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Effect of Cyclic GMP-Increasing Agents Nitric Oxide and C-Type Natriuretic Peptide on Bovine Chromaffin Cell Function: Inhibitory Role Mediated by Cyclic GMP-Dependent Protein Kinase

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SUMMARY

Both sodium nitroprusside (SNP), a nitric oxide (NO) generator, and C-type natriuretic peptide (CNP) have been found to raise cGMP levels in bovine chromaffin cells in a time- and concentration-dependent manner. The effect of these compounds on catecholamine secretion and calcium influx has also been studied, and both compounds were found to produce a slowly developing inhibitory effect on acetylcholine- or depolarization-stimulated catecholamine secretion and calcium increases without affecting the spontaneous release or the basal intracellular Ca^{2+} concentration. These inhibitory effects were observed only at high doses of acetylcholine or high levels of extracellular potassium and required concentrations of SNP or CNP very similar to those that increased cGMP levels. Preincubation with 100 μM zaprinast, a cGMP-phosphodiesterase inhibitor able to increase cGMP levels, mimicked the inhibitory effects of SNP and CNP. We investigated the effect of the soluble guanylate cyclase inhibitor methylene blue and the cGMP-dependent protein kinase (PKG) inhibitor 8-(4-chlorophenylthio)-guanosine 3′,5′-cyclic monophosphorothioate, Rp isomer, on inhibition by SNP or CNP. Although methylene blue (10 μM) partially prevented the inhibitory effect of SNP, it did not do so for that produced by CNP, thus indicating that SNP acts through cGMP produced by the NO-activated guanylate cyclase. 8-(4-Chlorophenylthio)-guanosine 3′,5′-cyclic monophosphorothioate, Rp isomer totally reversed both the SNP and CNP inhibitory effects. These results suggest that the activation of PKG mediates the inhibition induced by SNP and CNP. We successfully measured the PKG activity from cells preincubated with SNP or CNP, and our results show that this enzymatic activity increased with a time dependence very similar to the increase in the cGMP levels. Our results indicate that NO and CNP peptide inhibit secretagogue-stimulated catecholamine release via activation of soluble and particulate isoforms of the guanylate cyclase, respectively, presumably by inhibition of calcium entry through voltage-activated calcium channels. This inhibitory effects seems to be mediated by activation of the PKG.

The physiological function of cGMP in visual transduction has been unequivocally demonstrated (1), and the role of cGMP in relaxation of smooth muscle is rapidly being clarified (2). However, in the nervous system, although several lines of evidence suggest that cGMP might be involved in long-term potentiation in the hippocampus and in long-term depression in the cerebellum (3), the role of cGMP as an intracellular messenger for neurons in mammals is not fully understood.

Chromaffin cells from the adrenal medulla have many properties in common with neurons: they originate in the neural crest of the embryo, they function to release substances contained in the vesicles in response to specific stimuli, they are electrically excitable, and they are depolarizable by acetylcholine, the neurotransmitter released by the splachnic nerve terminals synapsing with chromaffin cells. Acetylcholine activates receptor-associated cation channels, which in turn leads to membrane depolarization and activation of voltage-sensitive calcium channels. Ca^{2+} entry through these channels triggers the exocytotic machinery to secrete catecholamines to the extracellular medium (4). The fact that these cells can be readily isolated in large numbers

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ABBREVIATIONS: NO, nitric oxide; SNP, sodium nitroprusside; CNP, C-type natriuretic peptide; $[Ca^{2+}]_i$, intracellular calcium concentration; $[Ca^{2+}]_o$, extracellular calcium concentration; PKG, cGMP-dependent protein kinase; DHBA, 3,4-dihydroxybenzylamine; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; Rp-8-pCPT-cGMPS, 8-(4-chlorophenylthio)-cyclic guanosine monophosphorothioate, Rp-isomer; NOS, nitric oxide synthase; 8-Br-cGMP, 8-bromo-cGMP; IBMX, 3-isobutyl-1-methylxanthine; PTX, pertussis toxin; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; HPLC, high performance liquid chromatography.

as a homogeneous cell population and maintained in culture makes them a good alternative model with which to investigate the modulation of neurosecretion by a second messenger like cGMP. Moreover, the adrenal medulla is of particular interest in the search for cGMP effects because of the special anatomic architecture of the organ. The adrenal medulla is a highly vascular endocrine organ in which chromaffin cells are grouped around capillary vessels. Endothelial cells that form the capillary walls can synthesize and release a wide range of bioactive substances, including NO (5). This molecule, which is generated during the conversion of L-arginine to citrulline by NOS, is an important mediator of cellular communication. Endothelium-derived relaxation of smooth muscle cells is thought to be mediated mainly through the release of NO from endothelial cells. NO possibly acts by activating a soluble guanylate cyclase, thereby causing a rise in intracellular cGMP (6). A functional interaction between adrenal chromaffin cells and endothelial cells has been suggested, and a NO/guanylate cyclase pathway seems to be implicated (7). Another potential source of NO in the adrenal medulla that must be taken into account is the innervation of the gland. Studies have shown that chromaffin cells into the adrenal medulla seem to be surrounded by NOS-immunoreactive fibers (8).

Previous studies have shown that chromaffin cells possess elements of the cGMP signaling system. First, cytosolic NO synthase activity has been described in the adrenal medulla; thus, chromaffin cells constitute not only receptor but also producer cells of NO (9–11). The two types of guanylate cyclase have also been described in chromaffin cells: the soluble form, which is activated by NO, and the particulate form, which is activated by natriuretic peptide ligands that bind to cell membrane receptors with transmembrane domains that are contiguous with intracellular guanylate cyclase (12, 13). CNP has been reported to exist in adrenal medullary cells and to be secreted from the cells during the secretory response (14). Moreover, like NO, CNP is produced in and secreted from endothelial cells (15). The most important receptor protein for cGMP in the majority of the studied cell types is PKG. Recently, we found PKG activity in bovine cultured chromaffin cells (16).

The literature suggests a dual role for cGMP in its effect on chromaffin cell function. cGMP has been reported to stimulate the catecholamine synthesis through activation of tyrosine hydroxylase, which catalyzes the conversion of tyrosine to dopa, the rate-limiting step in the synthesis (17). Also, cGMP plays an inhibitory role in the regulation of secretion from chromaffin cells (7, 18, 19). Recently, we showed that the membrane-permeable cGMP analogue 8-Br-cGMP produces an inhibition of catecholamine secretion and a reduction of calcium influx in bovine chromaffin cells and that these effects are mediated by activation of an endogenous PKG. The mechanism for these cGMP analogue effects seem to be a specific action on dihydropyridine-insensitive voltage-dependent calcium channels, which are present in chromaffin cells and actively participate in stimulus/secretion coupling (16, 20).

