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Effects of phorbol ester on carbachol-induced contraction in bovine ciliary muscle: possible involvement of protein kinase C

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Abstract

The aim of the research was to characterize muscarinic receptors of bovine ciliary muscle and to investigate the desensitization process. The role of protein kinase C was analyzed. The results show that muscarinic receptors of bovine ciliary muscle have the pharmacological characteristics of the M_3 subtype. Acute exposure to phorbol esters (1 μM phorbol 12,13-dibutyrate, PDB, or 0.1 μM phorbol 12-myristate 13-acetate, PMA, for 15 and 5 min, respectively) resulted in antagonism of muscarinic receptor-mediated contraction. Long-term pretreatment (18 h) with PMA to down-regulate protein kinase C resulted in potentiation of carbachol-induced contraction, reduction of agonist-induced desensitization and loss of phorbol ester-induced desensitization. Staurosporine (3 μM) and H_7 [1-(5-isoquinolinesulfonyl)-2-methyl-piperazine] (1 μM), protein kinase C inhibitors, produced a significant potentiation of the contractile effect of carbachol, reduced the desensitization produced by repeated addition of carbachol and suppressed that induced by phorbol esters. In vitro incubation with carbachol, PDB or PMA did not cause any modification of the binding of labeled [^{3}H]quinuclidinyl benzilate. In vitro incubation with PDB and PMA produced, as expected, a significant translocation of protein kinase C from the cytosol to the membrane. The incubation of the ciliary muscle with carbachol, using the protocol of exposure that induced maximal desensitization of contractile responses, produced a significant redistribution of the enzyme from the cytosol to the membrane. These findings suggest that agonist-induced modulation of functional cholinergic sensitivity in ciliary muscle is correlated, at least partially, to the translocation of protein kinase C from the cytosol to the membrane. The desensitization by phorbol esters is completely due to protein kinase C activation; during the desensitization process, direct modification of the density and affinity of muscarinic receptors is not involved. © 1997 Elsevier Science B.V.

Keywords: Ciliary muscle, bovine; Muscarinic receptor; Desensitization; Protein kinase C

1. Introduction

Muscarinic receptors have been established as stimulators of phosphatidylinositol bisphosphate (PIP_2) turnover, which leads to the formation of inositol trisphosphate and diacylglycerol (Berridge, 1987; Fisher and Agranoff, 1987). Like other receptor systems, muscarinic receptors undergo agonist-induced desensitization. The precise molecular and biochemical events underlying the desensitization process are still unclear; however, there is a good correlation between the efficacy of muscarinic agonists to stimulate PIP_2 hydrolysis and their ability to induce desensitization of receptor-mediated responses (Lippas et al., 1986). The fact that the breakdown of PIP_2 generates diacylglycerol, an endogenous activator of protein kinase C, has suggested an involvement of the enzyme in the process of desensitization of muscarinic receptors.

The tumor-promoting phorbol esters, which mimic the effect of endogenously produced diacylglycerol in activating protein kinase C (Castagna et al., 1982), have provided an important tool to investigate the role of protein kinase C in the regulation of muscarinic receptor function. Short-term activation of protein kinase C with phorbol esters results in the inhibition of muscarinic receptor agonist-induced phosphatidylinositol 4,5-bisphosphate hydrolysis and Ca^{2+} mobilization in different cell types (Labarca et al., 1984; Orellana et al., 1985; Vicentini et al., 1985; Xu and Chuang, 1987; Berridge, 1987; Abdel-Latif, 1989) and desensitization of muscarinic receptor-mediated responses.

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These observations suggest that protein kinase C may play a key role in the mechanisms of feedback regulation of the receptor-stimulated IP_3/Ca^{2+} pathway. This point is also supported by the observation that in some tissues the down-regulation of protein kinase C by means of chronic exposure to phorbol esters results in an increased receptor-stimulated PIP_2 hydrolysis, paralleled by a loss of phorbol ester responsiveness (Brown et al., 1987; Hepler et al., 1988; Fisher, 1995).

Phorbol esters reduce the affinity of muscarinic receptors for agonists, but not for antagonists (Serra et al., 1986). Whether these agents also cause internalization of muscarinic receptors is still controversial (Liles et al., 1986; Abdallah and El-Fakahany, 1991). Haga et al. (1990) have shown that porcine cerebral cortex purified muscarinic receptors are phosphorylated by protein kinase C purified from the same tissue, thereby contributing to desensitization.

The desensitization of muscarinic receptors that modulate the contractility of ciliary muscle is thought to be involved in the loss of outflow facility and accommodative responses, both mediated by ciliary muscle contraction, after prolonged topical treatment with cholinergic drugs in long-term therapy of glaucoma (Armaly et al., 1973).

Along this line, the purpose of the present study was to characterize, by means of functional and biochemical experiments, muscarinic receptors in bovine ciliary muscle and to investigate agonist-induced desensitization of muscarinic receptor responses in this tissue. In particular, the role of the diacylglycerol-protein kinase C pathway in the modulation of the contractility of the bovine ciliary muscle in response to muscarinic receptor stimulation and the possible involvement of protein kinase C in the mechanism of desensitization of muscarinic receptors induced by either receptor agonists or phorbol esters were investigated. An attempt was made to establish whether the desensitization induced by muscarinic receptor agonists, as well as by phorbol esters, is correlated with changes in the density and/or affinity of muscarinic receptors as well as in the distribution of protein kinase C in cytosolic and particulate fractions.

