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DESENSITIZATION OF ATRIOPEPTIN STIMULATED ACCUMULATION AND EXTRUSION OF CYCLIC GMP FROM A KIDNEY EPITHELIAL CELL LINE (MDCK)

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Abstract—Atriopeptin caused dose- (EC_{50} *ca.* 2×10^{-8} M) and time-dependent increases in the intracellular concentration of cyclic GMP in the MDCK kidney epithelial cell line; an effect potentiated by the phosphodiesterase inhibitor, IBMX. The atriopeptin-catalysed increase in cyclic GMP was transient and reached a maximum some 10–20 min after challenge of cells with atriopeptin. The basis for the transience of this increase was shown to be due to the densensitization of guanylate cyclase coupled with extrusion of cyclic GMP from the cells and the degradation of cyclic GMP by phosphodiesterase activity. Atriopeptin-catalysed extrusion of cyclic GMP was time- and dose-(EC_{50} *ca.* 1.5×10^{-8} M) dependent and was inhibited by probenecid but not by high external cyclic GMP concentrations. The extrusion process underwent apparent desensitization as did guanylate cyclase with similar half lives ($T_{\frac{1}{2}}$ of *ca.* 20 min). Desensitization was dose-dependent upon atriopeptin and did not appear to be mediated by elevated cyclic GMP concentrations as pre-incubation with 8-bromo cyclic GMP did not cause desensitization and the half-times for desensitization were similar whether or not IBMX was present. The majority of the cyclic nucleotide phosphodiesterase activity was found in the cytosol fraction of the cells and could be separated into two cyclic AMP specific forms and two cyclic GMP preferring forms.

Atrial natriuretic factor (ANF‡) is synthesized as a prepropeptide which is processed and stored in the atria as a propeptide until release into the circulation in the active form. This can occur in response to atrial distention or pressure, increases in sodium concentration or hormone induced increases in blood pressure [1, 2]. The biologically active form of ANF is the C-terminal fragment, ANF (99–126), with an intact disulphide bridge Cys^{105}—Cys^{212} and amino acids Phe^{124} and Arg^{125} being crucial for receptor recognition [3, 4]. ANF is believed to contribute to the regulation of blood pressure by promoting natiuresis and diuresis in the kidney and relaxation of vascular smooth muscle cells [1, 5–7]. In addition, ANF has been shown to inhibit the release of aldosterone from the adrenal glands [8, 9], vasopressin from the neurohypophysis [10, 11] and renin from the kidney [12, 13].

ANF binds to cell surface receptors which also express guanylate cyclase activity [14, 15]. Thus, in a variety of cell types [16–20], occupancy of ANF receptors leads to the accumulation of cyclic GMP. It is thus presumed that the biological action of ANF is mediated through the production of cyclic GMP which has been demonstrated to activate a cyclic GMP specific kinase and can also, in certain cells, regulate Ca^{2+}-channel opening [21]. Indeed, there is now good evidence to show that activation of a cyclic GMP-specific kinase [22], by elevated cyclic GMP concentrations, can modulate muscle tone in smooth muscle cells in a process involving the phosphorylation of specific proteins [23–25].

ANF injection/infusion *in vivo*, however, leads to a rapid and marked increase in plasma and urinary cyclic GMP in rats [26–28], monkeys [29], dogs [30] and humans [31]. This may result from the extrusion of cyclic GMP from cells, a feature shown originally [32] in liver challenged with nitrosamine although, more recently, observed for smooth muscle cells [20] challenged with ANF.

The kidney is a key organ which is directly involved in blood pressure regulation and is known to express receptors for ANF [15]. As a model system for evaluating ANF-stimulated cyclic GMP metabolism related to the kidney we have chosen to investigate a kidney epithelial cell line (MDCK). Here we show that atriopeptin, representing fragment 5–28 of ANF, can stimulate cyclic GMP formation in a dose- and time-dependent fashion through a process which undergoes desensitization. Desensitization of cyclic GMP production coupled with the extrusion of cyclic GMP from the intact cells and action of phosphodiesterase, leads to the intracellular levels of cyclic GMP being only transiently elevated upon atriopeptin challenge of these cells.

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‡ Abbreviations: ANF, atrial natriuretic factor; atriopeptin here refers to atriopeptin III being fragment 5–28 of ANF; EC_{50}, concentration at which 50% of the maximal effect was elicited.

MATERIALS AND METHODS

Materials. MDCK and MDBK cells were obtained from ECACC, PHLS Centre for Applied Microbiology and Research (Porton Down, U.K.). [^{125}I]Cyclic 3′5′ GMP radioimmunoassay assay kits and [^{14}C]guanine were purchased from Du Pont and [^{125}I]rat ANP (99–126) from Amersham International plc (Amersham, U.K.). Atriopeptin representing fragment 5–28 of ANF, was obtained from Cambridge Research Biochemicals (Cambridge, U.K.) and peptidase inhibitors, IBMX and Dowex resin from Sigma Chemical Co. (Poole, U.K.). All tissue culture media were purchased from Gibco Ltd (Uxbridge, U.K.) and foetal calf serum from Imperial Laboratories. Probenicid was a gift from Pfizer Central Research (Sandwich, U.K.).

Cell culture. MDCK cells were maintained in Dulbecco's Modification of Eagle Medium (DMEM), supplemented with 10% foetal calf serum, glutamine (2 mM) and penicillin/streptomycin (10 I.U./mL) in a humidified atmosphere of 95% air/5% CO_2 at 37°. For both cyclic GMP and cyclic AMP assays, the cells were seeded onto 24-well culture plates at a density of 2×10^4 cells per well and grown for 4–7 days. The medium was replaced with fresh medium on the day prior to assay.

Treatment of cells for determination of intracellular and extracellular cyclic GMP. The cells were washed three times with serum-free medium before incubation at 37° with atriopeptin (0.5 mL) at the concentrations and for the times specified in the figure legends. Atriopeptin was made up in serum-free medium containing trypsin inhibitor (5 mg/mL), aprotinin (1.1 I.U./mL) and pepstatin A (10 μg/mL). The reactions were stopped by removal of serum-free medium containing atriopeptin and cyclic GMP extracted from the cells with ethanol for determination of intracellular levels. When extracellular cyclic GMP was to be measured, the supernatants from the culture wells were collected and boiled for 3 min, with a 100 μL sample then being taken for assay. All samples were stored at −20° until assayed by radioimmunoassay. For each experiment, the cells from 3–6 wells were counted.

Determination of intracellular volumes was done on samples of *ca.* 2×10^5 cells suspended in 120 μL of medium containing 0.2 μCi/mL [^{3}H]H_2O and 0.4 μCi/mL U-[^{14}C]sucrose. This was layered on 0.5 mL silicone oil ($d = 1.045$) with an underlay of 100 μL 10% (v/v) TCA. Samples were centrifuged at 12,000 g_{av} for 20 sec and the upper phase and pellet removed for liquid scintillation counting. Counts in the pellet allowed for the determination of intracellular volume based upon [^{3}H]H_2O corrected for extracellular carry over of fluid as assessed by [^{14}C]sucrose. Eighty-seven per cent of the intracellular fluid was taken to be cytosolic.

