Differential regulation of natriuretic peptide receptors on ciliary body epithelial cells

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Atrionatriuretic peptide (ANP) lowers intraocular pressure in the eyes of humans and rabbits. We examined the effects of natriuretic peptides on cGMP formation and ^{125}I-labelled-ANP binding to cultured cells derived from ciliary body epithelium, the site of aqueous humour formation in the eye. ANP, brain natriuretic peptide (BNP) and C-natriuretic peptide (CNP) at 1 μM stimulated cGMP formation 8.2($\pm$1.2)-fold, 4.8($\pm$0.6)-fold and 87.3($\pm$12.1)-fold respectively. ^{125}I-ANP bound to intact cells at a single site, with a dissociation constant $K_D = 0.30 \pm 0.01$ nM. BNP was as effective as ANP in displacing ^{125}I-ANP, whereas CNP displaced label with a slightly higher IC_{50}. ^{125}I-ANP binding was displaced $> 95\%$ by c-ANP, a specific ligand for natriuretic peptide C receptors (NPR-C). Cross-linking of ^{125}I-ANP to cells labelled predominantly a protein of M_r 62000. These data suggest that ^{125}I-ANP binding was primarily to NPR-C, whereas cGMP stimulation occurred primarily via natriuretic peptide B receptors (NPR-B). Vasopressin and histamine, both activators of the inositol phosphate/diacylglycerol phosphate pathway in non-pigmented ciliary epithelial cells, inhibited CNP stimulation of guanylate cyclase (NPR-B) and ^{125}I-ANP binding (NPR-C) by 30–38 %. Inhibition was mimicked by PMA, dioctanoylglycerol and phorbol didecanoate, whereas 4α phorbol didecanoate had no effect. Staurosporine and bisindolylmaleimide both blocked inhibition of ^{125}I-ANP binding and cGMP formation by PMA. These results suggest that protein kinase C (PKC) down-regulates both NPR-B and NPR-C. PKC down-regulation of NPR-B varied inversely with CNP concentration. Inhibition by 1 μM PMA was 30.6($\pm$4.0) % with 500 nM CNP, but 83.4($\pm$8.8) % with 10 nM CNP, indicating that increasing CNP could partially overcome inhibition by PMA. Since extracellular CNP levels were not affected by PKC activation, the effect of PKC on NPR-B is best explained as a reduction in NPR-B affinity for CNP. NPR-C measured as ^{125}I-ANP binding was likewise reduced 36.4($\pm$5.1) % by exposure to PMA. In contrast with NPR-B inhibition, however, inhibition of NPR-C was due largely to a reduction in the number of receptor binding sites per cell rather than a reduction in receptor affinity for ligand. The data therefore suggest that both NPR-B and NPR-C are down-regulated by PKC, but that the mechanisms of down-regulation of the two receptors are different.

INTRODUCTION

Atrionatriuretic peptide (ANP) is a 28-amino-acid peptide synthesized primarily in the heart and is involved in vasodilation, natriuresis and diuresis [1]. ANP also lowers intraocular pressure in the eyes of rabbits and humans [2–7]. The lowering of intraocular pressure by ANP occurs in both normal and glaucomatous patients, unrelated to changes in blood pressure [6,7].

Brain natriuretic peptide (BNP) and C-natriuretic peptide (CNP) are related to ANP and also regulate natriuresis, diuresis and blood flow [1,8,9]. ANP and BNP are synthesized in the heart, adrenals and nervous tissue, and reach target tissues primarily via the blood supply (reviewed in [10]). CNP and CNP mRNA are found mainly in vascular endothelium [11] and neurons [12,13]. The latter finding, together with fact that CNP in human brain is 30–70 times more abundant than either ANP or BNP [13], suggests that CNP may function as a neuropeptide in humans. CNP was recently found to lower intraocular pressure more effectively than either ANP or CNP in rabbits [14].

The physiological actions of natriuretic peptides are mediated by activation of receptor-linked guanylate cyclases and production of cGMP [15,16]. Three natriuretic peptide receptor (NPR) subtypes, NPR-A, NPR-B and NPR-C, have been characterized [17–20]. NRP-A and NPR-B are approx. 1030-amino-acid transmembrane guanylate cyclases [17,18]. Distinct affinities for the three natriuretic peptides distinguish these receptor subtypes from one another: NPR-A is strongly stimulated by ANP, moderately by BNP, and minimally by CNP, whereas NPR-B is strongly stimulated by CNP but minimally by ANP and BNP [17,18]. In contrast, NPR-C does not possess a guanylate cyclase segment in its cytoplasmic domain [19,20]. Its function has been proposed to be a clearance mechanism for circulating ANP [21], although there is evidence that it may mediate effects of natriuretic peptides in other signalling pathways [22,23].