In the present study, we demonstrated that compounds that produce robust increases in chromaffin cGMP levels, such as SNP-generating NO or the physiological ligand CNP, are also able to inhibit both the acetylcholine- or depolarization-evoked catecholamine secretion and calcium influx. We hypothesized that natriuretic ligands and NO from chromaffin cells, adjacent endothelial cells, or even nerve terminals play an important physiological role in the modulation of the neurosecretion from the adrenal medulla and that such a role is mediated by the cGMP signaling system.

Experimental Procedures

Materials

Culture media and heat-inactivated fetal calf serum were obtained from GIBCO (Uxbridge, UK). Culture plates were obtained from Costar (Cambridge, MA). Collagen A was from Biochrom KG (Berlin, Germany). Collagenase A (EC 3.4.24.3) from *Clostridium histolyticum* and phenylmethylsulfonyl fluoride were purchased from Boehringer Mannheim (Mannheim, Germany). Urografin was from Schering España (Madrid, Spain). IBMX, SNP, acetylcholine chloride, epinephrine and norepinephrine bitartrate salts, DHBA, methylene blue, octanesulfonic acid, leupeptin, β-mercaptoethanol, histamine, and PTX from *Bordetella pertussis* were from Sigma Chemical Co. (St. Louis, MO). [γ-^{32}P]ATP, [^{3}H]cGMP, and AMP radioimmunoassay kits were from Amersham (Buckinghamshire, UK). Fura-2/AM was from Molecular Probes (Eugene, OR). CNP was from Peninsula Laboratories (Belmont, CA). IP_{20}-amide peptide inhibitor of protein kinase A and forskolin from *Coleus forskohlii* were from Calbiochem (San Diego, CA). Heptapeptide (RKRSRAE) was obtained from Promega Corporation (Madison, WI). Rp-8-pCPT-cGMPS was from Biolog (Bremen, Germany). Zaprinast (M&B 22,948) was kindly provided by Rhône-Poulenc Rorer Pharmaceuticals (Dagenham, UK). Protein was measured using the Bio-Rad protein assay from Bio-Rad (München, Germany). Inorganic salts were from Merck (Darmstadt, Germany).

Preparation of Chromaffin Cells

Chromaffin cells were obtained after digestion of bovine adrenal glands with collagenase in retrograde perfusion as described previously (16). Briefly, glands supplied by a local slaughterhouse were trimmed of fat, cannulated through the adrenal vein, and washed with Ca^{2+}/Mg^{2+}-free saline buffer containing 154 mM NaCl, 5.6 mM KCl, 3.6 mM $NaHCO_3$, 5 mM glucose, and 5 mM HEPES, pH 7.4. Digestion was performed with a 0.2% collagenase plus 0.5% bovine serum albumin solution in the above medium. After digestion, glands were halved, soft medulla were removed, and minced and dispersed cells were filtered through a nylon mesh. Cells were purified through a Urografin density gradient. Of the collected cells, >90–95% were chromaffin cells, as they were massively and clearly stained by neutral red. Occasionally, the culture purity was lower than this range; in this case, cells were further purified through differential plating. Purified chromaffin cells were suspended at a density of 10^6 cells/ml in Dulbecco's modified Eagle's medium containing 10% heat-inactivated fetal calf serum and standard antibiotics. Cells were plated onto collagen-treated 24-well Costar cluster dishes at a density of 10^6 cells/well for cGMP and cAMP determinations or 5×10^5 cells/well for catecholamine secretion in culture medium supplemented with 10 μM cytosinearabinofuranoside and 10 μM fluorodeoxyuridine, maintained at 37° in 5% CO_2/95% air. These cells were used during 3–5 days of cell isolation. For cytosolic calcium measurements and PKG determinations, cells were maintained in suspension and kept at 4°. These cells were used at 2–3 days of cell isolation.

Intracellular Cyclic Nucleotide Measurements

Cells were serum-deprived for 24 hr before cyclic nucleotide measurement and then washed twice with Locke's solution containing 140 mM NaCl, 4.4 mM KCl, 2.5 mM $CaCl_2$, 1.2 mM $MgSO_4$, 1.2 mM KH_2PO_4, 4 mM $NaHCO_3$, 5.6 mM glucose, and 10 mM HEPES, pH 7.4. After a 30-min preincubation at 37° in Locke's solution (plus 0.5 mM IBMX when used), cells were stimulated with agents in 0.5 ml of Locke's solution (with or without IBMX) for the indicated times.

Incubations were terminated through aspiration of the medium and the addition of 300 μl of 6% trichloroacetic acid. Cells were then scraped out of the wells and centrifuged. The supernatant fraction was neutralized with 3 M KOH and evaporated with a Speed-vac (Savant). The samples were resuspended in 4 mM EDTA, 50 mM Tris, pH 7.5, and the cGMP and cAMP contents were determined in the crude extracts with the use of commercial [^{3}H]cGMP and [^{3}H]cAMP radioimmunoassay kits (11).

Catecholamine Secretion Experiments

Secretion experiments. Cultured chromaffin cells in 24-well Costar cluster dishes at a density of 500,000 cells/dish were serum deprived for 24 hr before the experiments. The cells were washed twice with Locke's solution, and after 30 min of incubation with the same medium, they were incubated with the compounds to test their effect on catecholamine secretion during different experimental periods; they were then stimulated for 2 min with acetylcholine, high potassium solution, or Locke's solution (basal) in a final volume of 200 μl. Secretion was terminated through the aspiration of the medium. Catecholamines were assayed in both the supernatants and the cells.

In the catecholamine assay, two different methods were used to quantify catecholamines. For fluorimetric measurements, the supernatants containing the released catecholamines were maintained at 0° until the catecholamines were assayed (usually 15–30 min). The cellular catecholamine contents were extracted by adding 0.3 ml of 10% acetic acid to each well; cells were then scraped out of the wells and centrifuged. Catecholamines were assayed in both supernatants (that released to the medium and intracellular content) according to a trihydroxyindole fluorescence method (21). Epinephrine was used as standard. Catecholamine release is expressed as a percentage of the total catecholamine content (release plus content). The net secretion was obtained by subtracting the number of catecholamines spontaneously released from the number released by stimulation.

For HPLC measurements, catecholamines secreted into the medium and those remaining in the cells were separated through reverse-phase HPLC with electrochemical detection. Aliquots of the release media were mixed with an acidic solution to give 0.2 N $HClO_4$, 0.5 mM sodium metabisulfite, and 0.45 mM EDTA and centrifuged. Then, these supernatants were diluted to adjust the catecholamine quantities to the detector sensitivity by using the same solution containing DHBA as the internal standard. Catecholamine contents were extracted by adding 0.3 ml of perchloric/bisulfite/EDTA solution to each well; cells were scraped out of the wells and centrifuged. As with the released amines, the supernatants were diluted with the same solution plus DHBA. The HPLC isocratic mobile phase consisted of 25 mM citric acid, 25 mM Na_2HPO_4, 5 μM EDTA, and 0.12 mM octanesulfonic acid, pH 3.4 adjusted with phosphoric acid, at a flow rate of 1.2 ml/min. The HPLC stationary phase was a LC-18-DB, 15 cm × 4.6 mm, 5-μm column (Supelco, Bellefonte, PA). Detection was performed with a Metrohm 641 VA electrochemical detection set at a working potential of +0.8 V and a sensitivity of 50 nA. Epinephrine and norepinephrine release was calculated as a percentage of the total content of each amine.