Radioimmunoassay of cyclic GMP. Cyclic GMP was measured by a radioimmunoassay procedure using a kit from Du Pont as described in Ref. 33. Briefly, intracellular cyclic GMP samples were evaporated to dryness and resuspended in sodium acetate buffer, pH 6. Extracellular cyclic GMP samples were assayed directly. Samples were diluted as required and an aliquot (100 μL) was acetylated with a 2:1 mixture of triethylamine and acetic anhydride (5 μL), before incubation at 4° for 16–18 hr in the dark with cGMP anti-serum complex (100 μL) and succinyl cyclic GMP Tyrosine Methyl Ester-[^{125}I] in normal rabbit serum (100 μL). Following incubation, bound ligand was separated from free by addition of sodium acetate buffer (1 mL) and centrifugation for 15 min at 4° at approximately 15,000 *g*. Supernatants were decanted, pellets solubilized with 5 M NaOH and transferred to gamma counter tubes. Results are expressed as pmoles cyclic GMP formed per 10^6 cells.

[^{14}C]*Guanine labelling of cells for cyclic GMP determinations.* In order to determine changes in intracellular cyclic GMP concentrations and of extrusion in the presence of added extracellular cyclic GMP a [^{14}C]guanine labelling method was used [34]. MDCK cells were labelled with [^{14}C]guanine (2.5 μCi/well) in serum-free medium for 2 hr in order to achieve isotopic equilibrium. The cells were then washed and incubated with various concentrations of atriopeptin for 2 hr in the presence of peptidase inhibitors. Incubations were terminated by the removal of extracellular medium. [^{14}C]Cyclic GMP extruded into the media was purified on 1.2 × 4 cm Dowex columns (Dowex 50W, X2,200–400) by a modification of the procedures used to purify cyclic AMP. Briefly, columns were first equilibrated with 0.1 N HCl and washed with H_2O. Aliquots of 10% TCA (0.5 mL) and the internal standard [^{3}H]cyclic GMP were added to the extracellular samples before centrifugation in a microfuge for 5 min at 13,000 g_{av}. Supernatants were applied to the columns and washed with 4.4 mL HCl (0.1 N) and 1.5 mL H_2O. Cyclic GMP was then eluted with a further 4 mL of H_2O. GDP and GTP remaining in the eluates were precipitated with $CaCO_3$ and $FeSO_4$ (final concentration of both were 0.06 M). The samples were centrifuged for 5 min in a microfuge at 13,000 g_{av} and supernatants counted. Results were corrected for [^{3}H]cyclic GMP and expressed as dpm/10^6 cells.

Treatment of cells with 8-bromo cyclic GMP. MDCK cells were grown for 4 days in complete medium. The cells were then washed three times with serum-free medium and incubated with 8-bromo cyclic GMP (100 μM) in serum-free medium for 2 hr. Following 8-bromo cyclic GMP treatment, the cells were washed three times in serum-free medium and then incubated with atriopeptin (10^{-6} M) in the presence and absence of IBMX (0.5 mM) for 10 min. Atriopeptin was then removed, reactions stopped and intracellular cyclic GMP extracted by the addition of ethanol (1 mL) for determination by radioimmunoassay as described above. Intracellular and extracellular cyclic GMP accumulation were measured and the results expressed as pmol/10^6 cells.

[^{3}H]*Adenine labelling of cells for cyclic AMP determinations.* Measurement of cyclic AMP was achieved by labelling the cells with [^{3}H]adenine (1 μCi/well in 0.5 mL complete medium) for 2 hr at 37° in order to achieve isotopic equilibrium, prior to incubation with agonist/antagonist, made up in serum-free medium, at the concentrations and for the times specified in the text and legends. The agonist was then removed, reactions stopped and [^{3}H]cyclic AMP extracted with

5% TCA containing [^{14}C]cyclic AMP as an internal standard. [^{3}H]Cyclic AMP formed was purified by sequential chromatography on Dowex and alumina columns [35]. The results were expressed as dpm per 10^6 cells.

Incubation of cells with toxins. MDCK cells were seeded into 24-well culture plates and grown for 3 days in complete medium. The cells were then incubated for 17 hr in the presence of either cholera toxin (100 μg/mL) or pertussis toxin (1 μg/mL). Following toxin treatment, the cells were washed three times with serum-free medium before incubation with atriopeptin (10^{-6} M) for 10 min, in the presence of peptidase inhibitors as described above. Both intracellular and extracellular cyclic GMP formed were assayed and the results expressed as pmol cyclic GMP/10^6 cells.

Assay of cyclic nucleotide phosphodiesterase activity. This utilized a modified version [36], employing a freshly prepared slurry of Dowex:H_2O:ethanol (1:1:1) for extraction, of the two-step procedure of Thompson and Appleman [37] as referred to by us previously [38]. Assays were done at 30° using either [^{3}H]cyclic AMP or [^{3}H]cyclic GMP as substrate, at the indicated final concentrations of these cyclic nucleotides. Initial rates were evaluated from linear time-courses performed over 15 min.

Resolution of cyclic nucleotide phosphodiesterase activity by FPLC. This was performed as described in some detail by us previously for studies performed on liver [39], hepatocytes [39] and kidney [40]. In this instance, two roller bottle cultures of cells were homogenized in 20 mL of homogenization buffer (50 mM Tris–HCl final pH 7.5, 0.25 M sucrose, 5 mM benzamidine, 0.2 mM PMSF, 20 μM leupeptin, 0.1 mM EGTA and 0.1 mM dithiothreitol) using a teflon upon glass homogenizer. The homogenate was centrifuged at 100,000 g_{av} for 60 min and the supernatant applied at a rate of 1 mL/min to a Mono Q column (1 mL) which had previously been equilibrated in buffer B (50 mM Tris–HCl, 5 mM benzamidine, 0.2 mM PMSF, 2 μM leupeptin, 0.1 mM EGTA and 0.1 mM dithiothreitol, final pH 7.2). The column was then washed with 100 mL of buffer B at 1 mL/min. We assessed that over 95% of phosphodiesterase activity, using either cyclic GMP or cyclic AMP as substrate, had been bound to the column. Elution was then done at 1 mL/min using a NaCl gradient in buffer B at final pH 7.2, with gradient details being given in the legend to Fig. 6; over 93% of the bound phosphodiesterase activity was eluted under such conditions using either cyclic AMP or cyclic GMP as substrate.