The presence and the distribution of protein kinase C in cytosolic and particulate fractions prepared from bovine ciliary muscle were investigated under basal and stimulated (carbachol and phorbol esters) conditions by means of two different approaches: the measurement of the binding of labeled phorbol 12,13-dibutyrate, as index of tissue enzyme molecules availability, and the in vitro phosphorylation of the exogenous protein kinase C substrate, histone H_1, as probe for functional activity.

2. Materials and methods

Bovine eyes obtained from a slaughterhouse were enucleated within 5–10 min after death, placed immediately in an oxygenated modified Krebs solution at 4°C, and used for study within 30 min. The globe was opened equatorially, the zonule was cut and the lens was removed. Ciliary muscle was isolated under a binocular microscope. The ciliary body was dissected from the scleral spur, lens and choroid as previously described (Lograno and Reibaldi, 1986).

After the isolation, the tissues were immediately used or frozen on dry ice and stored at −80°C until assayed.

A series of tissues were pretreated either with phorbol esters (1 μM PDB for 15 min, 0.1 μM PMA for 5 min, 0.1 μM PMA for 18 h at 4°C) or carbachol (0.1 μM for 5 times, each of 30 s, every 3 min), and then used or frozen on dry ice and stored at −80°C until assayed.

2.1. Functional experiments

To investigate mechanical properties, ciliary muscle strips were set up under a tension of 200 mg in a 20 ml organ bath containing Krebs solution at 37°C, oxygenated with 95% O_2 and 5% CO_2. Contractile responses were measured isometrically by means of a force-displacement transducer (DY0, Ugo Basile, Italy), and recorded on a linear recorder coupled to a Grass polygraph. The tissues were allowed to equilibrate for 60 min before any drug addition. Atropine, pirenzepine, methoctramine, 4-diphenylacetoxy-*N*-methylpiperidine (4-DAMP), staurosporine, H_7 [1-(5-isoquinolinesulfonyl)-2-methyl-piperazine], phorbol 12,13-dibutyrate and phorbol 12-myristate 13-acetate were added to the Krebs solution to obtain the appropriate concentrations expressed as molar concentration. Muscarinic receptor antagonists were in contact with the tissues 30 min before carbachol; phorbol esters were in contact with the tissues for 15 min (short-term incubation with PDB at 37°C), 5 min (short-term incubation with PMA at 37°C) or 18 h (long term incubation with PMA at 4°C); staurosporine and H_7 were in contact with the tissues for 60 min; when more than one concentration of the antagonist was added, a control response to the agonist was recorded, in order to ensure that complete recovery from blockade had taken place.

Contractile responses are expressed as percentages of the maximal effect produced by carbachol in each control condition. In the case of overnight treatment (at 4°C) with 0.1 μM PMA (solubilized in Krebs solution), the control response was recorded from ciliary muscle strips maintained under the same conditions (at 4°C in Krebs solution) except for the presence of PMA in the incubation medium.

Differences were examined by Student's *t*-test for paired observations and were considered to be significant for $P < 0.05$.

pA_2 values were calculated from Arunlakshana and Schild (1959) plot; concentration ratios were calculated at the EC_{50} level.

The composition of the Krebs solution was as follows (mM): NaCl 136.8, KCl 5.4, $CaCl_2$ 2.7, $MgSO_4$ $7H_2O$ 0.8,

NaH_2PO_4 H_2O 1.4, $NaHCO_3$ 24, glucose 5 and Na-ascorbate 0.2 (pH 7.4).

2.2. Biochemical experiments

2.2.1. Muscarinic receptor characterization: membrane preparation and binding protocol

Tritiated quinuclidinyl benzilate ([^{3}H]quinuclidinyl benzilate, New England Nuclear, 43 Ci/mmol) was used. Ciliary muscle strips were minced with scissors and homogenized with a polytron homogenizer (setting 15; two times each of 30 s) in 20 volumes (based on the wet weight) of Na_2HPO_4/KH_2PO_4 buffer (50 mM; pH 7.4) containing 10 mM $MgCl_2$. The homogenate was filtered on a double-layer of cheese-cloth and centrifuged at $1000 \times g$ for 15 min. The supernatant was filtered and then centrifuged at $50\,000 \times g$ for 20 min at 2°C. The final pellet was resuspended with a polytron homogenizer in the incubation buffer to obtain 100 μg of protein in 200 μl homogenate. To each tube was added, in a final volume of 1 ml of Na_2HPO_4/KH_2PO_4 buffer (50 mM; pH 7.4) containing 10 mM $MgCl_2$, about 100 μg of protein suspension and increasing amounts of labeled quinuclidinyl benzilate. Specific binding was defined using 10^{-6} M atropine sulfate.

Inhibition curves were made with 300 pM [^{3}H]quinuclidinylbenzilate and 10 different concentrations of the following unlabeled drugs: atropine, pirenzepine, methoctramine and 4-DAMP. Samples were incubated at 30°C for 90 min and then filtered under vacuum through glass microfibre filters Whatman GF/B. Filters were washed three times with 4 ml of ice-cold Na_2HPO_4/KH_2PO_4 buffer and then placed in 8 ml of Formula 989 (New England Nuclear) scintillation cocktail overnight before scintillation counting.