Measurement of Cytosolic [Ca^{2+}]

Cytosolic [Ca^{2+}] was determined with the fluorescent indicator Fura-2. The cells were washed twice with Locke's solution, loaded with 2.5 μM Fura-2/AM for 45 min, and washed through centrifugation. The recordings were performed with a Perkin-Elmer LS-50 fluorimeter using 1-ml samples containing 10^6 cells in thermostated and stirred cuvettes. The excitation and emission wavelengths were set to 340 and 510 nm, respectively. When experiments were performed in the absence of $[Ca^{2+}]_o$, cells were resuspended in calcium-free EGTA-based Locke's medium calculated to give 100 nM $[Ca^{2+}]_o$. At the end of each experiment, the cells were lysed and the dye content was calibrated as described above (16). The cytosolic Ca^{2+} concentration ($[Ca^{2+}]_i$) was derived from fluorescence traces with the use of the equation of Grynkiewicz *et al.* (22).

Assays of PKG Activity

In experiments in which PKG was measured *in vitro* after specific treatments *in situ*, suspended chromaffin cells were washed twice with Locke's solution and then stimulated with 100 μM SNP or 100 nM CNP at 37°. Aliquots (500 μl) of chromaffin cell suspensions (~5 × 10^6 cells) were taken out at the indicated times. The cells were harvested by centrifugation (3000 rpm for 1 min). The supernatants were aspirated, and the cells taken for PKG activity were rapidly frozen by immersion of the tubes in liquid N_2. Then, 200 μl of a solution containing 10 mM potassium phosphate, 1 mM EDTA, 15 mM β-mercaptoethanol, 0.1 mM phenylmethylsulfonyl fluoride, and 10 μM leupeptin, pH 6.8, was added; cells were disrupted by sonication for 10 sec with a Braun Micro-Ultrasonic Cell Disrupter, and the suspensions were centrifuged at 13,000 rpm for 10 min at 4°. Portions (30 μl) of the supernatants containing 60–70 μg of protein (average, 67.34 ± 3.39 μg of protein) were taken for kinase activity determinations as described previously (23). Assays were carried out in a buffer consisting of 40 mM Tris·HCl, pH 7.4, 20 mM magnesium acetate, 0.2 mM [γ-^{32}P]ATP (200–300 cpm/pmol), 50 nM IP_{20}-amide peptide inhibitor of protein kinase A, and 10 μg of heptapeptide (RKRSRAE) as substrate of PKG. The reaction was initiated by the addition of 30 μl of soluble extract and incubated for 10 min at 30°. The reaction was terminated by pipetting an aliquot of the incubation mixture onto filter paper squares (Whatman P81 2.5 × 2.5 cm) and washing with 75 mM phosphoric acid (four times with 10 ml/paper). The filters were dried, and radioactivity was determined.

Analysis of Data

cGMP and cAMP experiments were performed in triplicate, and results are expressed as pmol/10^6 cells ± standard error. The data from the fluorimeter were interfaced to a computer to allow off-line analysis, and the numerical values presented are mean ± standard error of the magnitude of the calcium peak increase.

Concentration-response relationships were fitted to a sigmoidal model of the form log-concentration versus response. When the exponential decay of $[Ca^{2+}]_i$ toward basal level after a transient was examined, the recovery was fitted to a monoexponential decay of first order and the $t_{1/2}$ values calculated from this equation. Both types of curve-fitting were done with the program Parameter Fitter (Biosoft).

Results

SNP and CNP strongly increase cGMP levels in bovine adrenal chromaffin cells. cGMP levels in bovine chromaffin cells were increased by both SNP and CNP. Fig. 1 shows the time course of cGMP production in response to 100 μM SNP (Fig. 1A) or 100 nM CNP (Fig. 1B), in the absence and presence of the nonspecific phosphodiesterase inhibitor IBMX at a concentration of 0.5 mM. Preincubation with IBMX caused a 2-fold increase in the levels of cGMP in unstimulated cells and greatly potentiated the production on stimulation by the agents. Both SNP and CNP produced a time-dependent cGMP increase. In the absence of IBMX, SNP caused a continuous increase in cGMP formation for ≤15 min of incubation, after which the levels remained high. In the presence of IBMX, levels increased continuously, and equilibrium was not reached within the studied experimental times. Stimulation with CNP caused a time-dependent increase in cGMP levels, reaching an equilibrium at 5 min of stimulation in both the presence and absence of IBMX, and the levels were maintained high during CNP stimulation.