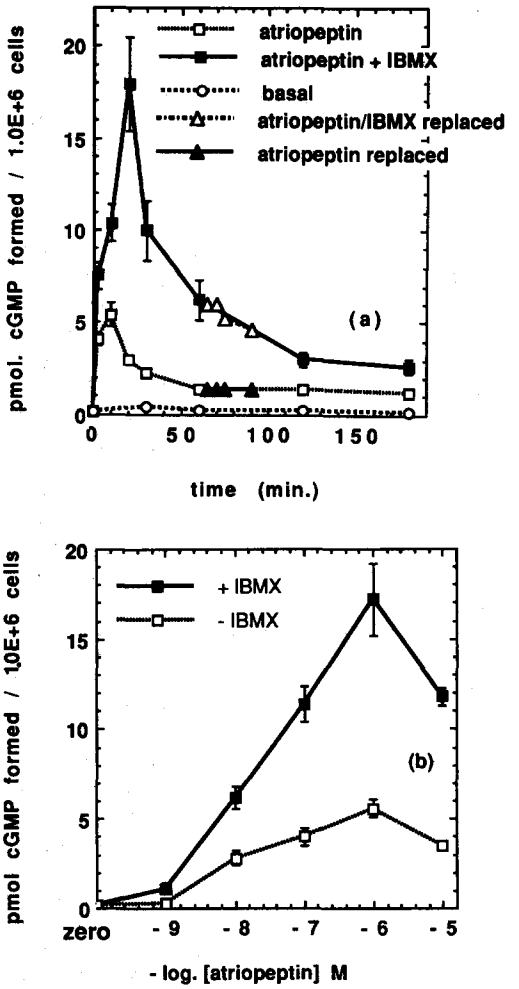

Fig. 1. Atriopeptin-stimulated intracellular cyclic GMP accumulation. (a) Time-course of atriopeptin (10^{-6} M)-stimulated intracellular cyclic GMP levels in MDCK cells done in the absence (····□ ····) and presence (—■—) of 0.5 mM IBMX as well as basal levels done in the absence of IBMX (---○---). For another portion of cells (atriopeptin + IBMX replaced), medium was removed some 60 min after challenged with those agents and fresh medium then added with sampling for the determination of intracellular cyclic GMP being made at the times indicated (--△--). For cells (pre-)incubated with atriopeptin alone then, over a 6 min period, they were washed three times with serum free medium, prior to addition of fresh atriopeptin and further incubation for the determinations of cyclic GMP accumulation made (—▲—). (b) Dose–effect curve of intracellular cyclic GMP accumulation into MDCK cells incubated with the indicated concentrations of atriopeptin for 10 min in the absence (···· □····) and presence (—■—) of 0.5 mM IBMX. These figures are representative of an experiment done on at least three separate occasions with triplicate determinations of cyclic GMP (means ± SD).

RESULTS

Intracellular accumulation of cyclic GMP in MDCK cells challenged with atriopeptin

Addition of atriopeptin (10^{-6} M) to MDCK cells initiated a rapid, transient increase in the intracellular concentration of cyclic GMP which attained maximal levels after about 10 min (Fig. 1a).

The presence of IBMX (0.5 mM) potently amplified the intracellular accumulation of cyclic GMP achieved by atriopeptin challenge (Fig. 1a). Again, however, the increase in intracellular cyclic GMP was transient, reaching a maximal value some 20 min after the addition of atriopeptin from which it rapidly declined (Fig. 1a). After 120 min the new steady state level of intracellular cyclic GMP was approximately double that achieved in the absence of IBMX (Fig. 1a). Maximal accumulation of cyclic GMP in the

presence of both atriopeptin and IBMX was some 2.5- to 4.0-fold greater than that achieved when challenging cells with atriopeptin alone (Fig. 1a). Addition of IBMX (0.5 mM) alone had little effect (less than 15%) on basal levels of cyclic GMP, indicating that basal guanylate cyclase activity was vanishingly small.

Atriopeptin stimulated the intracellular accumulation of cyclic GMP in a dose-dependent fashion with maximal effects occurring at 10^{-6} M (Fig. 1b). EC_{50} values for atriopeptin action were found to be $2.5 \pm 0.8 \times 10^{-8}$ M in the presence and $1.6 \times 0.5 \times 10^{-8}$ M in the absence of IBMX (errors are SD for N = 3 different experiments). When IBMX (0.5 mM) was added, this was done together with atriopeptin at the start of the experiment. No differences were detected upon pre-incubation of cells with IBMX for up to 16 min beforehand, suggesting that IBMX rapidly equilibrated with the cell interior.

Desensitization of intracellular cyclic GMP accumulation

If MDCK cells were first incubated with atriopeptin (10^{-6} M) and IBMX (0.5 mM), washed three times with serum-free medium and then re-challenged with atriopeptin (10^{-6} M) either in the presence or absence of IBMX (0.5 mM), then in neither instance did cells show any response in the form of elevation in intracellular cyclic GMP levels (Fig. 1a). We also 'pulsed' cells with micromolar additions of atriopeptin, at 5–10 min intervals, through the time-course. This had no effect on the transient nature of the accumulation of intracellular cyclic GMP despite the ability of these atriopeptin solutions to stimulate cyclic GMP accumulation in virgin cells (data not shown).

This desensitization was time-dependent (Fig. 2a), yielding half-times of $T_{\frac{1}{2}} = 29 \pm 5$ and 25 ± 8 min for experiments done in the presence and absence of 0.5 mM IBMX, respectively, and was dose-dependent (Fig. 2b), with EC_{50} values of $1.0 \pm 0.3 \times 10^{-8}$ M and $2.1 \pm 0.4 \times 10^{-8}$ M atriopeptin in the presence and absence of 0.5 mM IBMX, respectively (N = 3 separate experiments; errors are SD).

Extrusion of cyclic GMP from MDCK cells challenged with atriopeptin

Atriopeptin also caused the time-dependent extrusion of cyclic GMP from MDCK cells (Fig. 3a) with the addition of IBMX (0.5 mM) having little effect on the rate of extrusion.

In the absence of added atriopeptin we failed to observe the exit of cyclic GMP from the MDCK cells (rates being at least 100 times lower than those determined in the presence of atriopeptin). IBMX treatment alone did not cause stimulated exit of cyclic GMP in the absence of atriopeptin.

Atriopeptin elicited extrusion of cyclic GMP in a dose-dependent fashion (Fig. 3b) with EC_{50} values of $1.6 \pm 0.8 \times 10^{-8}$ M and $1.3 \pm 0.6 \times 10^{-8}$ M in the presence and absence of 0.5 mM IBMX, respectively (errors are SD for three separate experiments).

Over the time-course of incubation with atriopeptin over a 2 hr period the cells remained viable as indicated by trypan blue extrusion, maintenance of intracellular ATP levels and latent lactate dehydrogenase activity evidenced upon cell breakage.

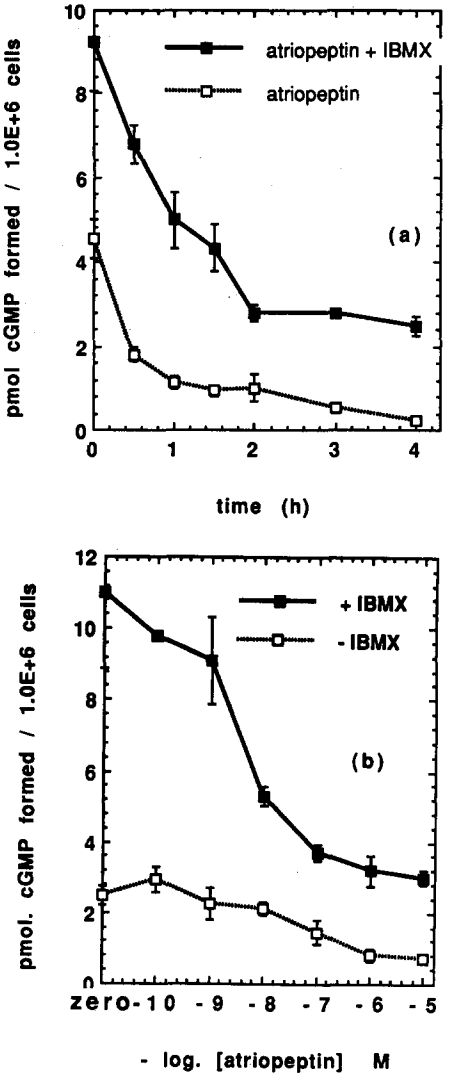

Fig. 2. Desensitization of atriopeptin-stimulated intracellular cyclic GMP accumulation. (a) Cells were incubated with atriopeptin (10^{-6} M) for the indicated times in either the absence (····□··· ·) or presence (—■—) of 0.5 mM IBMX. Cells were then washed rapidly three times with serum free medium and re-challenged with atriopeptin (10^{-6} M), either in the presence or absence of IBMX, for 10 min prior to determination of intracellular cyclic GMP accumulation. (b) MDCK cells were pre-incubated for 2 hr with the indicated concentrations of atriopeptin, either in the absence (····□··· ·) or presence (—□—) of IBMX (0.5 mM). Cells were then washed three times with serum free medium and re-challenged for 10 min with atriopeptin (10^{-6} M) in either the presence or absence of IBMX prior to determination of intracellular cyclic GMP. These figures are representative of an experiment done on three separate occasions with triplicate determinations of cyclic GMP (means ± SD).