Binding sites for ^{125}I-labelled ANP [24] and ANP stimulation of guanylate cyclase [25] have both been described in ciliary body epithelium, the site of aqueous humour formation in the eye [26]. The ciliary body epithelium is a double-layered structure composed of a pigmented epithelial layer overlain on the luminal side by a non-pigmented epithelial layer [26]. ANP has been reported to stimulate aqueous humour formation in primates [27], but the effects of BNP and CNP on this tissue have not been determined. The finding that CNP lowers intraocular pressure in rabbits

Abbreviations used: ANP, atrionatriuretic peptide; BNP, brain natriuretic peptide; CNP, C-natriuretic peptide; NPR-A, natriuretic peptide A receptor; NPR-B, natriuretic peptide B receptor; NPR-C, natriuretic peptide C receptor; PKC, protein kinase C; NPE, non-pigmented ciliary epithelial; DST, disuccinimidyl tartrate; BCS, bovine calf serum; IP, inositol phosphate; DAG, diacylglycerol; PDD, phorbol didecanoate.

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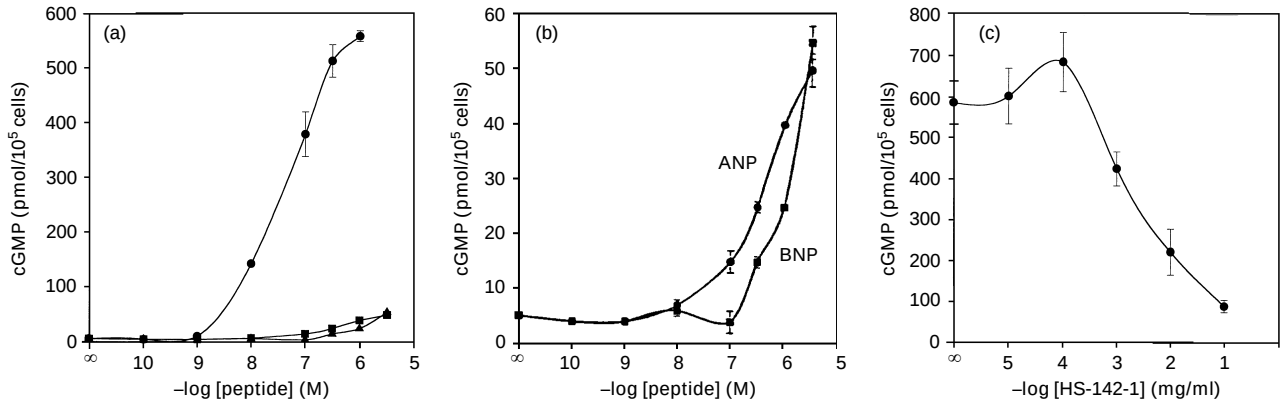

Figure 1 Stimulation of cGMP formation by natriuretic peptides

(**a**) Concentration dependence. Cells in test medium were given various concentrations of ANP (■), BNP (▲) or CNP (●) for 5 min at 37 °C. The reactions were then terminated and intracellular cGMP determined as described in the Materials and methods section. Data are from a representative experiment (of three with similar results) carried out in duplicate. Values are averages of duplicate determinations. (**b**) Enlargement of ANP and BNP curves from (**a**). (**c**) HS-142-1. Cells in test medium were given various concentrations of HS-142-1 for 5 min at 37 °C, followed by 0.5 μM CNP for 5 min. The reactions were then terminated and analysed as in (**a**). Data are from a representative experiment (of three with similar results) carried out in duplicate.

more effectively than either ANP or BNP [14] prompted us to examine the effects of the three natriuretic peptides on ^{125}I-ANP binding and guanylate cyclase activity in human non-pigmented ciliary epithelial (NPE) cells. The effects of histamine, vasopressin and PKC activation on these responses were also studied.

MATERIALS AND METHODS

Chemicals

ANP [ANP-(1–28), human] and bisindolylmaleimide were from Calbiochem (La Jolla, CA, U.S.A.). BNP-32 (human), CNP-22 (human) and Des-[Gln^{18},Ser^{19},Gly^{20},Leu^{21}Gly^{22}]atrionatriuretic factor-(4–23)-NH_2 (c-ANP) were from Peninsula Laboratories (San Carlos, CA, U.S.A.). PMA, 4β-phorbol didecanoate, 4α-phorbol didecanoate and dioctanoylglycerol were from Sigma Chemicals (St. Louis, MO, U.S.A.). Staurosporine was from Kamiya Chemical Co. (Thousand Oaks, CA, U.S.A.). Disuccinimidyl tartrate (DST) was from Pierce (Rockford, IL, U.S.A.). Molecular mass standards were from Bio-Rad (Richmond, CA, U.S.A.).^{125}I-ANP (2200 Ci/mmol) and ^{125}I-labelled CNP (2200 Ci/mmol) were from Amersham Corp. (Farmington, DE, U.S.A.). HS-142-1 was generously given by Dr. Yuzuru Matsuda (Kyowa Hakko Kogyo Co. Ltd., Tokyo, Japan) and A17915 by Dr. Thomas von Geldern (Abbott Laboratories, Abbott Park, IL, U.S.A.). All other chemicals were reagent grade from Fisher Chemical Company (Pittsburgh, PA, U.S.A.).

Cell culture

Human fetal NPE cells were isolated and cultured as previously described [28]. Cells were grown to confluence on 24-well multiwells or 35 mm dishes coated with extracellular matrix. Cells were grown in growth medium [Medium 199 (M199; Grand Island Biologicals, Gaithersburg, MD, U.S.A.) supplemented with 15 % (w/v) bovine calf serum (BCS; Hyclone, Salt Lake City, UT, U.S.A.), 2 mM glutamine, 20 μg/ml gentamycin, 0.5 μg/ml Fungizone and 500 ng/ml β fibroblast growth factor. Confluent cells were switched to maintenance medium (M199 supplemented with 10 % BCS, without fibroblast growth factor). Cells were fed every other day.