2.2.2. Protein kinase C determination: binding protocols for particulate and cytosolic fractions

Tritiated phorbol 12,13-dibutyrate ([^{3}H]PDB; New England Nuclear, 23 Ci/mmol) was used. Ciliary muscles were minced with scissors and then homogenized with a polytron (setting 15) for 30 s in 20 volumes (based on the wet weight) of ice-cold Tris-HCl buffer (50 mM, pH 7.7) containing 100 mM NaCl, 2 mM EDTA, 5 mM EGTA. The homogenate was filtered on a double-layer of cheese-cloth and centrifuged at $1000 \times g$ for 15 min. The supernatant was filtered and then centrifuged at $48\,000 \times g$ for 20 min. The supernatant constituted the cytosolic fraction and the pellet the particulate fraction.

When appropriate, a different protocol of tissue preparation, which allowed the recovery of the whole binding activity in the particulate fraction, was used. In this case, the homogenization buffer had the following composition: 50 mM Tris-HCl, 1 mM $CaCl_2$ and 100 mM NaCl (pH 7.7). With these homogenization conditions, the binding of [^{3}H]PDB to the membrane fraction represents almost 90% of total binding sites availability (cytosolic + particulate) (Battaini et al., 1990; Niedel et al., 1983).

2.2.3. Soluble fraction

To each tube was added, in a final volume of 1 ml of 20 mM Tris-HCl containing 5 mM $CaCl_2$, 50 mM β-mercaptoethanol, 1 mg/ml bovine serum albumin and 100 μg/ml of phosphatidylserine (pH 7.5), 50 μg of protein from cytosolic fraction and various amounts of [^{3}H]PDB. Specific binding was defined with 10 μM unlabeled PDB. Following 45 min at 25°C the incubation was stopped by adding 300 μl of a 10% suspension of Whatman DE_{52} resin in 10 mM Tris-HCl buffer (pH 7.5). Samples were transferred to an ice-cold bath, kept there for 20 min and then filtered under vacuum on Whatman GF/C glass fibre filters presoaked for 30 min in 20 mM Tris-HCl buffer (pH 7.5) containing 0.5% polyethylenimine to reduce filter binding. Filters were washed with 3 ml of ice-cold 20 mM Tris-HCl buffer (pH 7.5) for three times and then placed in 8 ml of Formula 989 (New England Nuclear) scintillation cocktail overnight before scintillation counting.

2.2.4. Particulate fraction

To each tube was added, in a final volume of 1 ml of 50 mM Tris-HCl containing 100 mM NaCl and 1 mM $CaCl_2$ (pH 7.7), 50 μg of protein suspension and various concentrations of labeled PDB. Specific binding was defined using 10 μM cold PDB. Samples were incubated at 25°C for 45 min and then filtered under vacuum on Whatman GF/C glass microfibre filters presoaked for 30 min in 50 mM Tris-HCl buffer (pH 7.7) containing 0.5% polyethylenimine. Filters were washed with 3 ml of ice-cold 50 mM Tris-HCl buffer (pH 7.7) for three times. The radioactivity retained was determined as described above.

With nanomolar concentrations of free ligand, specific binding represented about 80% of total binding. Binding specificity was confirmed by competition with active (PDB and PMA) and inactive (4α-phorbol) phorbol-related compounds. Freezing the tissue on dry ice and storage at −80°C for up to 2 weeks did not affect phorbol ester binding parameters. All radioligand binding assays were performed in triplicate.

Binding parameters were calculated from saturation experiments, using the computerized program 'LIGAND' (Munson, 1983). Comparison of the binding data was made using the *F*-test comparison between models, as indicated by Munson and Rodbard (1980). Values in the text are reported as means ± S.D.

In the competition experiments, inhibition constants were determined by means of the Cheng-Prusoff equation: $K_i = IC_{50}/1 + ([C]/K_d)$, where [C] = concentration of the radioligand and K_d = affinity value.

2.2.5. Measurement of protein kinase C activity in cytosolic and particulate fractions

Kinase activity of cytosolic and particulate fractions prepared from bovine ciliary muscle was determined ac-

cording to the method of Wehner et al. (1990), with some modifications.

Ciliary muscles were minced with scissors and homogenized with a Polytron (setting 15) for 30 s in 10 volumes (based on the wet weight) of ice-cold Tris-HCl buffer (20 mM, pH 7.1) containing 2 mM EDTA, 50 mM β-mercaptoethanol, 0.2 mM phenylmethylsulfonyl fluoride, 0.32 M sucrose and 20 μg/ml leupeptin (pH 7.1). The homogenate was filtered on a double-layer of cheese-cloth and centrifuged at $1000 \times g$ for 15 min. The supernatant, after filtration through a double-layer of cheese-cloth, was centrifuged at $100\,000 \times g$ for 30 min. The resulting supernatant served as the cytosolic fraction. The pellet was washed with homogenization buffer and recentrifuged at $100\,000 \times g$ for 30 min. The resulting pellet was resuspended by sonication. Triton X-100 was added to a final concentration of 0.2%. Samples were incubated on ice for 30 min, then centrifuged at $100\,000 \times g$ for 30 min. The resulting supernatant was used as the source of particulate protein kinase C.