Cyclic GMP can be extruded against a concentration gradient

Cells were loaded with [^{14}C]guanine and then challenged with various concentrations of atriopeptin in

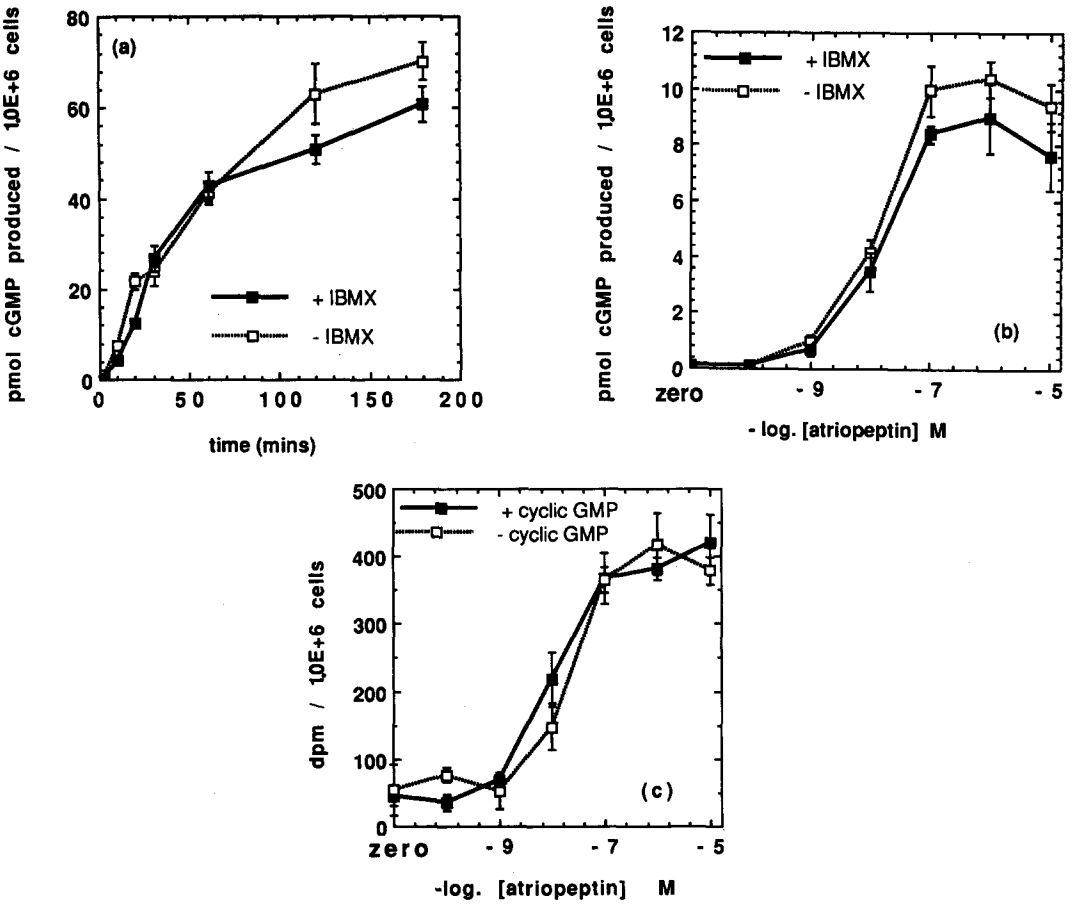

Fig. 3. Extrusion of cyclic GMP from MDCK cells challenged with atriopeptin. (a) Cells were challenged with atriopeptin (10^{-6} M) in either the absence (····□····) or presence (—■—) of 0.5 mM IBMX. Cyclic GMP in the medium was then evaluated at the indicated times. (b) Cells were challenged with the indicated concentrations of atriopeptin prior to harvesting after 10 min for determination of extracellular cyclic GMP concentrations. Under these conditions linear rates of extrusion were observed (data not shown). Experiments were done in either the absence (····□····) or the presence (—■—) of 0.5 mM IBMX. (c) In this instance extracellular cyclic GMP extrusion was evaluated by first labelling the cells with [^{14}C]guanine and then analysing the atriopeptin-stimulated egress of [^{14}C]cyclic GMP. This was done in the absence (····□····) and presence (—■—) of unlabelled extracellular cyclic GMP (5 mM), using the indicated concentrations of atriopeptin after a 2 hr incubation. These figures are representative of an experiment done on three separate occasions with triplicate determinations of cyclic GMP (means ± SD).

the presence of 0.5 mM IBMX prior to determining [^{14}C]cyclic GMP efflux. The dose–effect curve (Fig. 3c) obtained using this alternative assay procedure was comparable to those determined using the radioimmunoassay procedure (Fig. 3b) and yielded an EC_{50} value of $2.5 \pm 0.4 \times 10^{-8}$ M (N = 3 experiments, SD). The ability of atriopeptin to elicit the extrusion of cyclic GMP was unaffected by the inclusion of 5 mM cyclic GMP into the extracellular medium (Fig. 3c); the maximal level of extrusion was similar as was the EC_{50} value for activation at $1.1 \times 0.7 \times 10^{-8}$ M (N = 3 separate experiments, errors are SD).

Inhibition of cyclic GMP extrusion by probenecid

Probenecid caused the dose-dependent inhibition (Fig. 4a) of cyclic GMP extrusion both in the absence and presence of IBMX (0.5 mM), with EC_{50} values of $1.3 \pm 0.5 \times 10^{-3}$ M and $2.0 + 0.5 \times 10^{-3}$ M, respectively (three experiments; errors are SD). At the same time, probenecid elicited a marked increase in the intracellular accumulation of cyclic GMP (Fig. 4b). This was most marked in experiments where IBMX was present, to inhibit cyclic GMP phosphodiesterase activity, yielding an EC_{50} value for this process of $1.3 \pm 0.8 \times 10^{-3}$ M. In the absence of IBMX then probenecid also exerted a potentiation of the atriopeptin-elicited increase in levels of intracellular cyclic GMP (Fig. 4b) with an EC_{50} value for this process of $0.81 \pm 0.12 \times 10^{-3}$ M (N = 3 separate experiments; errors are SD).