^{125}I-ANP binding

Binding to intact NPE cells was carried out as described [29] with the following modifications. Confluent NPE cells in 24-well multiwells (Costar Corp, Cambridge, MA, U.S.A.) were washed once and then given 1 ml of labelling medium [M199 containing 0.5 % (w/v) bovine serum albumin] containing 5 fmol of ^{125}I-ANP and various concentrations of unlabelled peptide, and incubated at 4 °C for 17–20 h. The monolayers were then washed three times with 1 ml of ice-cold PBS, dissolved in 1 ml of 1 % (w/v) SDS and counted in a Beckman gamma counter. Non-specific binding was radioligand bound in the presence of 1 μM unlabelled ANP, and was routinely 10 % of total counts. Data are expressed as percentage of B_0, where B_0 is total specific binding (total-non-specific binding) in the absence of competing unlabelled ligand.

Affinity cross-linking

^{125}I-ANP was cross-linked to intact cells as described [29], with the following modifications. Confluent monolayers grown in 35 mm dishes (Costar Corp) were washed once and given 1.5 ml of Labeling Medium containing 20 fmol of ^{125}I-ANP and incubated for 45 min at 37 °C (maximal binding was achieved at 30 min; results not shown). The monolayers were then washed five times with ice-cold PBS and given 1 mM DST in 1.5 ml of PBS for 20 min at 4 °C. The cells were then washed three times with PBS and dissolved in 1 % SDS.

Gel electrophoresis

Immediately following addition of SDS, samples were heated to 95 °C for 10 min. Samples (1–10 μg of protein) were then electrophoresed on 7.5 % (w/v) acrylamide/SDS minigels made as described by Laemmli [30]. Gels were stained with Coomassie Blue, dried under vacuum and exposed to X-ray film (Eastman Kodak Co., Rochester, NY, U.S.A.) with two enhancing screens (Lightning Plus, Dupont, Wilmington, DE, U.S.A.) at −80 °C for 4–7 days. The film was then developed. Protein was determined by the method of Peterson [31].

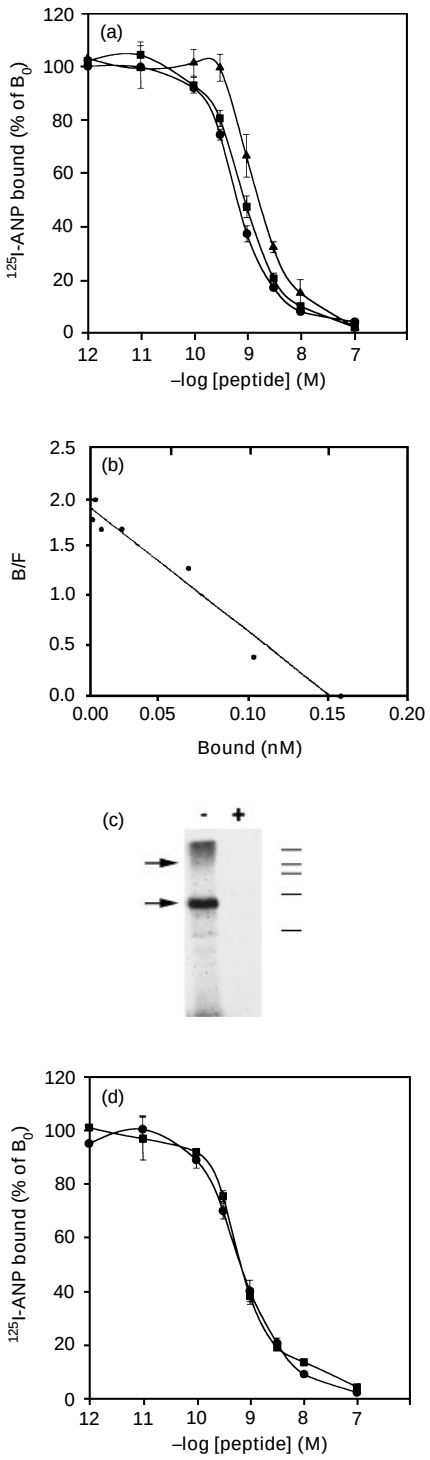

Figure 2 Binding of ^{125}I-ANP to NPE cells

(**a**) Displacement by ANP, BNP and CNP. Cells in binding medium given 5 pM ^{125}I-ANP were also given various concentrations of either ANP (■), BNP (▲) or CNP (●) and incubated for 60 min at 37 °C. The cells were then washed and counted as described in the Materials and methods section. Values shown were corrected for non-specific binding as described in the Materials and methods section. Data are means ± S.D. ($n = 4$). (**b**) Scatchard transform. The data in (**a**) were analysed by LIGAND [37]. B, bound ^{125}I-ANP; F, free ^{125}I-ANP. (**c**) Cross-linking of ^{125}I-ANP to intact NPE cells. Cells bound with ^{125}I-ANP in the absence (−) or presence (+) of 1 μM ANP were cross-linked, dissolved and lysates electrophoresed as described in the Materials and methods section. Lines at right are positions of M_r markers. From top down: myosin (200 000), B-galactosidase (116 250), phosphorylase B (97 400), bovine serum albumin (66 200) and ovalbumin (45 000). Top arrow at left indicates predicted position of NPR-A and NPR-B; bottom arrow indicates major cross-linked protein(s). (**d**) Effect of c-ANP. Cells given 5 pM ^{125}I-ANP were also given various concentrations of either c-ANP (■) or ANP (●) and incubated for 60 min at 37 °C. The cells were then washed and analysed as in (**a**). Data are means ± S.D. ($n = 3$).