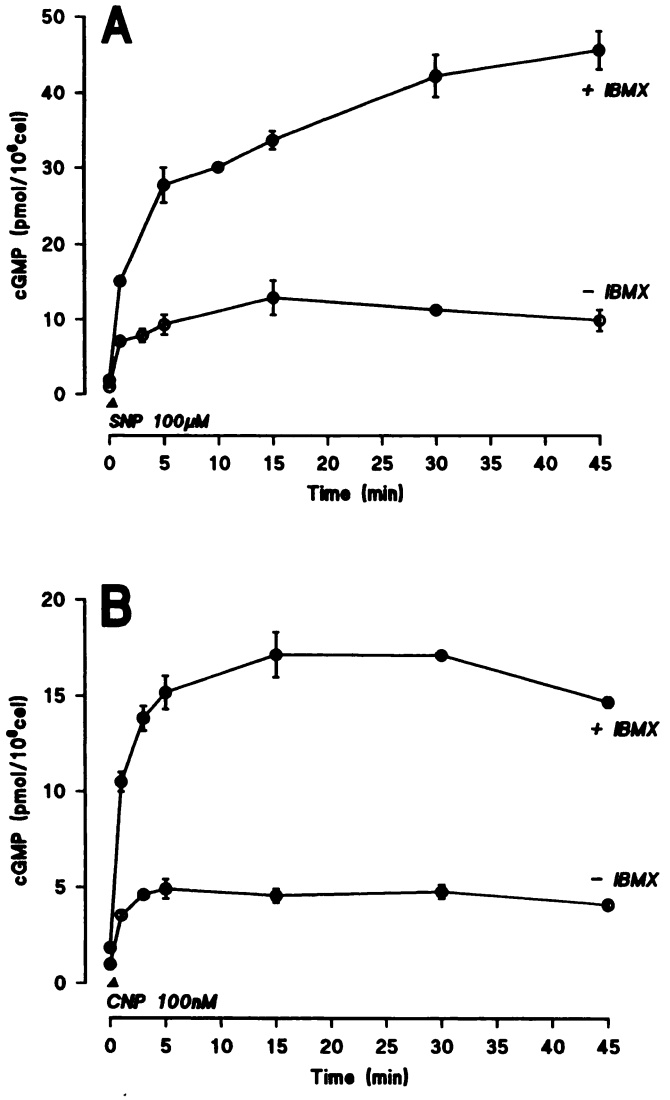

Fig. 1. cGMP production induced by SNP and CNP in bovine chromaffin cells. Time courses of cGMP formation elicited by 100 μM SNP (A) and 100 nM CNP (B) in the presence (●) and absence (○) of IBMX. Cultured bovine chromaffin cells (10^6 cells/dish) were preincubated with Locke's solution with or without 0.5 mM IBMX at 37° for 30 min. After preincubation, they were incubated for the indicated times with SNP or CNP (with or without IBMX). cGMP in the cells was assayed as described in Experimental Procedures. Experiments were performed in triplicate, and data, expressed in pmol/10^6 cells, are mean ± standard error from three different cellular preparations.

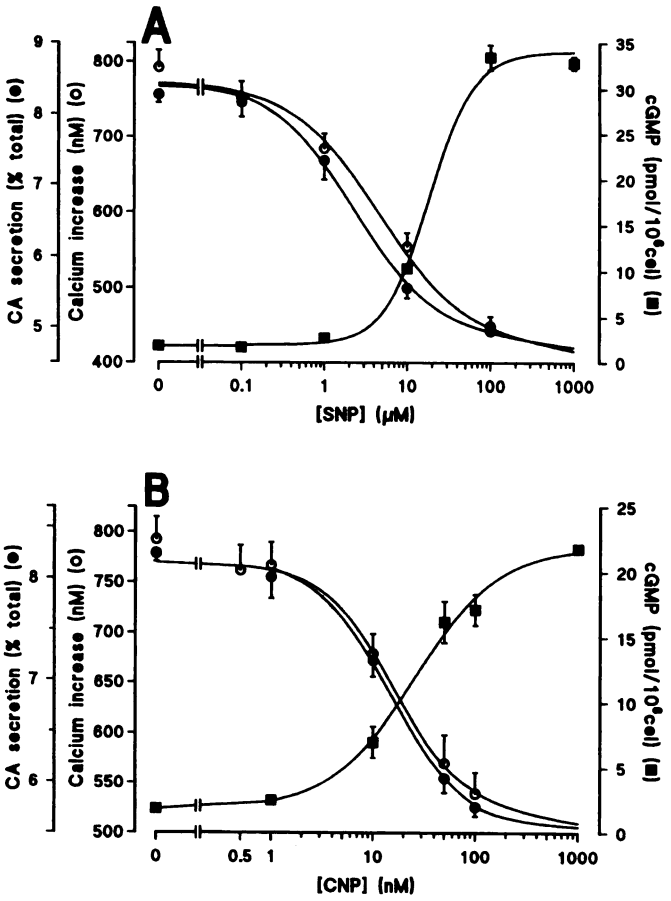

Fig. 2. Concentration-dependence curves for cGMP production and inhibition of catecholamine (CA) secretion and calcium increases induced by SNP (A) and CNP (B). For cGMP determinations (■), cells were preincubated for 30 min with 0.5 mM IBMX. After preincubation, they were incubated for 15 min with increasing concentrations of SNP or CNP. For catecholamine secretion (●) and $[Ca^{2+}]_i$ (○), cells were preincubated for 45 min with the different concentrations of SNP or CNP in the absence of the phosphodiesterase inhibitor and then stimulated with 50 μM acetylcholine. cGMP, catecholamine secretion, and calcium increases were measured as described in Experimental Procedures. *Curves* show cGMP expressed in pmol/10^6 cells, net catecholamine secretion as a percentage of total content (after subtraction of the basal release), and $[Ca^{2+}]_i$ measurements as increases in the calcium peaks. Experiments were performed in triplicate, and data are mean ± standard error from two different cellular preparations.

SNP was a more potent stimulator of cGMP production than CNP throughout the experimental time range.

Because the increases in the cGMP levels were greatly potentiated by the presence of IBMX, these experimental conditions were chosen to determine the concentration dependence of the cGMP increases evoked by both SNP and CNP. Fig. 2 shows that both compounds produced dose-dependent cGMP increases. The EC_{50} values for SNP and CNP obtained through measurement of the cGMP production at 15 min were 18.13 ± 2.99 μM and 25.63 ± 5.96 nM, respectively.

CNP and SNP produce increases in cGMP levels through different mechanisms. CNP activates a particulate guanylate cyclase, whereas SNP spontaneously releases NO in solution and activates the soluble guanylate cyclase. Thirty-minute incubations with SNP and CNP in the absence of IBMX produced 11- and 5-fold increases in cGMP formation, respectively. When chromaffin cells were incubated in the presence of 100 nM CNP plus 100 μM SNP, cGMP levels were increased 18-fold (basal, 0.976 ± 0.050; CNP, 4.774 ± 0.358; SNP, 11.277 ± 0.507; CNP plus SNP, 18.329 ± 1.361 pmol cGMP/10^6 cells). The effects of SNP and CNP on cGMP production at supramaximal concentrations were cumulative, confirming that each acts through a different mechanism.

cGMP may modulate the levels of the relative cyclic nucleotide cAMP and produce its biological effects by modifying the enzymatic activity of cGMP-stimulated or -inhibited cyclic nucleotide phosphodiesterases (24). To determine whether this type of modulation occurs in these cells, we examined the effect of SNP and CNP on cAMP biosynthesis in unstimulated cells and in forskolin-stimulated chromaffin cells. As shown in Table 1, basal cAMP levels were 7-fold higher than cGMP levels, and treatment with SNP or CNP did not modify the cAMP levels. Stimulation of chromaffin

TABLE 1

Cyclic nucleotide accumulation in response to combined incubations with cGMP-increasing agents and forskolin

Cultured chromaffin cells were preincubated with Locke's solution at 37° for 30 min. Then, they were incubated for 30 min with different drugs according to the treatment carried out, and the cAMP and cGMP levels were measured through radioimmunoassay as described in Experimental Procedures. Experiments were performed in triplicate, and data are mean ± standard error from two different cellular preparations.