Effect of re-challenging MDCK cells with atriopeptin on cyclic GMP extrusion

When cells were incubated with atriopeptin (10^{-6} M) for various times, washed in serum free

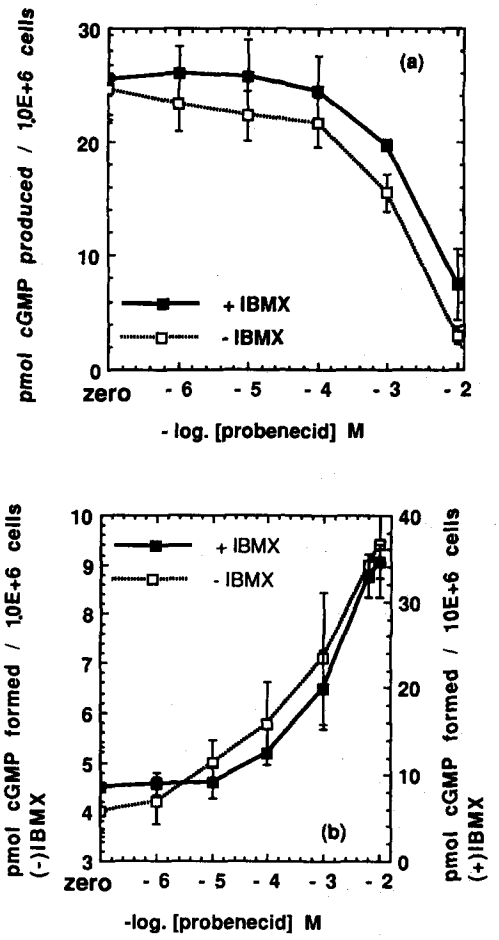

Fig. 4. Action of probenecid on the extrusion and accumulation of cyclic GMP. (a) MDCK cells were incubated with various concentrations of probenecid in either the presence (—■—) or absence (····□····) of 0.5 mM IBMX. In each case cells were challenged with atriopeptin (10^{-6} M) for 30 min prior to the determination of extracellular cyclic GMP. (b) Experiments done as in (a) but cells taken for the determination of intracellular cyclic GMP. These figures are representative of an experiment done on three separate occasions with triplicate determinations of cyclic GMP (means ± SD). In subsequent experiments we found that the addition of 40 mM probenecid did not cause any further inhibition.

medium and then re-challenged with atriopeptin (±IBMX) (Fig. 5) the rate of extrusion was markedly attenuated. This desensitization exhibited a half-life ($T_{\frac{1}{2}}$) of 20 ± 4 and 23 ± 5 min for experiments done in the presence and absence of IBMX, respectively (N = 3 separate experiments; errors are SD) and was dose-dependent, with EC_{50} values of 1.5 ± 0.8 × 10^{-8} M and 0.9 ± 0.5 × 10^{-8} M atriopeptin in the presence and absence of 0.5 mM IBMX, respectively (N = 3 separate experiments; errors are SD).

Atriopeptin-stimulated cyclic GMP production in MDCK cells pre-treated with cyclic GMP and other agents

MDCK cells were pre-incubated with 8-bromo cyclic GMP (100 μM) for 2 hr prior to its removal by washing. They were then challenged with atriopeptin (10^{-6} M) in the presence and absence of IBMX (0.5 mM) for 10 min. Intracellular accumulation in the presence of IBMX and atriopeptin was 10.2 ± 1.4 pmol/10^6 cells in control cells and 10.9 ± 1.7 pmol/10^6 cells in 8-bromo cyclic GMP pre-treated cells. For assays done in the absence of IBMX the respective values were 3.0 ± 0.4 and 3.2 ± 0.5 pmol/10^6 cells. Labelled cyclic GMP found extracellularly for cells challenged with IBMX and atriopeptin was 2.3 ± 0.7 pmol/10^6 cells using control cells and 2.9 ± 0.2 pmol/10^6 cells for cells which had been pre-treated with 8-bromo cyclic GMP (N = 3 separate experiments; errors are SD).

Incubation of cells with either forskolin or PGE_1 failed to exert any effect (less than 10%) on either the accumulation or extrusion of cyclic GMP elicited by atriopeptin (10^{-6} M) plus 0.5 mM IBMX. Treatment of cells with either cholera or pertussis toxin failed (less than 10% difference) to alter the atriopeptin (±IBMX)-induced accumulation or extrusion of cyclic GMP.

Cyclic nucleotide phosphodiesterase activity

Homogenates of MDCK cells exhibited both cyclic AMP and cyclic GMP phosphodiesterase activity. Over 90% of the cyclic GMP phosphodiesterase activity was found in the cytosol (100,000 *g* supernatant) fraction. This activity could be resolved on an FPLC Mono-Q column (Fig. 6), yielding an elution pattern similar to that we observed for kidney cytosol extracts [40]. This identified two cyclic AMP specific fractions eluting at the high ionic strength region of the gradient and two cyclic GMP preferring enzymes which eluted at lower ionic strength (Fig. 6). The activity of only the first eluted phosphodiesterase was stimulated (2.5–3.0-fold) by addition of Ca^{2+}

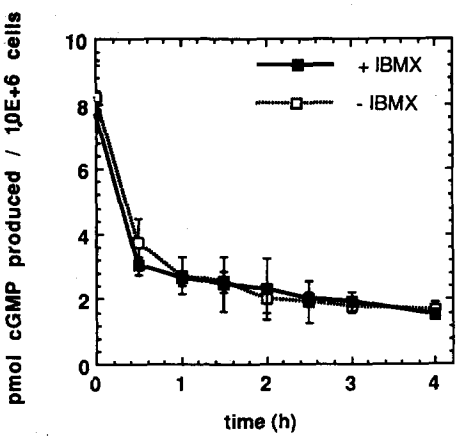

Fig. 5. Effect of re-challenge of MDCK cells with atriopeptin on cyclic GMP extrusion. Cells were incubated with atriopeptin (10^{-6} M) for the times specified prior to washing three times with serum-free medium, re-challenging with atriopeptin (10^{-6} M) for 10 min and determination of extracellular cyclic GMP. Experiments were done by pre-incubating and then incubating in the presence (—■—) and absence (····□····) of 0.5 mM IBMX. These figures are representative of an experiment done on three separate occasions with triplicate determinations of cyclic GMP (means ± SD).

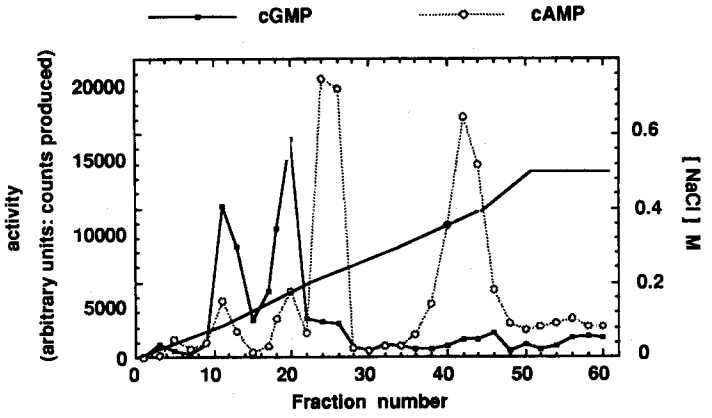

Fig. 6. FPLC separation on a Mono-Q column of MDCK cell cyclic nucleotide phosphodiesterase activity. MDCK cells were homogenized and subjected to centrifugation as described in Materials and Methods. A portion of the high speed supernatant was applied to a Mono-Q column whereupon all the cyclic GMP and cyclic AMP phosphodiesterase activity bound. Elution was done using the indicated NaCl gradient whilst following the cyclic AMP (····◇····) and cyclic GMP (—■—) phosphodiesterase activities at 0.1 μM substrate concentrations. Recovery of activities was greater than 90%. Data is a typical experiment of one done three times. These figures are representative of an experiment done on three separate occasions with triplicate determinations of cyclic GMP (means ± SD).