CNP assay

Extracellular CNP was directly measured with a radioimmunoassay kit according to the vendor's directions (Peninsula Laboratories).

Guanylate cyclase assay

Cell monolayers were incubated in Test Medium (Labeling Medium plus 1 mM isobutylmethylxanthine) for 5 min. Following addition of natriuretic peptides, cells were incubated at 37 °C in 5 % CO_2 for 5 min. Incubations were terminated by replacement of media with 1 ml of 6 % (v/v) trichloroacetic acid. Trichloroacetic acid was removed by extraction with 2 volumes of water-saturated ether followed by aspiration of the organic phase. Remaining ether was evaporated under a stream of N_2. cGMP assays were carried out using a radioimmunoassay kit (New England Nuclear, Wilmington, DE, U.S.A.) as suggested by the vendor.

Cell number was determined by Coulter automatic cell counter after release of cells by trypsinization for 5 min with 0.05 % (w/v) trypsin/0.02 % (w/v) EDTA in 0.9 % (w/v) NaCl.

RESULTS

Evidence for NPR-B and NPR-C

Stimulation of guanylate cyclase in NPE cells by ANP, BNP and CNP is shown in Figure 1(a). CNP (1 μM) caused a 87.3(± 12.1)-fold stimulation of cGMP formation, with an $EC_{50} = 40.8 \pm 3.4$ nM. ANP (1 μM) produced a 8.2(± 1.2)-fold, stimulation of cGMP. BNP was less potent ($P < 0.05$) with a 4.8(± 0.6)-fold stimulation. Maximal intracellular cGMP levels were attained within 4–5 min of treatment with each peptide (not shown). Extracellular cGMP determined at these times was < 5 % of intracellular cGMP (not shown).

HS-142-1, a non-protein antagonist of both NPR-A and NPR-B [32], inhibited guanylate cyclase activity (Figure 1c). Similar results were observed when cGMP formation was stimulated with 1 μM ANP (results not shown). However A71915, an ANP analogue that inhibits NPR-A activity in human neuroblastoma cells [33], had no inhibitory effect on CNP stimulation of cGMP (not shown). Taken together with the rank order of potencies of the three peptides from Figure 1 (CNP ⩾ ANP > BNP), these data indicate the presence of NPR-B on NPE cells.

Displacement binding of ^{125}I-ANP to NPE cells in the presence of unlabelled ANP, BNP or CNP is shown in Figure 2(a). ANP and BNP competed with radiolabelled ANP with apparent IC_{50} values of 0.70 ± 0.03 nM and 0.73 ± 0.09 nM, whereas CNP was somewhat less effective, with an IC_{50} of 1.3 ± 0.1 nM, although this difference was not significant ($P > 0.05$). Computer analysis of the data [34] indicated that ANP bound to NPE cells at a single site (Figure 2b), with a $K_D = 0.3 \pm 0.01$ nM. The number of ANP binding sites per cell under these conditions was calculated to be 180 000 ± 30 000.

Cross-linking studies of ^{125}I-ANP bound to NPE cells were carried out to further characterize ANP binding sites (Figure 2c). Specifically bound ^{125}I-ANP cross-linked to protein(s) migrating on denaturing gels with an M_r of approx. 62 000. This is approximately the size reported for NPR-C [18]. Little labelling could be

Table 1 Inhibition by histamine and vasopressin of cGMP stimulation by CNP

Cells in Test Medium were given either 10 μM histamine, 1 μM vasopressin, 10 μM carbachol or no additions for 15 min. CNP (100 nM) was then added for 5 min at 37 °C. The reactions were terminated and cGMP assayed as described in the Materials and methods section. Data are means $\pm$ S.D. ($n = 3$). [a]Differed significantly from unstimulated controls ($P < 0.05$).

Addition	cGMP (pmol/10^5 cells)	Percentage of control
None	342 $\pm$ 44	100.0
Histamine (100 μM)	211 $\pm$ 18	61.7[a]
Vasopressin (1 μM)	239 $\pm$ 31	69.9[a]
Carbachol (100 μM)	311 $\pm$ 48	90.9

seen at $M_r = 130000$, the size of NPR-A and NPR-B [10–13]. The label at the top of the gel was not seen in other experiments.

