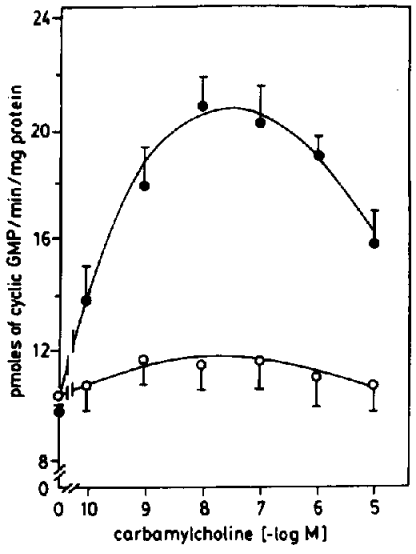

Fig.2. Effects of carbamylcholine (●—●) on guanylyl cyclase from plasma membrane (P-1). Effect of 1×10^{-6} M atropine (○—○) in the presence of carbamylcholine. Enzymatic activity was assayed in the presence of 200 μM GTP. Further details given in the text. Data are means ± SE of duplicate determinations from four separate experiments.

shown). Activation by either drug was blocked by 1 μM atropine.

3.3. *Effect of NaCl on stimulation of guanylyl cyclase by carbamylcholine*

Guanylyl cyclase in P-1 was inhibited at concentrations of NaCl or KCl above 100 mM as shown in fig.3. It should be borne in mind that this inhibition was observed only at low GTP levels (50–250 μM), and not at higher concentration (>500 μM). The maximal positive cooperativity of guanylyl cyclase activity to GTP was also only observed in the presence of 150 mM NaCl (not shown). Most significant was the stimulation of guanylyl cyclase by muscarinic agonists in a range from −9 to −8 (log M) which was only observed in the presence of NaCl (fig.4). Significantly, NaCl inhibition and muscarinic activation of guanylyl cyclase occur at intracellular levels of GTP (50–250 μM).

3.4. *Solubilization of guanylyl cyclase with octylglucoside*

In order to remove trapped soluble guanyl cyclase activity, P-1 was subjected to osmotic

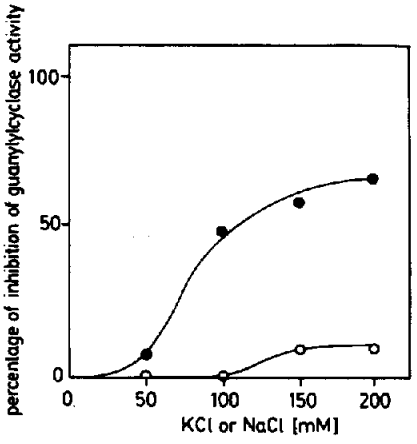

Fig.3. Effect of NaCl or KCl on membrane-bound and 1% octylglucoside-solubilized (OG-GC) guanylyl cyclase activities. Membrane-bound (●) and 1% OG-GC (○) enzyme assayed in the presence of 200 μM GTP and 3 mM Mn^{2+} as described in the text. Data are means of duplicate determinations from three separate experiments.

shock and extracted with 1% octylglucoside as described in section 2. The 1% OG solubilized guanylyl cyclase (OG-GC) activity was assayed at increasing concentrations of GTP and did not show the cooperativity described for native membrane-bound guanylyl cyclase (native GC) activity. In addition, the 1% OG-solubilized enzyme

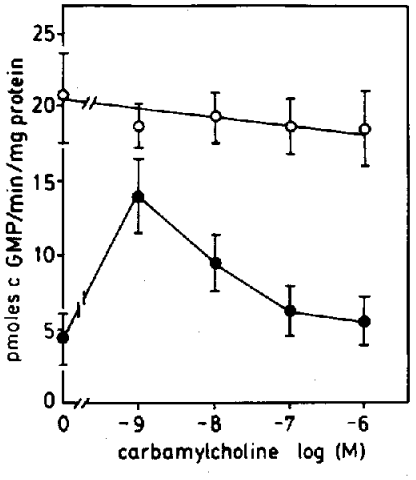

Fig.4. Effect of carbamylcholine (from −9 to −6 log *M*) on membrane-bound guanylyl cyclase activity in the presence of 154 mM NaCl (●—●) and without NaCl (○—○). Guanylyl cyclase was assayed at 200 μM GTP and 3 mM Mn^{2+} as described in section 2. Data are means ± SE of duplicate determinations from four separate experiments.

was insensitive to muscarinic agonists (1×10^{-8} M CC). The Hill coefficient was evaluated as n_H = 2.05 ± 0.05 for native GC activity, whereas the corresponding value for 1% OG-GC was n_H = 1.21 0.07 (not shown).