(50 μM) and calmodulin (200 nM). Neither of the activities of the cyclic AMP-specific enzymes, when assayed with 1 μM cyclic AMP, was inhibited by the presence of 2 μM cyclic GMP. Conversely, neither of the activities of the cyclic GMP-preferring enzymes, when assayed at 1 μM cyclic GMP, was inhibited by 2 μM cyclic AMP.

With 0.1 μM cyclic GMP as substrate then over 97% of cytosol phosphodiesterase activity was inhibited by 0.5 mM IBMX. This value fell to 88–92% (range, N = 3) employing 1 μM substrate and 72–76% (range, N = 3) employing 10 μM cyclic GMP as substrate. Determination of the intracellular volume of MDCK cells allowed us to calculate that some 20 min after exposure of MDCK cells to atriopeptin and IBMX, then intracellular cyclic GMP concentrations would be some 3–4 μM cyclic GMP, assuming equilibration throughout the cell. At such levels of cyclic GMP then it is evident that 0.5 mM IBMX would be capable of inhibiting phosphodiesterase activity only by *ca.* 80%.

When cells were incubated with 1 μM atriopeptin and 0.5 mM IBMX for periods of 10 to 60 min and then harvested and homogenized, we found no identifiable (less than 8%) difference in homogenates cyclic GMP phosphodiesterase activity.

Incubation of intact cells with [^{3}H]cyclic GMP (0.1–1 μM) failed to show degradation of this ligand, indicating the absence of extracellular phosphodiesterase activity.

Intracellular cyclic AMP accumulation in MDCK cells

Only when IBMX (0.5 mM) was present in the incubation medium did challenge of MDCK cells with either forskolin or PGE_2 elicit a marked time-dependent increase in the intracellular accumulation of cyclic AMP (Fig. 7). However, addition of 0.5 mM IBMX alone failed to increase intracellular cyclic AMP concentrations.

Atriopeptin addition over a range of concentrations (10^{-10}–10^{-5} M) failed to elicit any change in the intracellular concentration of cyclic AMP of cells challenged with PGE_1 together with IBMX.

DISCUSSION

Early studies have shown that cyclic GMP could be extruded from norepinephrine-stimulated pineal and posterior pituitary glands [41] and from carbamoyl choline-stimulated pancreatic lobules [42]. The first cell studies, however, were done on hepatocytes where *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine was shown to stimulate cyclic GMP production and extrusion [32]. More recently, however, cyclic GMP extrusion has been noted in vascular smooth muscle and endothelial cells challenged with either nitroprusside [43] or ANF [20]. Here we have shown that challenge of a dog kidney epithelial cell line (MDCK) with atriopeptin leads to a marked increase in intracellular cyclic GMP concentrations (Fig. 1). This increase in cyclic GMP is a transient phenomenon, peaking around 10–15 min after atriopeptin challenge and which can be dramatically amplified in the presence of the non-specific cyclic nucleotide phosphodiesterase inhibitor, IBMX (Fig. 1).

We believe that the transient nature of the cyclic GMP response to atriopeptin is due to a number of novel factors that we have identified. One of these is desensitization, which we can demonstrate by washing cells free of atriopeptin and re-challenging with this peptide. Using such a procedure we observed (Fig. 2) a time- and dose-dependent loss in the ability of atriopeptin to stimulate cyclic GMP accumulation. Furthermore, pre-incubation of cells with 8-bromo cyclic GMP did not mimic desensitization, indicating that it is unlikely to occur as a consequence of cyclic GMP elevation. Consistent with this are our observations that the half-times

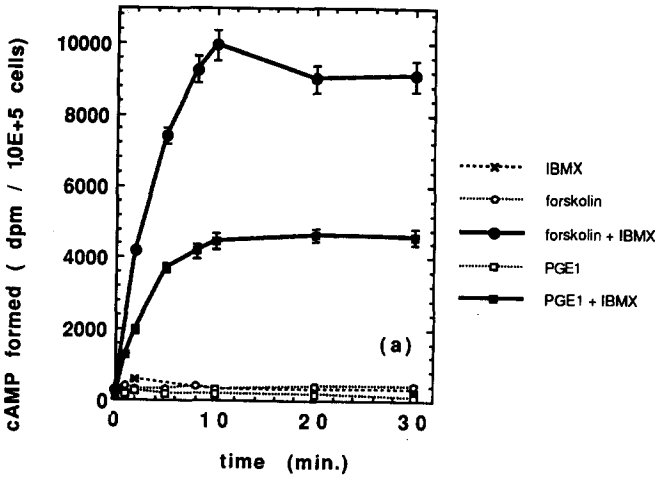

Fig. 7. Cyclic AMP accumulation in MDCK cells. Cells were pre-incubated with [^{14}C]adenine and the production of ^{14}C-labelled intracellular cyclic AMP determined. Cells were challenged with either 10^{-4} M forskolin (—●—, ····○····) or 10^{-5} M PGE_1 (—■—, ····□ ····) in the presence (—●—, —■—) or absence (···○···, ···□···) of 0.5 mM IBMX and then incubated for the indicated times prior to the determination of intracellular cyclic GMP concentrations. Basal cyclic GMP was also determined in the presence (– – × – –) of 0.5 mM IBMX. These figures are representative of an experiment done on three separate occasions with triplicate determinations of cyclic AMP (means ± SD).

for atriopeptin-catalysed desensitization were similar whether or not IBMX was added with atriopeptin despite such a treatment yielding very different increases in intracellular cyclic GMP concentrations (Fig. 2). It may be that desensitization results from agonist occupancy causing receptor down-regulation. We have, however, been unable to address this point due to the vanishingly small numbers of atriopeptin receptors on this cell line that have been observed by others [19] and ourselves (M. Woods and M. D. Houslay, unpublished).

A further contributing factor to the transient nature of cyclic GMP accumulation is our novel observation that, after challenge of cells with atriopeptin, cyclic GMP is actively extruded from the cell (Fig. 3) in a time- and dose-dependent fashion. In this instance, the initial rapid and linear rate of extrusion is tempered as intracellular levels of cyclic GMP begin to fall (Figs 2 and 3). This shows that the reduced intracellular accumulation seen over this period is not due to any activation of the extrusion system. We therefore suggest that the transient nature of intracellular accumulation is due to the concerted action of three processes namely desensitization, degradation by cyclic GMP phosphodiesterases and a continuing egression (transporter) activity.

The extrusion system identified in these cells appears to transport cyclic GMP actively out of the cell as egress of this cyclic nucleotide was unaffected (Fig. 3c) by the presence of a considerable concentration gradient of external cyclic GMP (5 mM). Furthermore, this process was inhibited in a dose-dependent fashion by the organic acid transport inhibitor probenecid (Fig. 4a). These features are similar to those noted using smooth muscle cells and hepatocytes [20, 32]. Consistent with blockade of egress, probenecid increased intracellular cyclic GMP concentrations (Fig. 4b). This was most marked when IBMX was present to prevent degradation of cyclic GMP.

Despite the fact that IBMX augmented the ability of atriopeptin to increase intracellular cyclic GMP accumulation it did not increase the rate of efflux of cyclic GMP observed (Fig. 3a). This suggests that the efflux system may be saturated at very low intracellular cyclic GMP concentrations. It is also possible, however, that IBMX exerts a small inhibitory effect on the carrier, as has been alluded to in studies done in other cells [20, 32].