Displacement binding of ^{125}I-ANP to NPE cells was carried out in the presence of c-ANP, an analogue of ANP which binds only to NPR-C [20]. c-ANP displaced $> 95\%$ of the specific ^{125}I-ANP binding to NPE cells (Figure 2d). ^{125}I-CNP binding to NPE cells was likewise almost completely displaced by c-ANP (results not shown). Taken together, these data indicate that binding of ^{125}I-ANP and ^{125}I-CNP is predominantly to NPR-C, suggesting that NPR-C is numerically the predominant NPR subtype on NPE cells.

Effects of histamine, vasopressin and phorbol esters on guanylate cyclase activity

Histamine, vasopressin and carbachol activate the inositol phosphate/diacylglycerol (IP/DAG) pathway in NPE cells [35]. When NPE cells were exposed to each compound before stimulation by CNP, 100 μM histamine or 1 μM vasopressin inhibited cGMP formation by approx. 30–38% (Table 1). Carbachol (100 μM) inhibited slightly, but this was not statistically significant ($P > 0.05$).

Activation of the IP/DAG pathway generates two second messengers: DAG, which activates protein kinase C (PKC); and inositol trisphosphate, which triggers elevation of intracellular calcium [36]. To investigate the effect of the DAG arm of the IP/DAG pathway on guanylate cyclase activation, we examined the effect of the PKC activator, PMA, on CNP-stimulated guanylate cyclase activity. Exposure to 1 μM PMA for 15 min resulted in a 30.6($\pm$4.0)% inhibition of cGMP formation stimulated by 500 nM CNP (not shown). After approx. 5 min of exposure to CNP, PMA-treated cGMP levels reached and sometimes exceeded control levels. When suboptimal CNP concentrations were used to stimulate guanylate cyclase, however, PMA inhibition was more pronounced. Stimulation with 100 nM CNP was inhibited by PMA by 56.1($\pm$6.2)%, and stimulation by 10 nM CNP was inhibited by 83.4($\pm$8.8)% (not shown). A dose–response curve of cGMP stimulation by CNP versus cGMP formation (Figure 3a) shows that PMA shifted the response curve to the right by 10.1($\pm$2.6)-fold.

At least two mechanisms could account for the shift of the CNP dose–response curve to the right by PMA: a reduction in extracellular CNP concentration, or a decrease in the affinity of NPR-B for CNP. Reduction of extracellular CNP could be caused by enhanced NPR-C activity, resulting in enhanced internalization of CNP bound to NPR-C ([37]; R. B. Crook and A. T. Chang, unpublished work), thereby removing the peptide from the medium. Alternatively, extracellular degradation of CNP, e.g. by enhanced neutral endopeptidase (protease 3.4.24.11) activity [38], could lower extracellular CNP. To test the first possibility, we treated cells with the NPR-C-inhibitor c-ANP. Figure 3(b) shows that increasing concentrations of c-ANP elevated CNP-stimulated cGMP formation ($P < 0.05$), suggesting that NPR-C activity does affect the extracellular CNP concentration. However, treatment with c-ANP had little effect on PMA inhibition of cGMP formation. To evaluate the possible role of neutral endopeptidase, which is found on many cell types and which degrades natriuretic peptides [38], stimulation of cGMP formation was carried out in the presence of phosphoramidon, a potent neutral endopeptidase inhibitor. No effect of

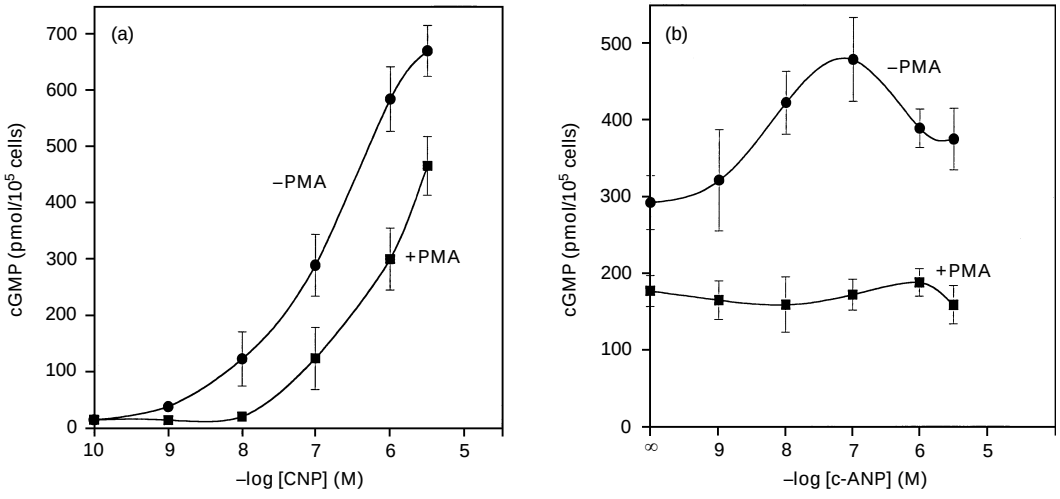

Figure 3 Effects of PMA on CNP and c-ANP modulations of cGMP formation

(**a**) CNP. Cells exposed to 1 μM (■) or no (●) PMA for 15 min at 37 °C were given the indicated concentrations of CNP for 5 min at 37 °C. The reactions were terminated and cGMP assayed as described in the Materials and methods section. Data are from a representative experiment (of four with similar results) carried out in duplicate. (**b**) c-ANP. Effect of c-ANP on PMA inhibition of cGMP stimulation by CNP. Cells exposed to 1 μM (■) or no (●) PMA for 15 min at 37 °C were given various concentrations of c-ANP for 5 min at 37 °C, followed by 0.5 μM CNP for 5 min at 37 °C. The reactions were analysed as in Figure 1(a). Data are from a representative experiment (of three with similar results) carried out in duplicate.