In order to measure protein kinase C activity, 20 μg of protein (from cytosolic or particulate fraction) was incubated at 37°C in 250 μl final volume of a buffer containing 5 μmol Tris-HCl (pH 7.5), 1.25 μmol magnesium acetate, 125 μg histone III-S; after 5 min preincubation, the reaction was started by adding 5 nmol [γ-^{32}P]ATP ($10–15 \times 10^4$ cpm/nmol) (Amersham, Arlington Heights, IL, USA, 3000 Ci/mmol). Stimulated activity was measured in the presence of 0.5 μmol $CaCl_2$ and 10 μg phosphatidylserine. Basal activity was measured in the presence of 0.5 μmol EGTA. The reaction was stopped after 5 min by 25% trichloroacetic acid precipitation and filtration through Millipore HA 0.45 μm filters under vacuum. Radioactivity retained by filters was determined by liquid scintillation using Formula 989, as described above.

The comparison between kinase activity values was made by means of the Student's *t*-test and differences were considered to be significant for $P < 0.05$.

2.2.6. Protein determination

Protein measurement was carried out by the method of Lowry et al. (1951), using bovine serum albumin as protein standard. In the experiments of protein kinase C activity determination, the method of Bradford (1976) was used.

2.3. Drugs used

Carbachol HCl, phorbol 12,13-dibutyrate (PDB), phorbol 12-myristate-13-acetate (PMA), 4α-phorbol didecanoate, staurosporine, atropine sulfate, H_7 [1-(5-isoquinolinesulfonyl)-2-methyl-piperazine], adenosine 5′-triphosphate, histone H1 III-S, phosphatidylserine, pirenzepine HCl, polyethylenimine and dimethyl sulfoxide were from Sigma (St. Louis, MO, USA). Methoctramine tetrahydrochloride and 4-DAMP methiodide were from Research Biochemicals International, Natick, MA, USA). Whatman DE 52 resin was from Carlo Erba (Milan, Italy).

All the other reagents were of analytical grade.

Stock solutions (5 mM) of phorbol esters and staurosporine were prepared in dimethyl sulfoxide (DMSO), stored at −20°C and diluted in the appropriate buffer at the time of the experiment; all samples contained the same amount of DMSO.

The experiments with phorbol esters were carried out in the dark.

3. Results

3.1. Functional experiments

The preparations of bovine ciliary muscle were allowed to equilibrate for a period of 60 min, during which they relaxed and reached a final length of about 8–10 mm. Spontaneous activity was absent. As already reported (Lograno et al., 1991), carbachol (from 1×10^{-10} M to 1×10^{-4} M) produced concentration-dependent contractile responses ($IC_{50} = 2 \times 10^{-8}$ M; maximal response achieved at 5×10^{-7} M) which were antagonized by atropine (5×10^{-9} M–5×10^{-8} M) in a competitive fashion. The concentration ratios of carbachol vs. atropine concentration resulted in a line with a slope of 0.9, from which a pA_2 value for atropine of 9.3 was calculated.

The selective antagonist of muscarinic M_3 subtype receptors, 4-DAMP (5×10^{-9} M–5×10^{-8} M), antagonized in a competitive manner the contractile responses to carbachol (Fig. 1); the concentration ratios of carbachol, when plotted as a function of 4-DAMP concentration, resulted in a line with a slope of 1.1, from which a pA_2 value of 9.8 was calculated (Fig. 1). Methoctramine (a muscarinic M_2 receptor antagonist) and pirenzepine (a muscarinic M_1 receptor antagonist) (from 5×10^{-9} M to 5×10^{-7} M) were almost completely ineffective (Fig. 2).

Repeated addition (every 3 min) of a single (submaximal) concentration of carbachol (0.1 μM) significantly desensitized the contractile responses.

PDB (1 μM) and PMA (0.1 μM), used as protein kinase C activators, incubated for 15 and 5 min, respectively, before carbachol, completely antagonized the contractile responses induced by the muscarinic agonist (number of experiments = 10); the effect was slowly reversible after washing (about 80 min). The data obtained after PMA are not shown.

Staurosporine and H_7 (3 and 1 μM respectively), perfused for 60 min before carbachol, reduced the desensitization induced by carbachol and suppressed the inhibitory effect of PDB and PMA on the carbachol response.

In some preparations, staurosporine and H_7 (from 1 to 30 μM) produced slowly developing tonic contractions that disappeared after a few minutes, although stau-

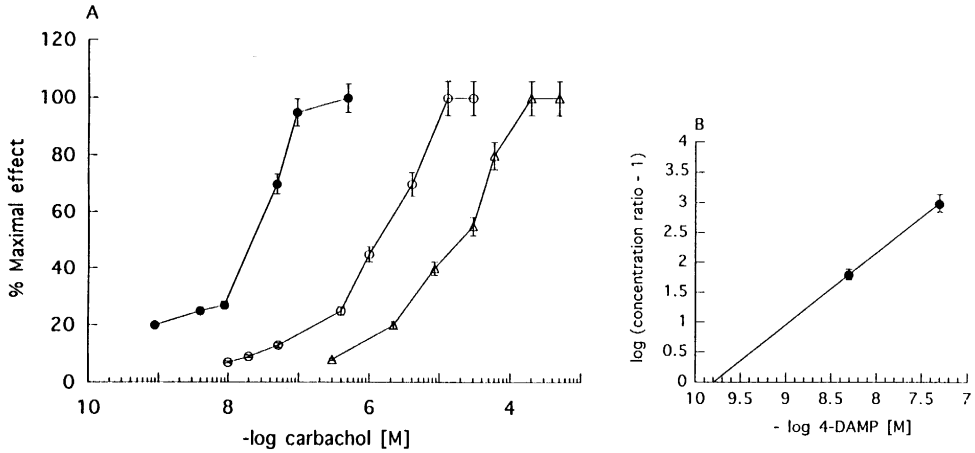

Fig. 1. Competitive antagonism by 4-DAMP of carbachol-induced contractions in bovine ciliary muscle. (A) Concentration-effect curves in the absence (●) and in the presence of 5×10^{-9} M (○) and 5×10^{-8} M (△) 4-DAMP. Each point represents the mean value with S.D. of 8 experiments ($P < 0.01$). (B) Schild plot for 4-DAMP with carbachol as agonist. Each point represents the mean value with S.D. of 8 experiments.

rosporine and H_7 were still present in the perfusion buffer (data not shown).

