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Mechanisms Involved in Desensitization of Particulate Guanylyl Cyclase in Human Airway Smooth Muscle: The Role of Protein Kinase C

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We recently showed that cultured human airway smooth muscle cells (HASMC) express both soluble and particulate guanylyl cyclases (GC) and that long term treatment with atrial natriuretic peptide (ANP) causes homologous desensitization of particulate GC. Here we determine if protein kinase C (PKC) activation would desensitize particulate GC and probe the role of PKC in particulate GC desensitization. Pretreatment of HASMC with phorbol 12-myristate 13-acetate (PMA), a PKC activator led to time and concentration-dependent desensitization of ANP-stimulated cGMP accumulation. GF109203X, a selective PKC inhibitor, blocked the PMA-induced desensitization, but did not block ANP-induced desensitization. In addition, desensitization by PMA and ANP showed an additive effect. These results suggest that PKC activation can desensitize particulate GC but that the desensitization induced by ANP is PKC-independent. © 1999 Academic Press

Cyclic GMP is produced from guanosine triphosphate (GTP) by the action of guanylyl cyclases (GC). GC exist in a soluble form and a particulate, membrane bound, form (1, 2). Membrane bound guanylyl cyclases are plasma membrane receptors for the natriuretic peptides and related hormones. We have previously characterised the types of GC present in cultured HASMC (3). These studies showed that both soluble and membrane bound forms of GC were present in HASMC with type A particulate GC (GC-A) is the most abundant. We subsequently showed that both forms could undergo homologous desensitization (4). ANP, the most potent ligand for particulate GC-A has several cGMP-mediated biological effects including relaxation of human airway smooth muscle in vitro (5), bronchodilatation in vivo (6, 7) and inhibition of HASMC proliferation (8). Natriuretic peptides such as ANP may, therefore, have a therapeutic role in asthma.

PKC exists in several isoforms many of which have been identified in HASMC (9). PKC may be activated directly by phorbol esters such as PMA which have therefore been used in examining the role of PKC. Processes for which roles of PKC have been postulated in airway smooth muscle include contraction and proliferation (10). Although the role of PKC in regulation of GC-A in ASM is yet not known, studies in other biological systems have suggested a regulatory role. Endothelin inhibits ANP-induced relaxation and cGMP accumulation in vascular smooth muscle by a PKC-mediated mechanism (11) and angiotensin II inhibits ANP-stimulated cGMP accumulation in cultured glomerular mesangial cells by PKC-mediated inhibition of GC-A (12).

As some of the effects of ANP were reported to be mediated via PKC activation (13), we hypothesised firstly that PKC may regulate the activity of GC-A in airway smooth muscle and secondly that PKC was involved in the homologous desensitization we previously demonstrated with ANP (4). Thus the current study was designed to determine whether pretreatment with PMA could desensitize GC-A in cultured HASMC. Furthermore, we used a PKC inhibitor to probe the role of PKC in PMA and ANP-induced desensitization.

MATERIALS AND METHODS

Materials. GF109203X were purchased from Calbiochem-NovaBiochem, (Nottingham, Nottinghamshire, UK), otherwise all chemicals were purchased from Sigma-Aldrich Company Limited (Poole, Dorset, UK). All concentrations of the reagents shown refer to the final concentration in the cell bathing solution. Plastic wares were purchased from Costar (Cambridge, MA).

Cell culture. Primary cultures of HASMC were prepared from explants of ASM according to methods previously described (3). Frozen aliquots of cells were thawed before use and plated at a density of 2×10^4 cell/well in 12-well culture plates containing DMEM with

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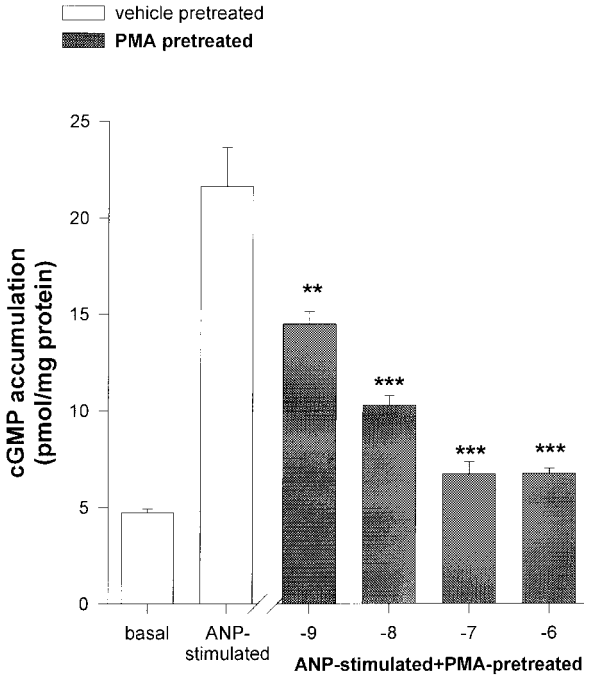

FIG. 1. Concentration response of PMA-induced desensitization. HASMC were treated with 10^{-9} M–10^{-6} M PMA for 8 h before challenging with 10^{-6} M ANP for 2 h in the presence of 10^{-3} M IBMX. Data represent the mean ± SEM of 8 observations from two independent experiments. Significantly different from ANP-stimulated values in the absence of PMA: **($p < 0.01$) and ***($p < 0.001$).