In order for atriopeptin to achieve a transient increase in the intracellular concentration of cyclic GMP when added to cells in the presence of IBMX, a markedly greater amount of cyclic GMP must be metabolized compared to that metabolized in cells which were challenged with atriopeptin alone (Fig. 1). It is clear that this is not achieved by enhanced extrusion (Fig. 3). We must conclude, therefore, that this must be due to metabolism by phosphodiesterase activity. Indeed, we can calculate that in cells challenged with atriopeptin + IBMX then intracellular cyclic GMP will attain levels of 3–4 μM, at which point it is likely that cyclic GMP phosphodiesterase activity will be only some 80% inhibited by the reversible inhibitor IBMX, thus allowing for considerable metabolism of this cyclic nucleotide. Whilst an IBMX-insensitive phosphodiesterase has been reported in hepatocytes [39] and uterus [44], this is a cyclic AMP specific enzyme. Indeed, we found no evidence for such a species in either MDCK cells or in kidney homogenates [40].

MDCK cells exhibit an adenylate cyclase activity that can be activated by a number of ligands [45]. We show here that significant accumulation of cyclic

AMP in response to such ligands only occurred when IBMX was present (Fig. 7a). It has been noted that atriopeptin can inhibit adenylate cyclase activity and cyclic AMP accumulation in atrial and ventricular cardiocytes [46] as well as artery preparations [47]. However, using a range of concentrations of atriopeptin, we failed to identify any inhibitory effect on PGE_1-stimulated cyclic AMP accumulation. This suggests that the atriopeptin receptor in kidney cells does not interact with the adenylate cyclase system and that elevation of intracellular cyclic GMP does not inhibit the breakdown of cyclic AMP. The latter observations are consistent with our findings that the major cyclic AMP metabolizing enzymes of MDCK cells appear to be specific for cyclic AMP (Fig. 6) and their activities are unaffected by cyclic GMP. Conversely, treatment of cells with agents such as PGE_1 and forskolin, which raise intracellular cyclic AMP levels in the presence of forskolin, failed to inhibit cyclic GMP accumulation or exit. This indicates a specificity for both degradation and extrusion. Nevertheless, that atriopeptin failed to inhibit intracellular cyclic AMP accumulation in the presence of IBMX, may indicate that inhibition of adenylate cyclase seen by other investigators is related either to a specific subtype or ANF receptor, as multiple mRNA species have been identified [15], or is a cell-specific phenomenon. Certainly ANF inhibition of adenylate cyclase is not a ubiquitous phenomenon as atriopeptin does not appear to inhibit adenylate cyclase in testis [47] despite the fact that ANF receptors are expressed there [48].

In summary, we have shown that a kidney epithelial cell line shows a transient accumulation of cyclic GMP in response to challenge with atriopeptin. The transient nature of this response is due to three factors; a rapid desensitization of guanylate cyclase, degradation of cyclic GMP by specific cyclic GMP phosphodiesterases and active extrusion of cyclic GMP. Intriguingly, inhibition of cyclic nucleotide phosphodiesterase activity enhanced accumulation of cyclic GMP but not its active egression. This suggests that this process was rapidly saturated and that the K_m for this carrier may be below 1 μM cyclic GMP. Estimation of the initial rates of atriopeptin catalysed accumulation and extrusion of cyclic GMP show these to be of the order of 0.6 and 0.9 pmol cyclic GMP/min/10^6 cells, respectively. At the same time, metabolism of cyclic GMP by phosphodiesterase action is occurring and this can be estimated as being around 0.5 pmol/min/10^6 cells, from analysis of initial rates done in the presence of IBMX. However, as detailed earlier, this is unlikely to lead to a complete inhibition of phosphodiesterase activity and the value given will thus be underestimated; probably by around 20% as gauged from our inhibitor studies using homogenates. Such data shows that the extrusion process has a similar capacity to the phosphodiesterase complement of MDCK cells in being able to remove cyclic GMP. The relevance of the extrusion process has yet to be determined but the potency of this effect might imply some physiological importance related to determining the length of time a kidney cell may have elevated intracellular cyclic GMP concentrations. It will be of interest to determine what effect selective inhibitors of either cyclic GMP phosphodiesterases or the active extrusion system have on biological systems and blood pressure.

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REFERENCES

1. de Bold AJ, Atrial natriuretic factor: a hormone produced by the heart. *Science* **230**: 767–769, 1985.
2. Maack T, Camargo MJF, Kleinert HD, Laragh JH and Atlas SA, Atrial natriuretic factor: structure and functional properties. *Kidney Int* **27**: 607–615, 1985.
3. Fethiere J, Meloche S, Nguyen JT, Ong H and De Lean A, Distinct properties of atrial natriuretic factor receptor sub-populations in epithelial and fibroblast cell lines. *Mol Pharmacol* **35**: 584–592, 1989.
4. Hirata Y, Tomita M, Takeda S and Yoshima H, Vascular receptor binding activities and cGMP responses by synthetic human and rat atrial natriuretic peptides (ANP) and receptors down-regulation by ANP. *Biochem Biophys Res Commun* **128**: 538–546, 1985.
5. Murad F, Cyclic guanosine monophosphate as a mediator of vasodilation. *J Clin Invest* **78**: 1–5, 1986.
6. Leckie B, How the heart rules the kidneys. *Nature* **326**: 644–645, 1987.
7. Weideman P, Saxenhofer H, Shaw SG and Ferier C, Atrial natriuretic peptide in man. *J Steroid Biochem* **32**: 229–249, 1989.
8. De Lean A, Racz K, Gutkowska J, Nguyen TT, Cantin M and Genest J, Specific receptor-mediated inhibition by synthetic atrial natriuretic factor of hormone-stimulated steroidogenesis in cultured bovine adrenal cells. *Endocrinology* **115**: 1636–1638, 1984.
9. Kudo T and Baird A, Inhibition of aldosterone production in the adrenal glomerulosa by atrial natriuretic factor. *Nature* **312**: 756–757, 1984.
10. Samson W, Atrial natriuretic factor inhibits dehydration and hemorrhage-induced vasopressin release. *Neuroendocrinology* **40**: 277–279, 1985.
11. Obana K, Naruse M, Naruse K, Sakurai J, Demura H, Inagami T and Shizume K, Synthetic rat atrial natriuretic factor inhibits *in vitro* and *in vivo* renin secretion in rats. *Endocrinology* **117**: 1281–1284, 1985.
12. Maack T, Mario DN, Camargo MJF, Kleinert HD, Laragh JH, Vaughan ED and Atlas SA, Effects of auriculin (atrial natriuretic factor) on blood pressure, renal function and the renin-aldosterone system in dogs. *Am J Med* **77**: 1067–1075, 1984.
13. Burnett JC, Granger JP, Opgenorth TJ, Effects of synthetic atrial natriuretic factor on renal function and renin release. *Am J Physiol* **247**: F863–F866, 1984.
14. Chinkers M, Garbers DL, Chang MS, Lowe DG, Chin H, Goeddel DV and Schulz S, A membrane form of guanylate cyclase is an atrial natriuretic peptide receptor. *Nature* **338**: 78–83, 1989.
15. Schulz S, Chinkers M and Garbers DL, The guanylate cyclase/receptor family of proteins. *FASEB J* **3**: 2027–2035, 1989.
16. Waldman SA, Rapport RM and Murad F, Atrial natriuretic factor selectively activates particulate cyclase and elevates cyclic GMP in rat tissues. *J Biol Chem* **259**: 14332–14334, 1984.
17. Wood KB and Ignarro LJ, Hepatic cyclic GMP formation is regulated by similar factors that modulate activation of purified hepatic soluble guanylate cyclase. *J Biol Chem* **262**: 5020–5027, 1987.
18. Leitman DC and Murad F, Atrial natriuretic factor receptor heterogeneity and stimulation of particulate guanylate cyclase and cyclic GMP accumulation. *Endocrinol Metab Clin N Am* **16**: 79–105, 1987.
19. Leitman DC, Andresen JW, Catalano RM, Waldman