Treatment	cAMP	Percent of basal	cGMP	Percent of basal
	pmol/10⁶ cells	%	*pmol/10⁶ cells*	%
Basal	6.820 ± 0.330	100	0.976 ± 0.050	100
CNP 100 nM	6.790 ± 0.110	99	4.774 ± 0.358	489
SNP 100 μM	6.811 ± 0.171	99	11.277 ± 0.507	1155
Forskolin 10 μM	10.918 ± 0.673	160	0.988 ± 0.133	101
CNP + forskolin	11.237 ± 0.789	164	5.089 ± 0.173	521
SNP + forskolin	10.768 ± 0.118	157	10.896 ± 0.250	1116

cells for 30 min with 10 μM forskolin increased cAMP levels 1.6 times but did not affect the cGMP levels. Although under our experimental conditions forskolin did not produce much increase in cAMP content, it must be taken into account that the results shown in Table 1 were obtained in the absence of any phosphodiesterase inhibitor. Other authors working with chromaffin cells have described very similar cAMP increases with the use of forskolin in the absence of IBMX (25). Moreover, combined incubations of chromaffin cells with forskolin plus SNP or CNP did not alter the increases in cAMP or cGMP elicited by each drug separately. Thus, at least in chromaffin cells, there seems to be no mechanisms controlling the cAMP levels through cGMP-regulated phosphodiesterases.

The increasing cGMP compounds, SNP and CNP, produced inhibition of secretagogue-stimulated catecholamine secretion. Incubation of chromaffin cells with SNP or CNP did not modify the basal catecholamine secretion rate (1.58 ± 0.06% in the absence of any drug, 1.46 ± 0.06% incubated with SNP, and 1.66 ± 0.18% incubated with CNP; basal secretion released within 2 min and is expressed as a percentage of total content) but did significantly reduce the secretion evoked by stimulation with the physiological secretagogue acetylcholine. Stimulation with 50 μM acetylcholine for 2 min caused a release of 8.15 ± 0.10% of the total catecholamine content. The activation of both the soluble and the membrane-bound guanylate cyclase by SNP and CNP in the absence of IBMX produced a time-dependent inhibition of acetylcholine-stimulated catecholamine secretion, as is shown in Fig. 3A. Preincubation with either 100 μM SNP or 100 nM CNP for 30–60 min produced a ~30% inhibition of acetylcholine-elicited catecholamine secretion. Similar results were obtained when 30 mM KCl was used to elicit the secretory response (results not shown). To study whether preincubation with SNP and CNP could be specifically affecting one of the secretory cell types (i.e., adrenergic and noradrenergic), both amines, epinephrine and norepinephrine, were separated and measured by HPLC coupled to electrochemical detection. Both SNP and CNP were able to inhibit both epinephrine and norepinephrine release in percentages in very similar manners (Fig. 3B). Thus, on preincubation with SNP and CNP, 50 μM acetylcholine-stimulated catecholamine secretion, although lower than in control conditions, presented the same epinephrine/norepinephrine ratio (~1.8–2.2, showing variability among cultures).

When low doses of secretagogues were used to stimulate chromaffin cells, neither SNP nor CNP was able to produce an effect. Fig. 4B shows a comparison of the effect of 45-min preincubation with 100 μM SNP or 100 nM CNP on the

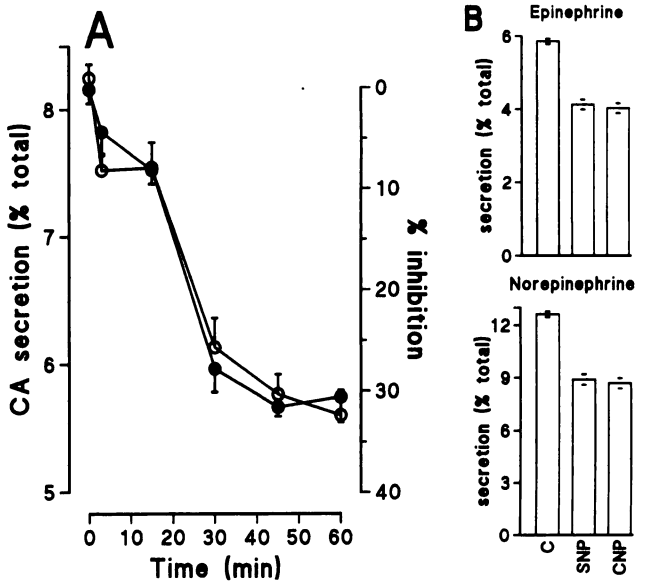

Fig. 3. Inhibitory effect of SNP and CNP on acetylcholine-evoked catecholamine (*CA*) secretion. A, Time course of the inhibitory effect of 100 μM SNP (●) or 100 nM CNP (○) on acetylcholine-evoked catecholamine secretion. B, Effect on epinephrine or norepinephrine release after preincubation with these compounds for 45 min. *C*, control. Cells were preincubated for the indicated times with SNP or CNP. After preincubation, the catecholamines released by 50 μM acetylcholine during a period of 2 min were measured as described in Experimental Procedures. The net secretion is expressed as a percentage of the total content (after subtraction of the basal release) and percentage of inhibition. Experiments were performed in triplicate, and data are mean ± standard error from four different cellular preparations.

catecholamine secretion evoked by 50 μM acetylcholine with that elicited by a secretagogue concentration of 10 μM. SNP and CNP inhibited the response to 50 μM acetylcholine, whereas they did not affect the response to 10 μM. Similar results were obtained with two different concentrations of KCl. At 30 mM KCl, SNP and CNP inhibited catecholamine release, but they did not modify the response to 18 mM KCl (results not shown).

The catecholamine content was slightly increased after preincubation with SNP or CNP (expressed as total catecholamines, 130.65 ± 5.68 nmol/10⁶ cells in control conditions and 169.37 ± 10.10 and 168.42 ± 6.74 nmol/10⁶ cells after 60-min preincubation with SNP and CNP, respectively).

We tested the range of SNP and CNP concentrations that produced increases in cGMP levels on the stimulated-catecholamine release. A 45-min preincubation with SNP or CNP produced dose-dependent inhibitory effects on catecholamine