Overnight incubation of the ciliary muscle at 4°C in Krebs solution produced a shift to the right of the concentration-response curve for carbachol ($IC_{50} = 2 \times 10^{-7}$ M; maximal response achieved at 5×10^{-5} M), compared to the response before the 18 h incubation. The down-regulation of protein kinase C, obtained by adding 0.1 μM PMA to the incubation buffer under the same conditions (18 h at 4°C), resulted in a shift to the left of the concentration-response curve with a significant potentiation of the maximal contractile response compared to the respective control condition (18 h at 4°C in Krebs solution) (Fig. 3). Long-term exposure to PMA reduced carbachol-induced desensitization and completely suppressed that induced by short-term exposure to PDB and PMA (data not shown). At concentrations up to 10 μM, 4α-phorbol, which is unable to activate protein kinase C, was effective against carbachol-induced contractions, ruling out a possible non-specific action of the phorbol structure.

The contractile responses to carbachol were potentiated by pretreatment of the ciliary muscle preparations with the two well-known protein kinase C inhibitors, staurosporine (3 μM) and H_7 (1 μM): the concentration-response curves

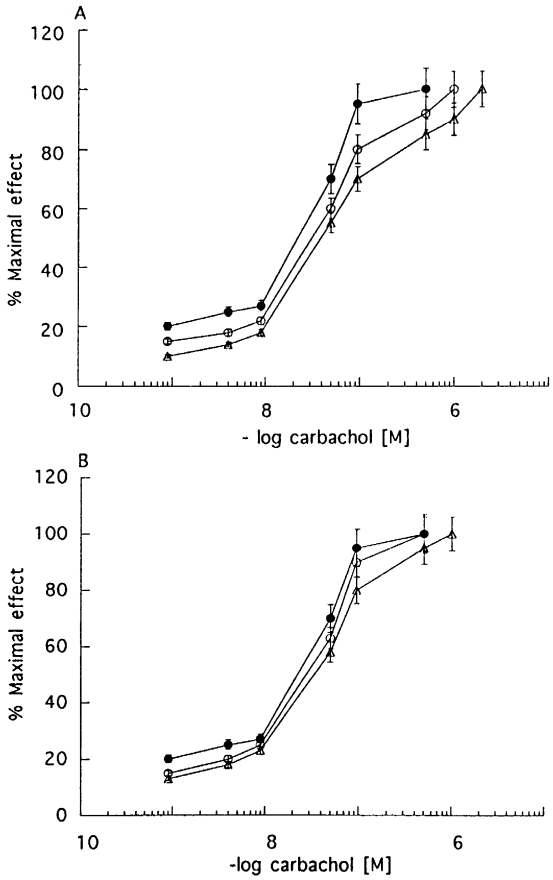

Fig. 2. Competitive antagonism by methoctramine (A) and pirenzepine (B) of carbachol-induced contractions in bovine ciliary muscle. Concentration-effect curves in the absence (●) and in the presence of 5×10^{-9} M (○) and 5×10^{-7} M (△) methoctramine or pirenzepine. Each point represents the mean value with S.D. of 7 experiments ($P > 0.05$).

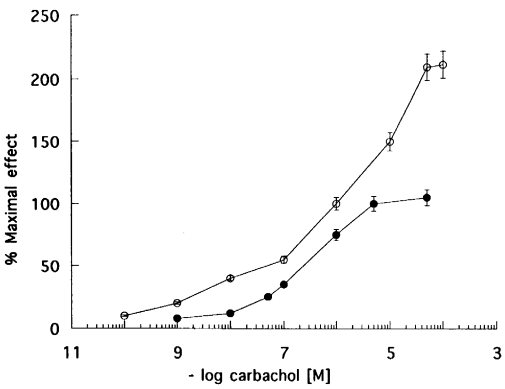

Fig. 3. Potentiation of the carbachol-induced contraction by overnight (18 h) pretreatment of the ciliary muscle with 0.1 μM PMA. Concentration-response curves before (●) and after (○) PMA treatment. Each point represents the mean value ± S.D. of three experiments ($P < 0.01$).