SA, Tuan JJ and Murad F, Atrial natriuretic peptide binding, cross-linking and stimulation of cyclic GMP accumulation and particular guanylate cyclase activity in cultured cells. *J Biol Chem* **263**: 3720–3728, 1988.
20. Hamet P, Pang SC and Tremblay J, Atrial natriuretic factor-induced egression of cyclic guanosine 3',5' mono-phosphate in cultured vascular smooth muscle and endothelial cells. *J Biochem Chem* **264**: 12364–12369, 1989.
21. Houslay MD, Rennaissance for cyclic GMP. *Trends Biochem Sci* **10**: 465–466, 1986.
22. Francis SH, Noblett BD, Todd BW, Wells JN and Corbin JD, Relaxation of vascular and tracheal smooth muscle by cyclic nucleotide analogues that preferentially activate purified cGMP-dependent protein kinase. *Mol Pharmacol* **34**: 506–517, 1988.
23. Casnellie JH, Ives HE, Jamieson JD and Greengard P, Cyclic GMP-dependent protein phosphorylation in intact medial tissue and isolated cells from vascular smooth muscle. *J Biol Chem* **255**: 3770–3776, 1980.
24. Parks TR, Nairn AC, Greengard P and Jamieson JD, The cyclic nucleotide-dependent phosphorylation of aortic smooth muscle membrane proteins. *Arch Biochem Biophys* **255**: 361–371, 1987.
25. Lincoln TM and Johnson RM, Possible role of cyclic GMP-dependent protein kinase in vascular smooth muscle function. *Adv Cyclic Nucleotide Protein Phosphorylation Res* **17**: 285–296, 1984.
26. Hamet P, Tremblay J, Thibault G, Garcia R, Cantin M and Genest J, ANF-induced increases in plasma and urinary cyclic GMP levels. *Endocrinology* **112**: 289–294, 1983.
27. Hamet P, Tremblay J, Pang SC, Garcia R, Thibault G, Gutkowska J, Cantin M and Genest J, Effect of native and synthetic atrial natriuretic factor on cyclic GMP. *Biochem Biophys Res Commun* **123**: 515–527, 1984.
28. Pang SC, Hoang MC, Tremblay J, Catin M, Garcia R, Genest J and Hamet P, Effect of natural and synthetic atrial natriuretic factor on atrial blood pressure, natriuresis and cyclic GMP excretion in spontaneously hypertensive rats. *Clin Sci* **69**: 721–726, 1985.
29. Hamet P, Tremblay J, Pang SC, Skuherska R, Schifrin EC, Garcia R, Cantin M, Genest J, Palmour R, Ervin FR, Martin S and Goldwater R, Alterations in plasma cGMP concentrations effected by ANF in monkeys. *Hypertension* **4** (Suppl 2): S49–S56, 1986.
30. Seymour AA, Blaine EH, Mazak EK, Smith SG, Stabilito II, Haley AB, Napier MA, Whinnery MA and Nutt RF, Renal and systematic effects of synthetic atrial natriuretic factor. *Life Sci* **36**: 33–44, 1985.
31. Gerzer R, Witzgall H, Tremblay J, Gutkowska J and Hamet P, Rapid increase in plasma and urinary cyclic GMP after bolus injection of atrial natriuretic factor in man. *J Clin Endocrinol Metab* **61**: 1217–1219, 1985.
32. Tjornhammar ML, Lazaridis G and Bartfai T, Cyclic GMP efflux from liver slices. *J Biol Chem* **258**: 6882–6886, 1983.
33. Steiner AL, Parker CW and Kipnis DM, Radioimmunoassay for cyclic nucleotides. Preparation of antibodies and iodinated cyclic nucleotides. *J Biol Chem* **247**: 1106–1112, 1972.
34. Richelson E, Prendegast FG and Divinetz-Romero S, Muscarinic receptor-mediated cyclic GMP formation by cultured nerve cells—ionic dependence and effects of local anaesthetics. *Biochem Pharmacol* **27**: 3039–3048, 1978.
35. Clark RB, Su YF, Ortmann K, Cubeduo X, Johnson GL and Perkins JP, Factors influencing the effect of hormones on the accumulation of cyclic AMP in cultured human astrocytoma cells. *Metabolism* **24**: 343–350, 1975.
36. Rutten WJ, School BM and DuPont JSH, Adenosine 3',5'-monophosphate phosphodiesterase assay in tissue homogenates. *Biochim Biophys Acta* **315**: 378–383, 1973.
37. Thompson WJ and Appleman MM, Multiple cyclic nucleotide phosphodiesterase activities from rat brain. *Biochemistry* **10**: 311–316, 1971.
38. Marchmont RJ and Houslay MD, A peripheral and an intrinsic enzyme constitute the cyclic AMP phosphodiesterase activity of rat liver plasma membranes. *Biochem J* **187**: 381–392, 1980.
39. Lavan BE, Lakey T and Houslay MD, Resolution of soluble cyclic nucleotide phosphodiesterase isoenzymes from liver and hepatocytes identifies a novel IBMX-insensitive form. *Biochem Pharmacol* **38**: 4123–4136, 1989.
40. Hoey M and Houslay MD, Identification and selective inhibition of four distinct soluble forms of cyclic nucleotide phosphodiesterase activity from kidney. *Biochem Pharmacol* **40**: 193–202, 1990.
41. O'Dea RF, Gagnon C and Zatz M, Regulation of guanosine 3',5' cyclic monophosphate in the rat pineal and posterior pituitary glands. *J Neurochem* **31**: 733–738, 1978.
42. Kapoor CL and Krishna G, Hormone-induced cyclic guanosine monophosphate secretion from guinea-pig pancreatic tubules. *Science* **196**: 1003–1005, 1977.
43. Schini V, Grant NJ, Miller RC and Takeda K, Morphological characterisation of cultured bovine aortic endothelial cells and the effects of atriopeptin II and sodium nitroprusside on cellular and extracellular accumulation of cyclic GMP. *Eur J Cell Biol* **47**: 53–61, 1988.
44. Hardman JG and Wells JN, Resistance of a separated form of canine ureteral phosphodiesterase activity to inhibition by xanthines and papaverine. *Biochem Pharmacol* **30**: 2371–2374, 1981.
45. Rich KA, Codina J, Floyd G, Sekura R, Hildebrandt JD and Iyenger R, Glucagon-induced heterologous desensitisation of the MDCK cell adrenylyl cyclase. *J Biol Chem* **259**: 7893–7901, 1984.
46. Anand-Srivastrava MB and Cantin M, Atrial natriuretic factor receptors are negatively coupled to adenylate cyclase in cultured atrial and ventricular cardiocytes. *Biochem Biophys Res Commun* **138**: 427–436, 1986.
47. Anand-Srivastrava MB, Franks DJ, Cantin M and Genest J, Atrial natriuretic factor inhibits adenylate cyclase activity. *Biochem Biophys Res Commun* **121**: 855–862, 1984.
48. Marala RB and Sharman RK, Characterisation of atrial-natriuretic factor-receptor-coupled guanylate cyclase from rat and mouse testes. *Biochem J* **251**: 301–304, 1988.