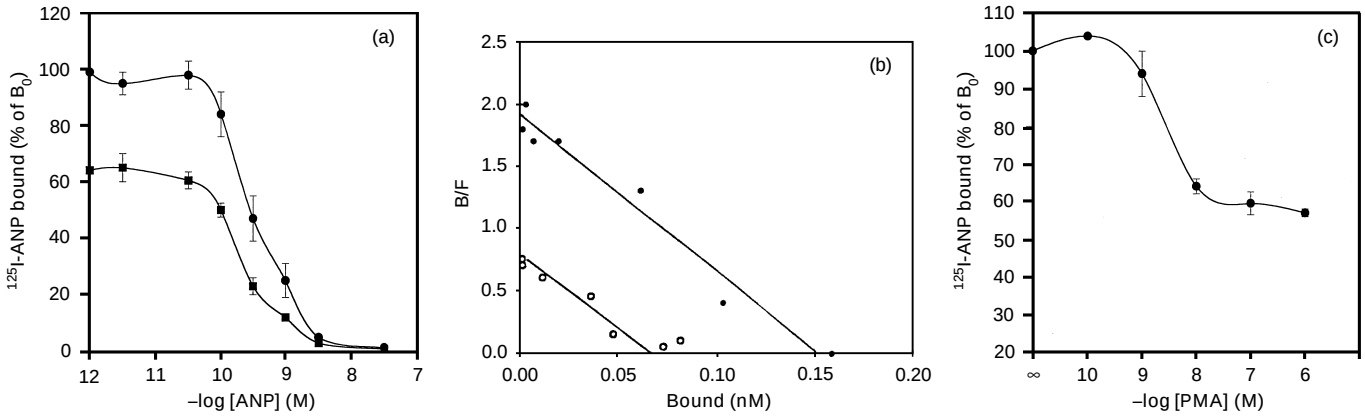

Figure 4 Effect of PMA on specific ^{125}I-ANP binding to NPE

(**a**) Cells exposed to 1 μM (■) or no (●) PMA for 15 min at 37 °C were given 5 pM ^{125}I-ANP and various concentrations of unlabelled ANP for 60 min at 37 °C. Data are means ± S.D. ($n = 4$). (**b**) The data were converted to the Scatchard format using the LIGAND program [34]: 1 μM (○) or no (●) PMA. (**c**) PMA dose–response. Cells exposed to various concentrations of PMA for 15 min at 37 °C were given 5 pM ^{125}I-ANP and either 1 μM or no ANP for 60 min at 37 °C. The cells were then analysed as in Figure 4(**a**). Data are means ± S.D. ($n = 3$).

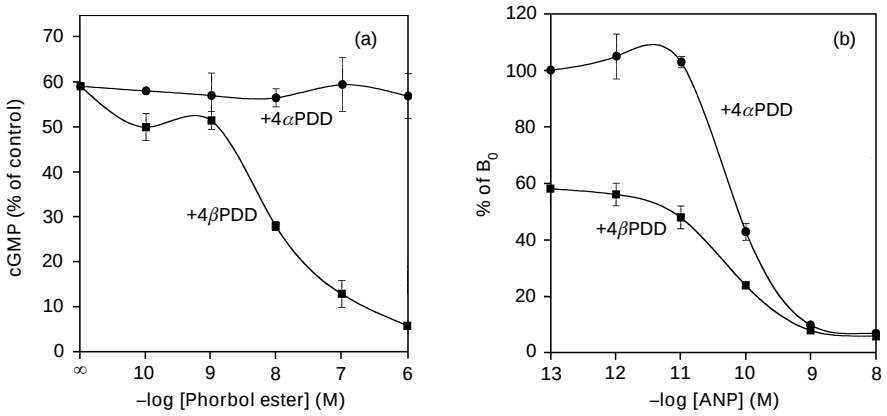

Figure 5 Effect PDDs on cGMP formation and specific ^{125}I-ANP binding

(**a**) cGMP formation. Cells exposed to various concentrations of either 4β PDD or 4α PDD for 15 min at 37 °C were given 0.5 μM CNP for 5 min at 37 °C. The reactions were terminated and cGMP assayed as described in the Materials and methods section. Data are from a representative experiment (of three with similar results). (**b**) ^{125}I-ANP binding. Cells exposed to 1 μM 4β PDD (■) or 1 μM 4α PDD (●) for 15 min at 37 °C were given 5 pM ^{125}I-ANP and various concentrations of unlabelled ANP for 60 min at 37 °C. The cells were then washed and analysed as in Figure 4(a). Data are means ± S.D. ($n = 3$).

phosphoramidon at concentrations from 0.1 nM to 1 μM was observed (not shown). Finally, direct measurement of extracellular CNP detected no reproducible difference in the extracellular media of cells treated or not with PMA (not shown). These data suggest that cell-mediated lowering of extracellular CNP did not contribute to the PMA shift of the CNP dose–response curve.