Table 1
Characterization of [^{3}H]quinuclidinyl benzilate binding to bovine ciliary muscle membranes, before and after in vitro incubation with 1×10^{-7} M carbachol, 1×10^{-6} M PDB and 1×10^{-7} M PMA

Treatment	B_{max} (pmol/mg protein)	K_d (nM)
Control	3.0 ± 1.6	0.020 ± 0.009
PDB	4.1 ± 0.5	0.040 ± 0.01
PMA	3.6 ± 0.8	0.032 ± 0.01
Carbachol	4.0 ± 1.2	0.028 ± 0.08

Each value represents the mean ± S.D. of five experiments using at least six concentrations of labeled quinuclidinyl benzilate with samples in triplicate.

were shifted to the left and the maximal response was significantly increased (from 100% to about 200%). The EC_{50} values for the contractile effect of carbachol in the presence of 3 μM staurosporine and 1 μM H_7 were lower (1.7×10^{-9} M and 2×10^{-9} M respectively) than those in untreated preparations (2×10^{-8} M) (number of experiments = 8).

3.2. Biochemical experiments

3.2.1. Muscarinic receptor characterization

Under equilibrium conditions, the binding of [^{3}H]quinuclidinyl benzilate to ciliary muscle membranes increased with increasing amounts of added protein and was linear at 20–200 μg of protein per tube. Saturation experiments revealed that in ciliary muscle membranes the specific binding of labeled quinuclidinyl benzilate increased with the radioligand concentration up to 2 nM. The calculated K_d and B_{max} are reported in Table 1.

Competition curves were made by using atropine (10^{-10}–10^{-6} M), pirenzepine (10^{-10}–10^{-6} M), methoctramine (10^{-10}–10^{-6} M) and 4-DAMP (10^{-10}–10^{-6} M). Atropine and 4-DAMP fully displaced the specific binding, with the following pK_i values (calculated by means of the Cheng-Prusoff equation): atropine 10.2 and 4-DAMP 10.7. Methoctramine inhibited about 30% of the binding at 10^{-6} M; pirenzepine displaced 10% of the binding only at the higher concentrations tested.

Table 2
Inhibition of tritiated PDB binding to membranes prepared from bovine ciliary muscle

Drug	pK_i (M)
Phorbol 12,13-dibutyrate	8.2
Phorbol myristate acetate	9.1
4α-Phorbol	> 5
Staurosporine	> 5
H_7	> 5
Carbachol	> 5

Radioligand binding studies were performed as described in Section 2. For inhibition experiments, a protocol of tissue preparation, which allowed the recovery of whole binding activity in the particulate fraction, was used. IC_{50} values were determined from inhibition curves using six triplicate concentrations of each inhibitor. Apparent inhibition constants, assuming competitive inhibition, were determined using the Cheng-Prusoff equation.

In vitro incubation with carbachol, PDB (1 μM for 15 min) or PMA (0.1 μM for 5 min), following the same protocols of exposure that induced contractile response desensitization, did not modify quinuclidinyl benzilate binding to ciliary muscle membranes (Table 1).

3.3. Protein kinase C characterization

3.3.1. PDB binding

The specificity of phorbol ester to displace [^{3}H]PDB binding to membranes from bovine ciliary muscle is reported in Table 2. Active phorbol esters such as PDB and PMA displaced the binding with a nanomolar K_i, while 4α-phorbol, a compound unable to activate protein kinase C, was ineffective. Drugs known to modify protein kinase C activity, but which do not directly act on phorbol ester sites, were also inactive in binding experiments.

[^{3}H]PDB binding to cytosolic and particulate fractions from bovine ciliary muscle increased with increasing

Table 3
Distribution of protein kinase C in soluble and particulate fractions prepared from bovine ciliary muscle following in vitro incubation with 1×10^{-7} M carbachol, 1×10^{-6} M PDB and 1×10^{-7} M

Treatment	Fraction	B_{max} (pmol/mg protein)	K_d (nM)	% of translocated protein kinase C
Control	Soluble	6.2 ± 0.7	12.7 ± 1.8	–
	Particulate	24.2 ± 8.2	16.8 ± 1.7	–
PDB	Soluble	n.d.	n.d.	n.d.
	Particulate	35.7 ± 3.7 [a]	17.0 ± 2.6	47.5 ± 15.0
PMA	Soluble	n.d.	n.d.	n.d.
	Particulate	38.5 ± 4.2 [a]	18.4 ± 2.5	59.0 ± 17.0
Carbachol	Soluble	1.7 ± 0.3 [b]	14.8 ± 2.2	72.5 ± 5.0
	Particulate	45.0 ± 11.2 [a]	19.0 ± 3.6	86.0 ± 45.0

The columns from left to right indicate: the treatments, the fraction of protein kinase C in the cellular membrane and cytosol, the B_{max}, which is the maximal receptor concentration/mg of protein, the K_d, which is the dissociation constant, the % values of protein kinase C translocated that have been calculated as the difference between the pmol/mg (B_{max}) of control (assumed as 100%) and the fraction of soluble and particulate for each different treatment. Values are means ± S.D. of 4 experiments, each using at least 6 different concentrations of labeled PDB with samples in triplicate. n.d. stands for not detectable. Significant differences: [a] $P < 0.01$ vs. control (particulate fraction); [b] $P < 0.01$ vs. control (soluble fraction).

amounts of added protein and was linear at 20–150 μg of protein per tube. Saturation experiments revealed that the specific binding of [^{3}H]PDB increased with the radioligand concentration up to 30 nM, both in cytosolic and in particulate fractions. The calculated K_d (nM) and B_{max} (pmol/mg protein) were as follows: cytosolic fraction K_d 13.2 ± 1.5, B_{max} 7.5 ± 0.9; particulate fraction K_d 15.2 ± 2.1, B_{max} 25.2 ± 6.5.