10% FCS, penicillin G, streptomycin, amphotericin B and L-glutamine. All experiments were performed in confluent HASMC which had been growth arrested for 24 h by serum deprivation. Cells were used between passage 2–5.

cGMP assay. Cells were washed with sterile PBS at the end of the pretreatment period, then incubated with 10^{-6} M ANP for 2 h in the presence of 10^{-3} M IBMX. cGMP was measured as described previously (3). Briefly, cells were removed with trypsin (0.25%). cGMP was extracted by adding 1 ml of ice cold 0.1M hydrochloric acid to the cell suspension. The resulting suspension was freeze-dried (SB9, Lab Plant Ltd., Huddersfield, Yorkshire, UK) prior to the measurement of cGMP content using a commercially available ELISA kit, RPN 226 (Amersham LTD, Little Chalfont, Buckinghamshire, UK). The samples were first acetylated with a mixture of acetic anhydride and triethylamine to increase the sensitivity of the assay to 2 fmol/50 μl. All samples were assayed in duplicate. The cells from 6 wells, not used for cGMP assay, were used to give a representative estimate of protein content in each experiment by the method of Bradford using bovine serum albumin as a standard (14).

Cell viability. Cell viability was assessed by (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide; Thiazolyl blue) (MTT) assay (4). HASMC were grown in 96-well plates till confluence then treated with either DMEM (control), 10^{-6} M PMA or 10^{-6} M ANP for 24 h. At the end of the treatment period, 20 μl of 5 mg/ml MTT were added to each well for 1 h at 37°C. The media was discarded and 200 μl of DMSO was added to each well to solubilize the blue formazan product and the plates were shaken for 5 min. The optical density at 630/570 nm was compared with control using a plate reader (MR 5000, Dynatec, West Sussex, UK). Viability was set as 100% in control cells.

Statistical analysis. Results are shown as means ± SEM of the indicated number of individual observations. Data are expressed as pmol cGMP/mg protein. The significance of drug effect was assessed by one way ANOVA followed by Student's *t* test using the SPSS software program (SPSS Inc., Chicago, IL). A *p* value <0.05 was regarded as significant.

RESULTS

Effect of PMA Pretreatment on ANP-Stimulated cGMP Accumulation

Concentration response. ANP stimulated cGMP accumulation in intact HASMC (Fig. 1). Pretreatment of HASMC with PMA (10^{-9}–10^{-6} M) for 8 h led to a concentration-dependent inhibition of subsequent ANP-stimulated cGMP accumulation ($p < 0.001$ for 10^{-9} M concentration and <0.001 for the higher concentrations, Fig. 1).

Time course. Cells treated with 10^{-6} M PMA exhibited a time-related inhibition of ANP-stimulated cGMP accumulation which was seen as early as 30 min, was significant between 30 min and 8 h ($p < 0.001$) but had worn off by 24 h (Fig. 2).

Effect of 4-α phorbol pretreatment. Pretreatment with 4-α phorbol, an inactive form of phorbol ester, for 8 h had no effect on ANP-stimulated cGMP accumulation (data not shown).

Additive effect of ANP and PMA-induced desensitization. Incubation of HASMC with 10^{-6} M PMA and/or 10^{-6} M ANP for 8 h decreased subsequent ANP-