Effect of phorbol esters on ^{125}I-ANP binding

We next investigated the effects of phorbol esters on NPR-C by measuring ^{125}I-ANP binding to NPE cells. Exposure of NPE to 1 μM PMA caused a 36.4(±5.1)% reduction in specific ^{125}I-ANP binding (Figure 4a). A Scatchard transform of these data [34] (Figure 4b) indicated that this was due primarily to a decrease in receptor binding sites, with little change in apparent K_D. Figure 4(c) shows that PMA caused a reduction in ^{125}I-ANP binding to NPE cells, with $IC_{50} = 2.6 \pm 0.3$ nM.

Involvement of PKC in phorbol ester effects

Phorbol esters have been reported to have effects on cells that do not involve PKC [39]. 4α-Phorbol didecanoate (4α PDD) does not bind to PKC and is therefore an ineffective activator of the enzyme, while the 4β isomer activates effectively [40]. Both isomers were tested for inhibition of cGMP stimulation and ^{125}I-ANP binding. 4α PDD had little effect on either stimulation of cGMP formation (Figure 5A) or ^{125}I-ANP binding at 1 μM (Figure 5B). However, 4β PDD inhibited both functions. To confirm that PMA inhibition of peptide binding and cGMP stimulation were due to activation of PKC, dioctanoylglycerol, an analogue of DAG, the physiological activator of PKC [41], reduced cGMP formation ($IC_{50} = 0.50 \pm 0.07$ μM, $n = 3$) and ^{125}I-ANP binding by 27.8(±3.6)% ($n = 3$) at 10 μM (not shown).

Finally staurosporine, a protein kinase inhibitor [42], was tested. When given to cells 5 min before PMA, concentrations of approx. ⩾ 50 nM staurosporine completely reversed PMA in-

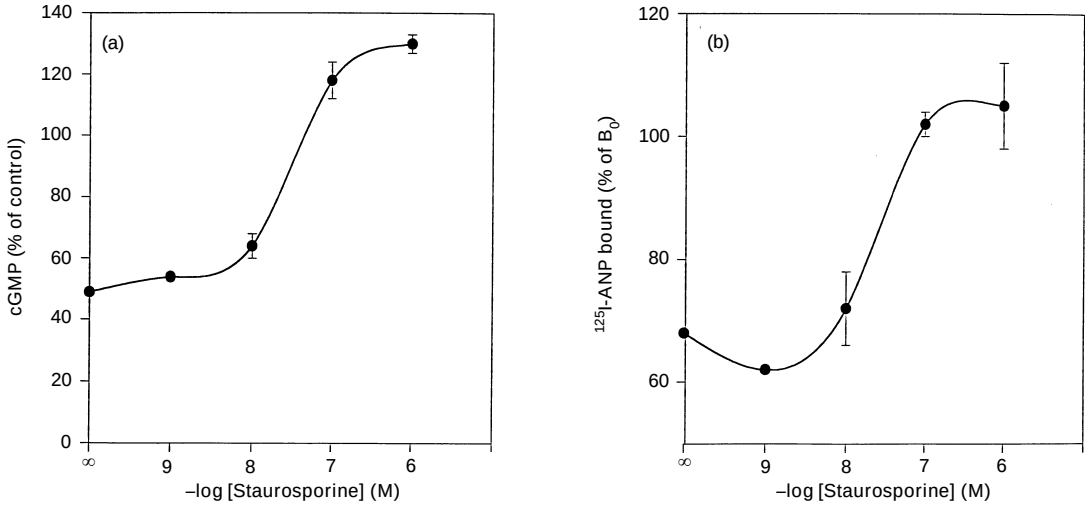

Figure 6 Effect of staurosporine on PMA

Down-regulation of cGMP formation and specific ^{125}I-ANP binding. (**a**) cGMP. Cells exposed to various concentrations of staurosporine for 15 min followed by either 1 μM or no PMA for 15 min at 37 °C were then given 1 μM CNP for 5 min at 37 °C. The reactions were terminated and cGMP assayed as described in the Materials and methods section. Data are expressed as a percentage of controls containing staurosporine but lacking PMA. Data are from a representative experiment (of three with similar results) carried out in duplicate. (**b**) ^{125}I-ANP binding. Cells were exposed to various concentrations of staurosporine for 15 min followed by either 1 μM or no PMA for 15 min at 37 °C and were then given 5 pM ^{125}I-ANP and various concentrations of unlabelled ANP for 4 h at 4 °C. The cells were then analysed as in Figure 4(a). Data are means ± S.D. ($n = 3$).

hibition of cGMP formation (Figure 6a) and completely blocked PMA-induced loss of ANP binding sites (Figure 6b). Complete inhibition of both functions was also seen with 0.5 μM bisindolylmaleimide, a specific PKC inhibitor [43] (not shown).