An interesting finding was that the activity of the extract was insensitive to NaCl or KCl, in contrast to the native GC shown in fig.3. The kinetic parameters (apparent K_m, V_{max} and n_H = 2.0) for Mn^{2+} in the cases of 1% OG-GC and native GC remained unchanged under conditions of saturating amounts of 1 mM GTP (not shown). It must be pointed out that the positive cooperativity to Mn^{2+} displayed by the native enzyme is the same in the OG-GC.

4. DISCUSSION

4.1. *Cooperativity studies with particulate guanylyl cyclase*

Kinetic analyses of particulate guanylyl cyclase suggest complex mechanisms for catalytic regulation. Curvilinear plots of the kinetics show positive cooperativity to GTP [29,30], which has been described for several mammalian particulate guanylyl cyclase preparations [31–35]. This behaviour can be altered by different treatments. Thus, detergent extraction of the particulate enzyme from rat lung [35] and mouse mammary gland ([36] and this paper) usually yields a solubilized enzyme exhibiting classical Michaelis-Menten kinetics with Hill coefficients of about 1.0. Also, proteolytic treatment of the particulate guanylyl cyclase yields a 'solubilized' enzyme that displays typical linear kinetic behaviour with respect to GTP [37]. Thus, positive cooperativity appears to be a characteristic of guanylyl cyclase in its native membrane environment. It was shown recently that highly purified particulate guanylyl cyclase from sea urchin spermatozoa retained positive cooperativity kinetics [38]. Such behaviour could be subsequently converted to that for Michaelis-Menten kinetics by dephosphorylation. These data suggest that the cooperative interactions of GTP-binding sites are regulated by the phosphorylation state of the enzyme in this biological system. The role of phospho/dephosphorylation mechanisms in the regulation of mammalian particulate guanylyl cyclase remains to be studied.

4.2. *Effect of NaCl and KCl on particulate guanylyl cyclase*

Membrane-bound guanylyl cyclase is inhibited by chloride salts at low GTP concentrations (50–250 μM), however, at higher levels of the nucleotide (>500 μM), inhibition by such salts does not occur (not shown). The salt effect on basal enzyme activity may be explained by the existence of an 'anion-binding site' on the structure of the guanylyl cyclase, where anions are capable of interacting and, thereby, regulating the catalytic site. This possibility seems to be unlikely, since the 1% OG solubilized enzyme is not modified by the salts; however, one cannot dismiss the possibility that the solubilization procedure may alter the enzyme structure masking the anion-binding site. Alternatively, the salt effects may be mediated, in part, by activation of G-proteins. Such heterotrimers may be associated with the particulate guanylyl cyclase and act as modulators in analogy with the hormone-stimulated adenylyl cyclase [39]. Purified G-proteins (G_o, G_s) are activated by physiological concentrations of chloride without subsequent hydrolysis of GTP in the absence of Mg^{2+} [40]. The latter experimental conditions are very similar to ours. In this sense, G_s has been postulated to be responsible for chloride activation of adenylyl cyclase via this mechanism [41–43]. Nevertheless, more research should be performed using highly purified G-proteins in order to elucidate more fully this (anion?) chloride inhibition of particulate guanylyl cyclase.

4.3. *Muscarinic activation of membrane-bound guanylyl cyclase*

Muscarinic cholinergic agents have been shown to increase cGMP levels in different types of smooth muscle [3–5,44,45], inducing contraction that is dependent on extracellular calcium [5]. In several tissues, guanylyl cyclase is present in both soluble and particulate forms [29]. Particulate guanylyl cyclase [13,18], but not the soluble form [12], is stimulated by muscarinic agonists. Here, we have presented additional evidence indicating that such muscarinic activation appears to operate via modifications in the K_m increasing the affinity of the enzyme for GTP at intracellular nucleotide concentrations. However, muscarinic agonists exert biphasic effects on the particulate guanylyl cyclase. Activation of this membrane-bound en-

zyme takes place at low agonist concentration (1×10^{-9}–1×10^{-7} M), which may be explained on the basis of activation of high- (H) or super-high- (SH) affinity muscarinic receptors [46]. However, a decrease in activation of the particulate form at high agonist concentrations ($1 \times 10^{-7} \times 10^{-5}$ M) is observed, which might result from activation of a low-affinity- (L) muscarinic receptor population [46] or a desensitization process [47]. Our results indicate that muscarinic agents are able to release the membrane-bound enzyme from inhibition imposed by Cl^- via an unknown mechanism. This activation mediated by muscarinic agents must arise from muscarinic receptors on the structure of the plasma membrane, which can interact with the guanylyl cyclase system via the following coupling mechanisms: Direct interactions between muscarinic receptor and guanylyl cyclase in the same fashion as described for ANF (atrial natriuretic factor)-stimulated guanylyl cyclase [48,49]. Interactions of muscarinic receptors with the particulate enzyme through a coupling process mediated by G-proteins as described for G_i, which has been reported to be directly involved in the transducing mechanism of muscarinic inhibition of adenylyl cyclase [50,51]. However, another type of indirect interaction may be possible through products associated with muscarinic receptor activation such as those from PIP_2 metabolism [52,53], arachidonic acid-related compounds [45,54] and lysophosphatides [55,56]. Arachidonic acid metabolites [54,57] and lysophosphatides [55,56] have been postulated to be involved in activation of particulate guanyl cyclase; however, the activation process in these cases appears to be a consequence of the use of high concentrations of lysophosphatides and arachidonate-related compounds, which when present at such levels may disrupt the membrane structure and induce nonspecific detergent-like activation of the particulate form.