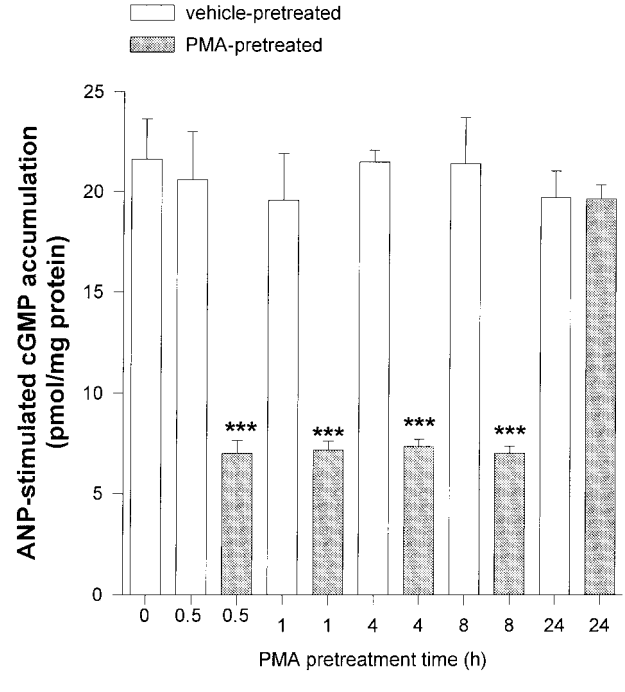

FIG. 2. Time course of PMA-induced desensitization. HASMC were treated with 10^{-6} M PMA for 0.5–24 h before challenging with 10^{-6} M ANP for 2 h in the presence of 10^{-3} M IBMX. Data represent the mean ± SEM of 8 observations from two independent experiments. Significantly different from ANP-stimulated values in the absence of PMA: ***($p < 0.001$).

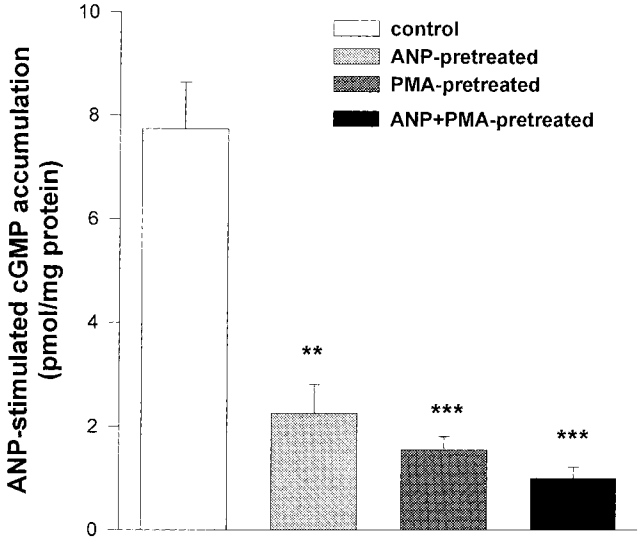

FIG. 3. Effect of pretreatment with PMA and/or ANP on ANP-stimulated cGMP accumulation. HASMC were treated with 10^{-6} M PMA and/or 10^{-6} M ANP for 8 h before challenging with 10^{-6} M ANP for 2 h in the presence of 10^{-3} M IBMX. Data represent the mean ± SEM of 4 observations from a single experiment. Significantly different from control: **($p < 0.01$) and ***($p < 0.001$).

stimulated cGMP accumulation ($p < 0.01$, 0.001 and 0.001 for cells pretreated with 10^{-6} M ANP, 10^{-6} M PMA and 10^{-6} M ANP + 10^{-6} M PMA respectively, Fig. 3).

Effect of the PKC Inhibitor, GF109203X, on ANP and PMA-Induced Desensitization

GF109203X had no effect on basal cGMP levels however it had a small nonsignificant effect on ANP-stimulated cGMP accumulation ($p = 0.09$). Treatment of HASMC with GF109203X blocked PMA-induced desensitization ($p < 0.05$, Fig. 4). In contrast, treatment with GF109203X had no effect on ANP-induced desensitization ($p = 0.7$, Fig. 5).

DISCUSSION

We previously showed that long term exposure of cultured HASMC to ANP causes cGMP-independent homologous desensitization of particulate guanylyl cyclase (4). Here we show that ANP-induced desensitization of particulate GC in HASMC is PKC-independent. In addition we showed that activation of PKC using PMA could cause desensitization of particulate GC. This latter observation suggests that particulate GC may be desensitized by stimuli which activate PKC in chronic asthma.

We used the PKC inhibitor GF109203X to probe the role of PKC in ANP-induced desensitization of particulate GC. The lack of effect of GF109203X suggests that PKC was not involved in ANP-induced desensitization as the concentration of GF109203X we used has been shown to inhibit PKC in other biological systems (15). The fact that pretreatment with PMA and ANP showed an additive effect is also consistent with these agents using disparate pathways to produce their effects as is the difference in their time course of action. PMA-induced desensitization occurred as early as 30 min whereas in previous studies with ANP we have shown that longer incubation (8–16 h) was required to observe ANP-induced desensitization (4). Collectively, these findings strongly suggest that ANP-induced desensitization is PKC-independent. These findings are consistent with reports in vascular smooth muscle (16).