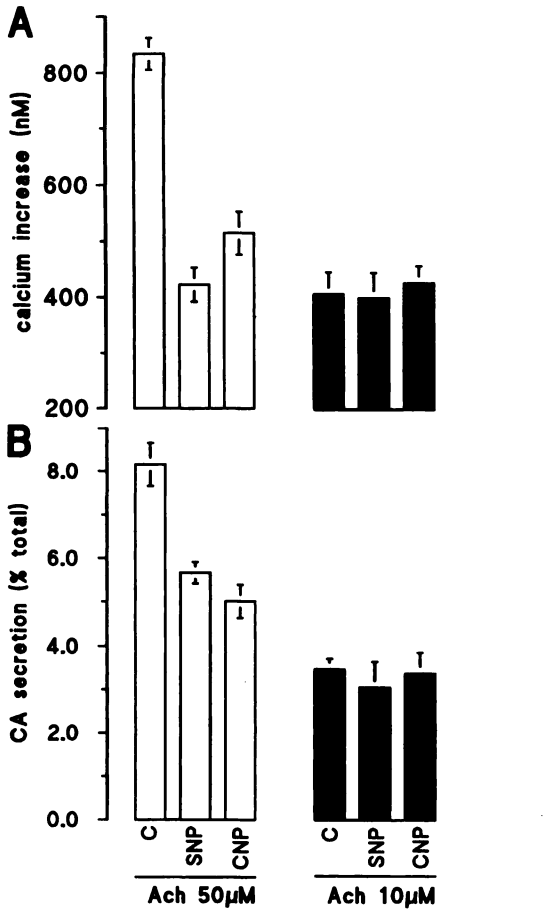

Fig. 4. Effect of SNP and CNP on catecholamine secretion elicited by two different doses of acetylcholine (*Ach*). Cells were preincubated for 45 min with 100 μM SNP or 100 nM CNP. After preincubation, cells were stimulated with 50 μM or 10 μM acetylcholine, and calcium increases (A) and catecholamine (*CA*) secretion (B) were measured as described in Experimental Procedures. *C*, control. Catecholamine secretion is expressed as a percentage of the total content (after subtraction of the basal release), and $[Ca^{2+}]_i$ measurements are expressed as increases in the calcium peaks. Experiments were performed in triplicate, and data are mean ± standard error from two different cellular preparations.

secretion with IC_{50} values of 2.40 ± 1.06 μM and 19.00 ± 5.76 nM, respectively (Fig. 2).

SNP and CNP also produced inhibition of the calcium influx. Challenging chromaffin cells with 50 μM acetylcholine or 30 mM KCl induced a rapid and transient rise in cytosolic calcium followed by a slow decline to a plateau level (Fig. 5). Both the direct and indirect depolarizations evoked by high extracellular potassium levels and through nicotinic receptor activation, respectively, led to activation of voltage-dependent calcium channels, which provide the rise in $[Ca^{2+}]_i$ (4).

Fig. 5 also shows that SNP (like CNP; data not shown) failed to elicit any change in $[Ca^{2+}]_i$ levels in adrenal chromaffin cells. The resting $[Ca^{2+}]_i$ level was unaffected by these two compounds, and its concentration remained at ~150–170 nM. Cells not responding to SNP or CNP were challenged with 50 μM acetylcholine or 30 mM KCl and always responded to these stimuli. However, when cells were preincubated for longer times (30–60 min) with 100 μM SNP or 100 nM CNP, an inhibitory action on both acetylcholine- and KCl-evoked $[Ca^{2+}]_i$ rise was observed (Fig. 5, A–D). These actions were studied in the absence of the phosphodiesterase inhibitor IBMX to avoid a nonspecific effect of this compound.

Fig. 5, A and B, shows typical records of $[Ca^{2+}]_i$ increases elicited by 50 μM acetylcholine or 30 mM KCl measured with the use of Fura-2 in cells preincubated for different times with 100 μM SNP. Fig. 5C shows the time courses for inhibitory effects of SNP and CNP on KCl-evoked $[Ca^{2+}]_i$ increases. The maximal effect was achieved at a 60-min exposure to SNP or CNP (44.20 ± 2.68% and 34.69 ± 3.33%, respectively). In Fig. 5D, the effects are shown of SNP and CNP on acetylcholine-evoked $[Ca^{2+}]_i$ increase. SNP and CNP inhibited the acetylcholine-evoked calcium response by 49.32 ± 3.53% and 38.31 ± 3.35%, respectively. This inhibition was calculated as a reduction in peak response.

When 10 μM acetylcholine was used to elicit calcium increases, the cGMP-increasing agents SNP and CNP were not able to produce any effect (Fig. 4A). Similar results were obtained when 18 mM KCl was used as a stimulator (results not shown).

On examination of the calcium signals in detail, the shape of the 50 μM acetylcholine- or 30 mM KCl-evoked $[Ca^{2+}]_i$ peaks was not altered by SNP or CNP pretreatment. The exponential recovery of $[Ca^{2+}]_i$ toward basal levels was not altered by pretreatment with SNP or CNP. The $t_{1/2}$ for the decay phase of the acetylcholine-evoked $[Ca^{2+}]_i$ peak was 14.50 ± 1.11 sec. This value was not altered by SNP or CNP preincubation, being 14.71 ± 0.44 sec and 14.72 sec in the presence of SNP or CNP, respectively.

Although acetylcholine also activates muscarinic receptors coupled to Ca^{2+} mobilization from internal stores, results obtained in the absence of $[Ca^{2+}]_o$ (Fig. 6) indicate that the contribution of this release to calcium increase in bovine tissue is very small (<5%) compared with the calcium influx. The slight response to acetylcholine in Ca^{2+}-free medium cannot be accounted for by nonfunctional calcium stores because histamine, a well-known agonist that mobilizes calcium stores in these cells (4), was able to produce significant increases in $[Ca^{2+}]_i$.

Fig. 6 also shows that SNP or CNP preincubation did not affect the calcium release response to histamine. Thus, both drugs were only able to modify the calcium entry evoked by activation of voltage-dependent calcium channels and did not produce an effect on the calcium release response to histamine.

The concentration dependence was studied of this inhibitory effect on calcium entry. Fig. 2 shows that SNP and CNP inhibited stimulated calcium influx with IC_{50} values of 6.94 ± 2.41 μM and 11.01 ± 2.11 nM, respectively. These results, together with the EC_{50} values for cGMP production and the IC_{50} values for catecholamine secretion, show a clear parallel between the concentrations required to elicit cGMP increases and those that inhibit catecholamine secretion and calcium influx. This behavior indicated that an intracellular signaling pathway based on cGMP may cause the inhibitory effect on catecholamine secretion by acting on calcium channels.

Involvement of PKG in the action of SNP and CNP on $[Ca^{2+}]_i$ and catecholamine secretion. Although SNP and CNP elevate cGMP levels through different mechanisms by activation of different guanylate cyclases, the inhibitory pathway converges after cGMP production in an unique way because combined incubations for 60 min of both compounds did not reduce the acetylcholine-evoked catecholamine secre-