Thus far, precise details at the molecular level of the mechanism of muscarinic activation of membrane-bound guanylyl cyclase remain to be determined, further work being required in order to elucidate this problem.

Acknowledgements: We thank Mrs V. Herrera for technical assistance, and Dr W. Ghiorse and Professor E. Racker for critical reading of the manuscript. This work was supported by grants CONICIT S1-1933 and CDCH M10.21-87 (to I.L.B.), M10.4.81 (to M.A.) and M10.6.81 from Consejo de Desarrollo Científico y Humanístico de la Universidad Central de Venezuela (UCV). A.E.P.A. was a Graduate Student at Curso de Postgrado en Ciencias Fisiologicas-UCV supported by Universidad de Oriente (UDO).

REFERENCES

[1] Ferrendelli, J.A., Steiner, A.L., McDougal, D.B. and Kipnis, D.M. (1970) Biochem. Biophys. Res. Commun. 41, 1061–1067.
[2] George, W.J., Polson, J.B., O'Toole, A.G. and Golberg, N.D. (1970) Proc. Natl. Acad. Sci. USA 66, 398–403.
[3] Lee, T.P., Kuo, J.F. and Greengard, P. (1972) Proc. Natl. Acad. Sci. USA 69, 3287–3291.
[4] Murad, F. and Kimura, H. (1974) Biochim. Biophys. Acta 343, 275–286.
[5] Schultz, G., Hardman, J.G., Schultz, K., Barid, C.E. and Sutherland, E.N. (1973) Proc. Natl. Acad. Sci. USA 70, 3889–3893.
[6] Diamond, J. and Hartle, D.K. (1976) J. Cyclic Nucl. Res. 2, 179–188.
[7] Ohkubo, H., Takayanagi, I. and Takagi, K. (1976) Jap. J. Pharmacol. 26, 65–71.
[8] Leiber, D., Vesin, M.F. and Harbon, S. (1978) FEBS Lett. 86, 183–187.
[9] Axelsson, K.L., Anderson, R.G.G. and Wikberg, J.E.S. (1980) Acta Pharmacol. Toxicol. 47, 328–334.
[10] Fiscus, R.R., Torphy, T.J. and Mayer, S.E. (1984) Biochim. Biophys. Acta 805, 382–392.
[11] Howell, S.L. and Montague, W. (1974) Biochem. J. 142, 379–384.
[12] Limbird, L.E. and Lefkowitz, R.J. (1975) Biochim. Biophys. Acta 377, 186–196.
[13] St. Louis, P.J. and Sulakhe, P.V. (1976) Biochem. J. 158, 535–541.
[14] Strange, P.G., Birdsall, N.J.M. and Burgen, A.S.V. (1977) Biochem. Soc. Trans. 5, 189–191.
[15] Coult, D.B. and Howells, D.J. (1979) Biochem. Pharmacol. 28, 2673–2675.
[16] Hisayama, T. and Takayanagy, I. (1981) J. Pharm. Dyn. 4, 738–741.
[17] Takayanagy, I., Hisayama, T. and Kotsugai, T. (1981) Jap. J. Pharmacol. 31, 831–834.
[18] Lippo de Becemberg, I., Herrera, V., Perez-Ayuso, E. and Alfonzo, M. (1982) FEBS Lett. 137, 303–306.
[19] Alfonzo, M., Adjounian, H. and Lippo de Becemberg, I. (1980) Acta Cient. Venez. 31, 18 (Abstr.).
[20] Becemberg, I.L., Ponte-Sucre, A. and Alfonzo, M. (1987) Arch. Venez. Farm. Terap. 5, 244–256.
[21] Touster, O., Aronson, N.N., Dulaney, J.T. and Hendrickson, H. (1970) J. Cell Biol. 47, 604–617.
[22] Fiske, C.H. and Subbarow, Y. (1925) J. Biol. Chem. 66, 375–400.
[23] Penefsky, H.S. (1979) Methods Enzymol. 54, 527–530.
[24] Fields, J.Z., Roeske, W.R., Morkin, E. and Yamamura, H.I. (1978) J. Biol. Chem. 253, 3251–3258.
[25] Bensadoun, A. and Weinstein, D. (1976) Anal. Biochem. 70, 241–250.