The mechanism of PMA-induced desensitization of particulate GC is likely to be PKC-mediated as it was inhibited by GF109203X. Consistent with this 4-α phorbol, an inactive analogue that does not activate PKC was without effect. The effect of PMA on particulate GC is similar to the effect of phorbol esters on adenylyl cyclase activity in ASM. This later effect is due to PKC-mediated phosphorylation of the β-adrenoceptor leading to uncoupling from adenylyl cyclase activation. Since particulate GC contains both the receptor and GC in the same molecule, it is unlikely that PKC activation by PMA phosphorylates the ANP receptor to uncouple it from particulate GC. It is unlikely that PKC was acting by altering cGMP phosphodiesterase activity as cGMP accumulation was performed

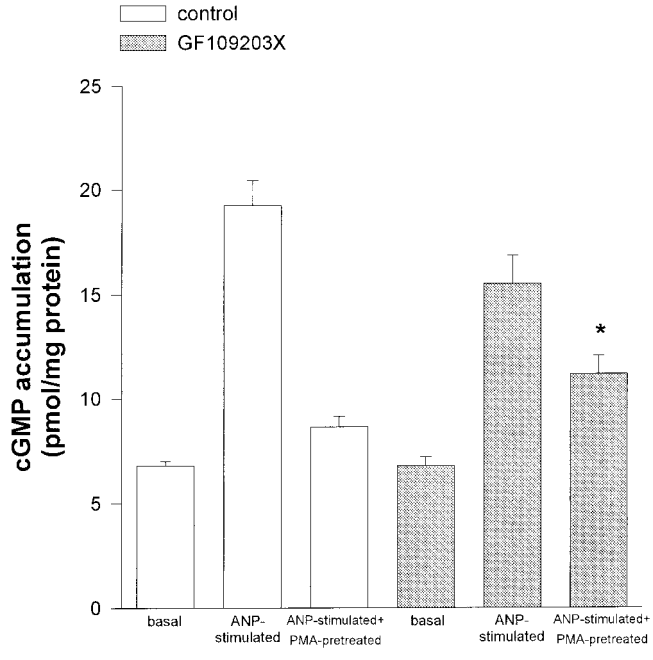

FIG. 4. Effect of GF109203X on PMA-induced desensitization. HASMC were treated with 10^{-6} M PMA for 30 min in the absence or the presence of 10^{-6} M GF109203X before challenging with 10^{-6} M ANP for 2 h in presence of 10^{-3} M IBMX. Data represent the mean ± SEM of 4 observations from a representative experiment that was repeated twice with similar results. Significantly different from values in the absence of GF109203X: *($p < 0.05$).

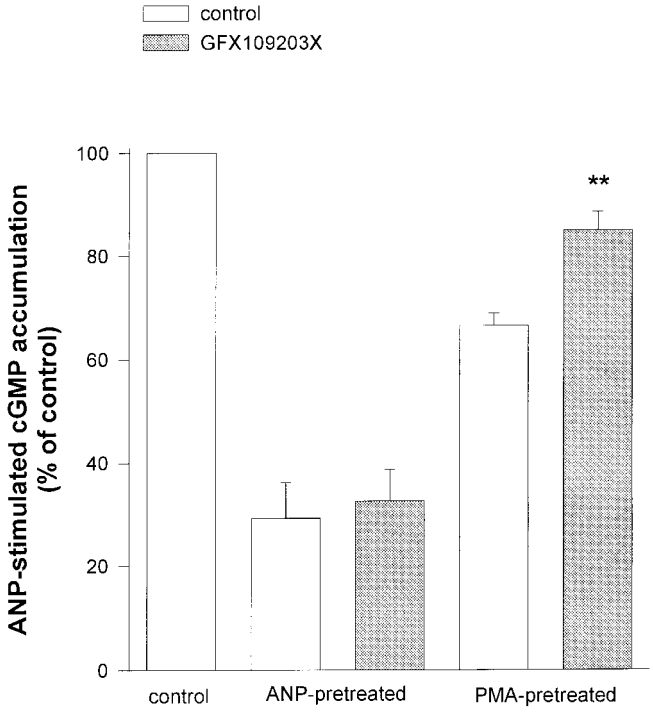

FIG. 5. Effect of GF109203X on ANP and PMA-induced desensitization. HASMC were treated with 10^{-6} M PMA or 10^{-6} M ANP for 8 h in the absence or the presence of 10^{-6} M GF109203X before challenging with 10^{-6} M ANP for 2 h in the presence of 10^{-3} M IBMX. Data represent the mean ± SEM of 4 observations from a single experiment. Significantly different from values in the absence of GF109203X: **($p < 0.01$).

in the presence of IBMX, a phosphodiesterase inhibitor, in our experiments. The most likely explanation is that PKC decreases particulate GC activity possibly by dephosphorylation although this requires further study (17).

In conclusion, we have shown that PKC activation can desensitize particulate GC in HASMC but that this is not involved in ANP-induced homologous desensitization. PKC activation by cytokines released in chronic asthma may desensitize particulate GC in ASM thus impairing the relaxant effect of endogenous ANP and so contributing to the bronchial hyperresponsiveness.

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