PDB (1 μM for 15 min) and PMA (0.1 μM) were used as protein kinase C activators; at the concentrations used in these experiments, PDB and PMA induce maximal activation of protein kinase C in various cell types (Nishizuka, 1984).

In vitro incubation of bovine ciliary muscle with 1 μM PDB or 0.1 μM PMA induced, as expected, a significant translocation of the enzyme from the cytosolic to the particulate fraction; no modification in the affinity values was observed (Table 3). Following treatment with PDB or PMA, specific binding in the cytosolic fraction was still present; however, it was so low that it became undetectable.

In vitro incubation of bovine ciliary muscle with carbachol, according to the same protocol that produced functional desensitization, induced a significant elevation in PDB binding to the membrane fraction. No significant difference was observed in K_d value. Concurrent with the elevation in membrane-associated protein kinase C, there was a significant reduction in the cytosolic fraction of the enzyme (Table 3). Control tissues were processed in the same way as treated tissue, except for the presence of carbachol or phorbol esters in the medium.

3.3.2. Kinase activity

Table 4 reports protein kinase activity in cytosolic and membrane fractions from ciliary muscle under control conditions and after in vitro treatment with PDB, PMA or carbachol. PDB and PMA treatment produced a significant increase (+48% and +65% respectively) in phosphorylating activity in the particulate fraction. Under this condition, the activity of the cytosolic fraction was not detectable.

Table 4
Effect of in vitro incubation with PDB, PMA and carbachol on cytosolic and membrane-associated protein kinase C activity in bovine ciliary muscle

Treatment	Protein kinase C activity (nmol/min per mg protein)	
	Particulate	Soluble
Control	2.9 ± 0.50	1.3 ± 0.2
PDB	4.3 ± 0.52 [a]	n.d.
PMA	4.8 ± 0.22 [a]	n.d.
Carbachol	5.4 ± 0.48 [a]	0.40 ± 0.15 [a]

Data are means ± S.D. of three experiments with samples in triplicate.
[a] $P < 0.05$ compared to control value (Student's *t*-test).

In vitro treatment with carbachol increased protein kinase C activity in the particulate fraction by 86% and this was paralleled by a significant reduction in the cytosolic fraction.

Long-term PMA treatment resulted in 90–100% loss of measurable protein kinase C activity both in the cytosolic and in the particulate fractions (data not shown).

4. Discussion

Chronic agonist activation of muscarinic receptors results in desensitization of receptor-mediated responses in different cell types. The molecular mechanisms underlying the desensitization process are so far not completely known; however, a primary role of protein kinase C has been suggested. This view is supported by the results obtained in the present investigation.

To pharmacologically characterize muscarinic receptors of bovine ciliary muscle, functional as well as biochemical experiments were performed. The results show that the carbachol-induced contractile effect was competitively antagonized by atropine, a non-selective antimuscarinic agent, and by 4-DAMP, a selective muscarinic M_3 receptor antagonist, and was insensitive to pirenzepine, a muscarinic M_1 receptor antagonist, and methoctramine, a muscarinic M_2 receptor antagonist.

In the same way, the binding of quinuclidinyl benzilate, a non-subtype-specific muscarinic antagonist, was fully displaced by atropine and 4-DAMP, but not by pirenzepine and methoctramine. The rank order of potency of 4-DAMP and atropine in displacing quinuclidinyl benzilate was the same as that in antagonizing the contractions elicited by carbachol. pK_i values were comparable with pA_2 values. Differences in absolute values may be explained on the basis of the different potency of the antagonists to compete for binding sites in partially purified membranes when compared with their ability to reach the active sites in the isolated organ; the ratio of activity atropine/4-DAMP was the same in binding and in functional studies. All together, these results indicate that muscarinic receptors from bovine ciliary muscle belong predominantly to the muscarinic M_3 receptor subtype. Previous studies from our laboratory (Lograno et al., 1991) indicated that prolonged exposure of bovine ciliary muscle tissue to carbachol caused desensitization of the contractile responses. Furthermore, preincubation of the ciliary muscle with PDB at low concentrations, which are specific for the activation of protein kinase C (Nishizuka, 1984), produced desensitization of muscarinic receptor-mediated responses. This finding led us to postulate that agonist-induced modulation of functional cholinergic sensitivity may be due, at least in part, to a protein kinase C-mediated mechanism.

Given the premise that phorbol esters have a time-dependent dual action on KCl- or Ca^{2+}-induced contraction

in smooth muscle (Itoh et al., 1986), they were used for a short-time exposure in order to activate protein kinase C. Secondly, in central nervous system phorbol esters have been shown to induce a positive short-term activation of protein kinase C, thereafter followed by a negative action, owing to protein kinase C down-regulation, due to a preferential degradation of activated protein kinase C by calpain (Nishizuka, 1988). On this basis, a long-term exposure to phorbol esters was applied as well. To further define the contribution of protein kinase C to the modulation of cholinergic-mediated contractility of the ciliary muscle, its activity was blocked using pharmacological agents as inhibitors.

As shown in Section 3, the short-term exposure of the ciliary muscle to phorbol esters resulted in antagonism of muscarinic receptor-mediated contraction. In contrast, long-term pretreatment caused a marked potentiation of carbachol responsiveness, as well as a reduction of agonist-induced desensitization and loss of short-term phorbol ester-induced desensitization. These data support the hypothesis that down-regulation of protein kinase C prevents protein kinase C-mediated negative feedback inhibition of responsiveness.