DISCUSSION

NPR-B and NPR-C on NPE cells

The present data suggest that human ocular NPE cells possess NPR-B and NPR-C. The evidence for NPR-B is, first, that CNP evoked an approx. 10-fold greater response of guanylate cyclase than equivalent concentrations of either ANP or BNP; and second that the NPR-A antagonist, 17915, did not inhibit CNP- or ANP-stimulated cGMP formation in NPE cells, whereas HS-142-1, an antagonist for both NPR-A and NPR-B, inhibited well. Koller et al. [44] reported that COS cells transfected with NPR-B cDNA were approx. 10-fold more responsive to CNP than to either ANP or BNP.

NPR-B is the major guanylate cyclase-containing NPR subtype in choroid plexus [45], pituitary and brain [12], as well as in ciliary epithelium. CNP, the primary ligand for NPR-B, is found primarily in neurons [12,13] and is the most common natriuretic peptide in the brain and in cells from the neural crest [13]. Although CNP localization has not been undertaken in the eye, our results suggest the possibility that CNP will be found in nerves supplying the anterior ocular segment.

^{125}I-ANP binding to NPE cells detected primarily NPR-C, based upon the following observations: first, cross-linked ^{125}I-ANP bound predominantly to a protein(s) of M_r 62000 on denaturing gels, approximately the size of NPR-C receptors [18]; second, over 95% of ^{125}I-ANP binding to NPE cells was displaceable by the NPR-C-specific peptide, c-ANP. Third, all three natriuretic peptides bound to binding sites on NPE cells with high affinity, which is characteristic of NPR-C but not NPR-A or NPR-B [44]. Fourth, binding of ^{125}I-CNP showed similar characteristics to ^{125}I-ANP binding. Thus NPE cells appear to be similar to several cell types in which NPR-C is the numerically predominant NPR subtype [46]. However, cultured aortic smooth-muscle cells have been reported to possess more NPR-C than cells freshly isolated from the same tissue [47]. Thus it remains to be determined whether the relatively high concentration of NPR-C on NPE cells reflects the situation *in vivo*. Binding to NPR-B presumably accounts for $< 5\%$ of total binding in cultured NPE cells.

The fact that both NPR-B and NPR-C are down-regulated by PKC, as well as by histamine and vasopressin (which activate the IP/DAG pathway [35]), suggests that stimulation of the DAG arm of the IP/DAG pathway mediates down-regulation of both NPR subtypes. Other examples of heterologous regulation of NPRs have been described in several tissues [48,49]. Data are discussed below that suggest that PKC down-regulates NPR-B and NPR-C by different mechanisms.

Down-regulation of NPR-B by PKC

The finding that the extent of PKC inhibition of NPR-B varied with the CNP concentration indicated that increased extracellular CNP could partially offset PMA inhibition. This could be due to either (i) reduction of the extracellular concentration of CNP; or (ii) a decreased binding affinity of NPR-B for CNP.

Two mechanisms by which reduction of extracellular CNP could occur are (1) enhanced NPR-C activity resulting in increased clearance (and degradation) of CNP [37]; and (2) increased neutral endopeptidase activity resulting in extracellular hydrolysis of CNP. NPR-C appears to play a small role since c-ANP had no effect on inhibition of cGMP formation by PMA. It did, however, cause an increase in control cGMP formation, suggesting that NPR-C activity may influence NPR-B under normal circumstances. Neutral endopeptidase likewise appears not to play a role in PMA inhibition of guanylate cyclase activity, since phosphoramidon had no detectable effect on PMA inhibition of CNP-stimulated cGMP formation. Finally, direct measurement of CNP in the extracellular medium failed to detect

changes in CNP concentration as a function of PMA treatment. Taken together, these findings suggest that neither NPR-C nor neutral endopeptidase strongly influence the effect of PKC on CNP-stimulated guanylate cyclase.

If PKC does not exert its effects on NPR-B by altering the extracellular CNP concentration, it might do so via a modification of NPR-B itself. A model for NPR-A inactivation has been proposed by several laboratories [50–52]. A salient feature of this model is that molecular events subsequent to ligand binding to NPR-A decrease the affinity of the receptor for ligand. NPR-A has also been reported to be inactivated by PKC-dependent dephosphorylation of the receptor [52]. We speculate that NPR-B may also be covalently modified by PKC, leading to a reduced binding affinity of NPR-B for CNP. Since CNP also binds strongly to NPR-C (Figure 3a), and since NPR-C comprises the overwhelming majority of NPR sites on NPE cells, we have been unable to directly measure the K_D for ^{125}I-CNP binding to NPR-B. However, abrogation of receptor inhibition by increased ligand concentration would be very difficult to explain by a mechanism involving loss of NPR-B binding sites. On the other hand, a mechanism involving reduction of NPR-B binding affinity for CNP would predict such a result.

Down-regulation of NPR-C by PKC

In contrast with the effect of PMA on NPR-B, inhibition of NPR-C by PMA did not involve altered receptor affinity for ligand. Rather, a decrease in NPR-C binding sites resulted in reduced ligand binding. Although down-regulation of NPRs has been described in other tissues [48,49], this to our knowledge is the first report of differential regulation of NPR subtypes in a single cell type.

In summary, the data suggest that NPR-B and NPR-C are both down-regulated by PKC, but by different mechanisms. NPR-B appears to be down-regulated via decreased affinity of receptor for ligand. NPR-C down-regulation involves reduction of the number of NPR-C binding sites, with little change in ligand affinity for this receptor subtype.

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