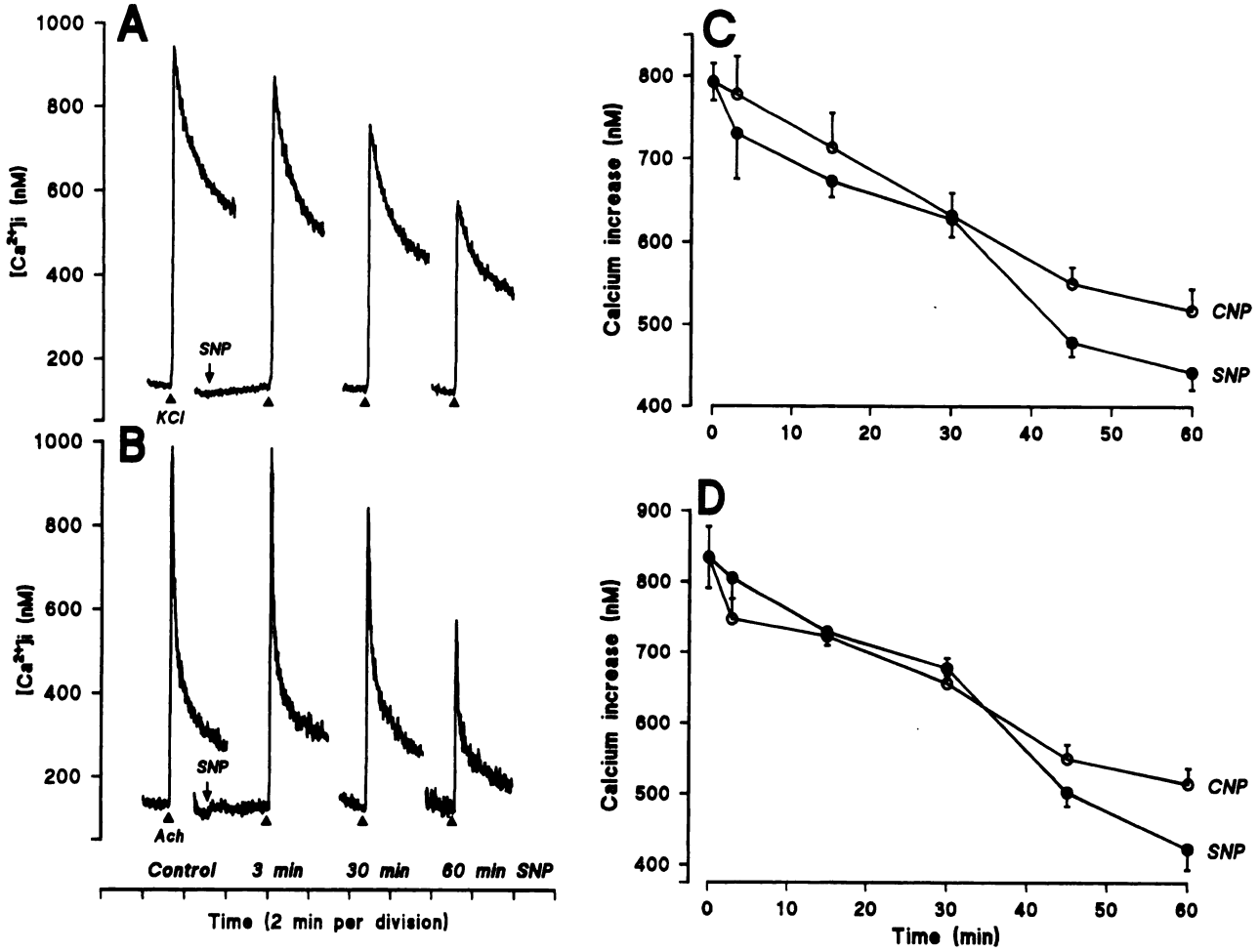

Fig. 5. Time-dependent inhibitory effect of SNP and CNP on acetylcholine (*Ach*)- and KCl-evoked calcium increases. Cells loaded with Fura-2 were preincubated for the indicated time with 100 μM SNP or 100 nM CNP. Representative records show the calcium transients evoked by 30 mM KCl (A) or 50 μM acetylcholine (B) under control conditions or after 3-, 30-, and 60-min preincubation with SNP. Time courses show the magnitude of calcium increases evoked by 30 mM KCl (C) or 50 μM acetylcholine (D) after preincubation for the indicated times with SNP (●) or CNP (○). Experiments were performed in triplicate, and data in C and D are mean ± standard error from four different cellular preparations.

tion and calcium influx to a greater extent than did each drug separately (data not shown).

To gain additional information about the role of the cGMP signaling pathway in regulating catecholamine secretion and calcium influx, the effect of several agents on these processes was examined. Fig. 7 shows the effect of zaprinast, an inhibitor of the cGMP phosphodiesterase, on cGMP levels (Fig. 7A) and on both acetylcholine-evoked $[Ca^{2+}]_i$ increase (Fig. 7C) and catecholamine release (Fig. 7B). Zaprinast (100 μM) doubled the cGMP levels and was able to inhibit both calcium increase and catecholamine release elicited by acetylcholine, mimicking the effects of SNP or CNP. As was expected, methylene blue (10 μM), a well-known soluble guanylate cyclase inhibitor, effectively inhibited the cGMP increase elicited by SNP (78.47 ± 6.26% of inhibition) and did not affect the cGMP production elicited by CNP (data not shown). As shown in Fig. 8, simultaneous incubation with methylene blue and CNP did not modify the extent of inhibition caused by CNP on acetylcholine-evoked $[Ca^{2+}]_i$ transient and catecholamine release. Nevertheless, simultaneous incubation with methylene blue and SNP limited the inhibitory effect of SNP on acetylcholine-evoked catecholamine release and $[Ca^{2+}]_i$ increases. The partial reversion caused by methylene

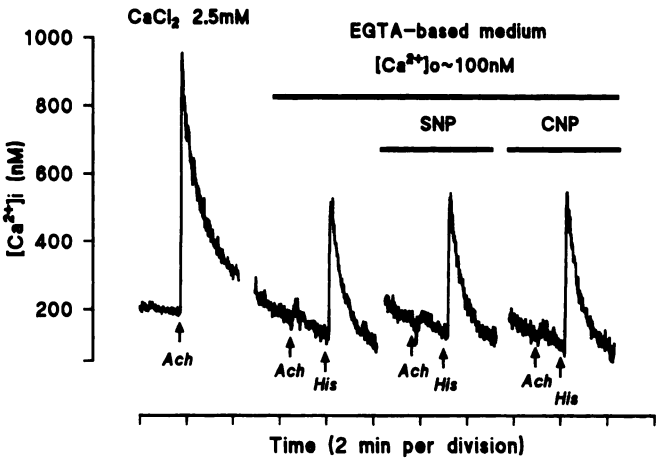

Fig. 6. Effect of SNP and CNP pretreatment on calcium release from internal stores. Adrenomedullary chromaffin cells loaded with Fura-2 were stimulated with 50 μM acetylcholine (*Ach*) in normal medium including 2.5 mM $CaCl_2$ and in calcium-free EGTA-based medium with 100 nM $[Ca^{2+}]_o$. The effect of 100 μM histamine (*His*) was studied in calcium-free medium. Cells were preincubated for 45 min with 100 μM SNP or 100 nM CNP. Each tracing shows individual results from representative experiments.