[26] Marquardt, D.L., Motulsky, H.J. and Wasserman, S.I. (1982) J. Appl. Physiol. Respir. Environ. Exercise Physiol. 53, 731–736.
[27] Murlas, C., Nadel, J.A. and Roberts, J.M. (1982) J. Appl. Physiol. Respir. Environ. Exercise Physiol. 52, 1084–1091.
[28] Segel, I.H. (1975) in: Enzyme Kinetics, pp.365, 429, Wiley, New York.
[29] Waldman, S.A. and Murad, F. (1987) Pharmacol. Rev. 39, 163–196.
[30] Chrisman, T.D., Garbers, D.L., Parks, M.A. and Hardman, J.C. (1975) J. Biol. Chem. 250, 374–381.
[31] Siegel, M.I., Puca, G.A. and Cuatrecasas, P. (1976) Biochim. Biophys. Acta 438, 310–323.
[32] Kimura, H. and Murad, F. (1974) J. Biol. Chem. 249, 6910–6916.
[33] Sulakhe, S.J., Leung, N.L. and Sulakhe, P.V. (1976) Biochem. J. 157, 713–719.
[34] Waldman, S.A., Lewicki, J.A., Chang, L.Y. and Murad, F. (1983) Mol. Cell Biochem. 57, 155–166.
[35] Rillema, J. and Linebaugh, Z.E. (1978) Horm. Metab. Res. 10, 331–336.
[36] Waldman, S.A., Lewicki, J.A., Chang, L.Y. and Murad, F. (1982) J. Cyclic Nucleotide Res. 8, 359–370.
[37] Ben Salah, A., Ebrentz-L'Homme, C., Lacombe, M. and Hanoune, J. (1983) J. Biol. Chem. 258, 887–893.
[38] Ramarao, C.S. and Garbers, D.L. (1988) J. Biol. Chem. 263, 1524–1529.
[39] Northup, J.K. (1985) in: Molecular Mechanisms of Transmembrane Signalling (Cohen, P. and Houslay, M.D. eds) pp.91–116, Elsevier, Amsterdam.
[40] Higashijima, T., Fergusson, K.M. and Sternweis, P.C. (1987) J. Biol. Chem. 262, 3597–3602.
[41] Johnson, R.A., Pilkis, S.J. and Hamet, P. (1975) J. Biol. Chem. 250, 6599–6607.
[42] Svoboda, M. and Christophe, J. (1978) FEBS Lett. 86, 230–234.
[43] Roy, C., Lebars, N.C. and Jard, S. (1977) Eur. J. Biochem. 78, 325–332.
[44] Nilsson, K.B. and Andersson, R.G.G. (1977) Acta Physiol. Scand. 99, 246–253.
[45] Lieber, D. and Harbon, S. (1982) Mol. Pharmacol. 21, 654–663.
[46] Birdsall, N.J.M. and Hulme, E.C. (1983) Trends Biochem. Sci. 4, 459–463.
[47] Sokolovsky, M. and Bartfai, T. (1981) Trends Biochem. Sci. 6, 303–305.
[48] Nambi, P., Aiyar, N.V. and Sharma, R.K. (1982) FEBS Lett. 140, 98–102.
[49] Kuno, T., Andresen, J.W., Kamasaki, Y., Waldman, S.A., Chang, L.Y., Saheki, S., Leitman, D.C., Nakane, M. and Murad, F. (1986) J. Biol. Chem. 261, 5817–5823.
[50] Gilman, G.A. (1987) Annu. Rev. Biochem. 56, 615–649.
[51] Mattera, R., Pitts, B.J.R., Entman, M.L. and Birnbaumer, L. (1985) J. Biol. Chem. 260, 7410–7421.
[52] Hepler, J.R., Hughes, A.R. and Harden, T.K. (1987) Biochem. J. 247, 793–796.
[53] Meurs, H., Roffel, A.F., Posteman, J.B., Timmermans, A., Elzinga, C.R.S., Kauffman, H.F. and Zaagsma, J. (1988) Eur. J. Pharmacol. 156, 271–274.
[54] Wallach, D. and Pastan, I. (1976) J. Biol. Chem. 251, 5802–5809.
[55] White, A.A. and Lad, P.J. (1975) Fed. Proc. 34, 232–237.
[56] Shier, W.T., Baldwin, J.H., Nielsen-Hamilton, M., Hamilton, R.T. and Thanassi, N.M. (1976) Proc. Natl. Acad. Sci. USA 73, 1586–1590.
[57] Gruetter, D.Y. and Ignarro, L.J. (1979) Prostaglandins 18, 541–556.