Staurosporine and H_7 caused a significant potentiation of the carbachol-mediated contractile effect and reduced the desensitization produced by repeated exposure to carbachol, while suppressing the inhibitory action produced by short-term exposure to phorbol esters. In view of these findings, it is conceivable that the desensitization by phorbol esters is mostly due to protein kinase C activation, whereas that induced by receptor agonists appears to be only partially mediated by mechanisms involving protein kinase C activation.

To establish whether the desensitization phenomenon was associated to a direct modification of muscarinic receptors, binding experiments were performed. The binding of quinuclidinyl benzilate was unaffected by repeated exposure to carbachol, or by preincubation with PDB or PMA. Due to the low affinity of carbachol for the muscarinic M_3 receptor (which seems to be the prevalent subtype in the bovine ciliary muscle), direct measurement of agonist affinity was technically impossible, so that the antagonist binding data presented in Table 1 may be considered an indirect indicator that the receptor binds ligands normally following the pretreatment protocols.

Inasmuch as the density and the affinity of muscarinic receptors were not altered by conditions that induced desensitization of the functional responses, the inhibition might occur at the level of receptor coupling to its transductional mechanisms.

Bearing in mind the results of the present functional studies and the data from the literature, which suggest the involvement of protein kinase C in the desensitization of muscarinic receptors, we investigated the distribution and the activity of protein kinase C in order to characterize the protein kinase C system in bovine ciliary muscle. Finally, attempts were made to establish whether the desensitization induced by the muscarinic receptor agonist carbachol, as well as by phorbol esters, was correlated with changes in the distribution and activity of protein kinase C in cytosolic and particulate fractions.

As shown, bovine ciliary muscle was found to contain protein kinase C in the cytosolic and in the membrane fractions. However, there was considerably more protein kinase C in the membrane fraction (about 80% of the total content) than in the cytosolic fraction. Thus, it appears that even under unstimulated conditions, bovine ciliary muscle contains considerable quantities of protein kinase C associated with the membrane, in agreement with studies for other smooth muscles, for example bovine tracheal muscle (Langlands and Diamond, 1992).

During stimulation with phorbol esters, as expected, a further association of the enzyme with the membrane occurred. Concurrent with the elevation in membrane-associated protein kinase C there was a reduction in cytosolic protein kinase C. In fact, the specific binding in the cytosolic fraction was so low that it became undetectable. With the protocol that induced maximal functional desensitization, a significant translocation of protein kinase C from the cytosol to the membrane also occurred after stimulation with the muscarinic agonist carbachol. Together with the increase of PDB binding, a significant augmentation of enzyme activity in the particulate fraction was observed after phorbol ester or carbachol exposure.

All together, our results are consistent with the view that the responsiveness of the ciliary muscle to cholinergic stimulation is regulated, at least in part, by the activation of protein kinase C, through its redistribution from the cytosol to the membrane and consequent feedback inhibition of responsiveness. One can envision that the prolonged stimulation of muscarinic receptors, via the hydrolysis of phosphoinositides and the generation of diacylglycerol, strongly activates protein kinase C which, in turn, phosphorylates muscarinic receptors, contributing to their desensitization. This may occur without affecting the binding parameters of muscarinic receptors. Haga et al. (1990) have demonstrated that muscarinic receptors from porcine cerebral cortex are phosphorylated by protein kinase C purified from the same tissue, and sites for this phosphorylation have been described (Uchiyama et al., 1990). Another possibility is that the desensitization process is mediated by protein kinase C-induced modulation of phospholipase C activity and IP_3 generation or hydrolysis (Fisher, 1995). In this context, there is evidence indicating that phorbol esters induce a decrease in the accumulation of IP_3 in response to muscarinic receptor agonists (Labarca et al., 1984; Orellana et al., 1985; Vicentini et al., 1985; Hepler et al., 1988), and this may represent a physiological mechanism for feedback regulation of the turnover of phosphatidylinositol.

Muscarinic receptor desensitization may occur by more than one mechanism: changes in the number of cellular

receptors and receptor phosphorylation or compartmentation, all resulting from chronic exposure of the receptor to agonist, may contribute to the modulation of the signal transduction pathway. Although we cannot exclude the possibility that other kinases such as the cAMP-dependent protein kinase A (PKA) may contribute to the mechanisms of desensitization of muscarinic M_3 receptor induced by PDB, to date there is no evidence that this mechanism is present in the bovine ciliary muscle studied here.

It is known that a cross-talk mechanism exists between protein kinase C and protein kinase A pathways in other smooth muscles, such as iris sphincter (Yoshimura and Cooper, 1993; Tachado et al., 1993). However, this cross-talk mechanism is strictly dependent on the different isoforms of the enzymes involved, on the species specificity, and/or on the subcellular fraction of tissue under study (Husain and Abdel-Latif, 1996).

Taken as a whole, the data reported point out a relevant role for protein kinase C in the desensitization of muscarinic receptor-mediated responses and indicate that, as in other tissues, muscarinic receptors of bovine ciliary muscle operate under a close feedback regulation mediated by the second messengers generated as result of receptor activation.

This has important implications if we consider that protein kinase C activated by persistent muscarinic receptor stimulation leads in turn to desensitization of the stimulated receptor, thus protecting its biochemical and functional apparatus from abnormal stimulation, which is a potentially harmful event for the cell system.

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