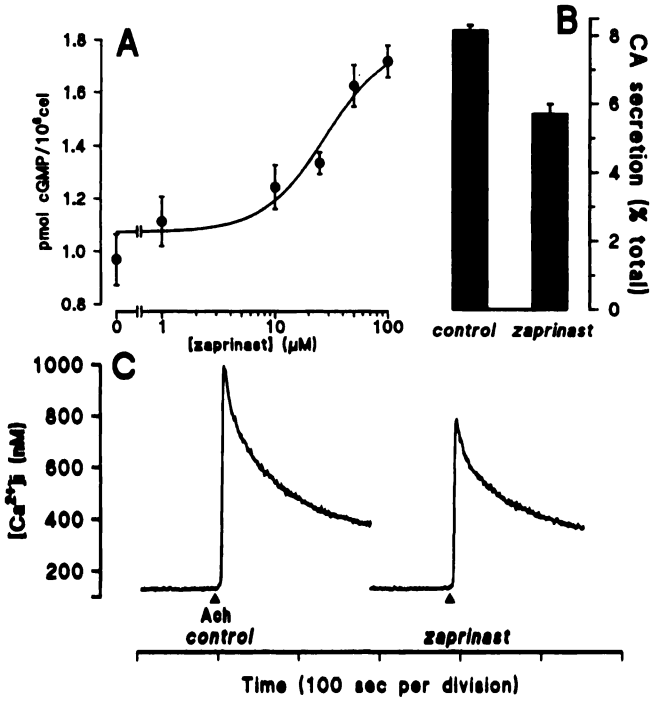

Fig. 7. Effect of the cGMP-phosphodiesterase inhibitor zaprinast on cGMP levels and on acetylcholine (*Ach*)-stimulated catecholamine (*CA*) secretion and calcium increases. A, Concentration-dependence curve for cGMP levels from chromaffin cells incubated for 30 min with increasing concentrations of zaprinast. B, Histogram showing the effect of 30-min preincubation with 100 μM zaprinast on 50 μM acetylcholine-stimulated catecholamine secretion. C, $[Ca^{2+}]_i$ traces from representative records showing the effect of 30-min preincubation with 100 μM zaprinast on 50 μM acetylcholine-stimulated calcium increases. Experiments were performed in triplicate, and data in A and B are mean ± standard error from two different cellular preparations.

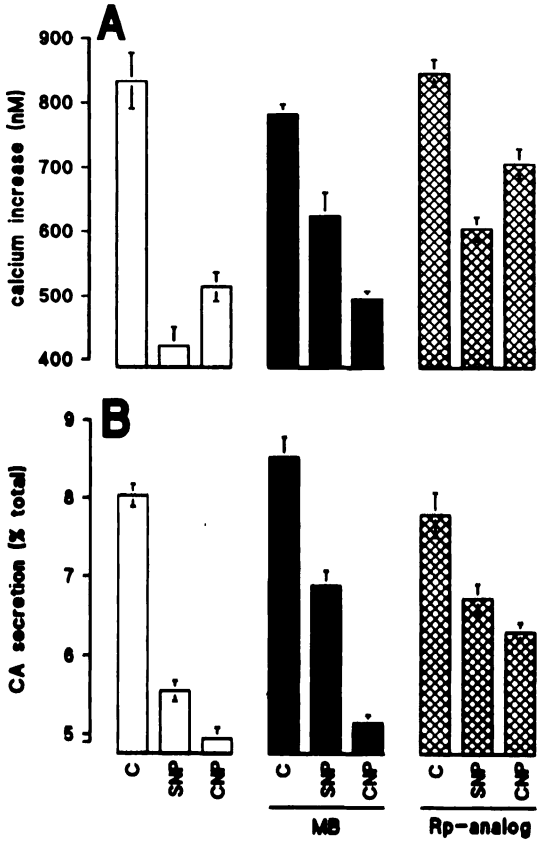

Fig. 8. Effects of the soluble guanylate cyclase inhibitor methylene blue and the PKG inhibitor Rp-8-pCPT-cGMPS on the inhibition by SNP and CNP of acetylcholine-evoked catecholamine (*CA*) secretion and calcium increases. Cells were preincubated for 45 min with SNP or CNP in the presence and absence of 10 μM methylene blue (*MB*) or 5 μM Rp-8-pCPT-cGMPS (*Rp-analog*). *C*, control. After preincubation, calcium increases (A) and catecholamine secretion (B) were determined as described in Experimental Procedures. Experiments were performed in triplicate, and data are mean ± standard error from three different cellular preparations.

blue could be accounted for by the fact that SNP-evoked cGMP production in the presence of 10 μM methylene blue is still sufficient to produce a small inhibitory effect. The specific inhibitor of PKG, Rp-8-pCPT-cGMPS) (26), was able to limit the inhibitory effects of both SNP and CNP on $[Ca^{2+}]_i$ and catecholamine release (Fig. 8). This PKG inhibitor in a dose-dependent manner totally reversed the observed inhibitory effect of cGMP-increasing compounds (shown for CNP, Fig. 9). The IC_{50} value for this compound for the reversion of the inhibitory effect produced by CNP on acetylcholine-evoked catecholamine secretion was 4.34 ± 1.87 μM. Although other mechanisms cannot be ruled out, these results argue for the involvement of cGMP through PKG activation in the mechanisms by which both SNP and CNP act.

To further examine the involvement of PKG in the inhibitory actions of CNP and SNP, chromaffin cells were treated with SNP (100 μM) and CNP (100 nM) for different periods, and PKG activity was measured through phosphorylation of an exogenous substrate. These agents enhanced the activity of PKG measured *in vitro* as a function of time (Fig. 10). For comparison, Fig. 10, A and B, show the time courses of cGMP production and the inhibition of calcium influx and catecholamine secretion for both SNP and CNP. It can be observed that on incubation with SNP or CNP, a well-defined sequence of events occurs: cGMP production is accompanied by the triggering of PKG activity, reaching maximal kinase activity after the cGMP production has reached its maximal level for both SNP and CNP. Maximal inhibitory effects on calcium influx and catecholamine secretion require longer incubation times and appear with a significant delay compared with the earlier phenomena.

Although relatively long periods of incubation with SNP and CNP were required to inhibit $[Ca^{2+}]_i$ peaks and catecholamine secretion, this does not imply that the continuous presence of these compounds is needed for such effects. After a brief 5-min pulse with SNP (sufficient to increase cGMP levels), a 55-min wash period made the cGMP levels drop to basal level, whereas an inhibitory effect on calcium influx and secretory response was still produced and persisted throughout the experimental time, although this inhibitory effect was not as great as that observed in the continuous presence of SNP (data not shown). These results suggest that intracellular signaling requires short intervals of activation by agents that increase cGMP to produce long term effects on secretory response.

The involvement of G proteins in the PKG calcium and cation channel modulation mechanism has been shown in several cell types (27). For this reason, we wondered whether G proteins could be involved in the cGMP increase-mediated inhibitory mechanism. Fig. 11 shows the effect of PTX on

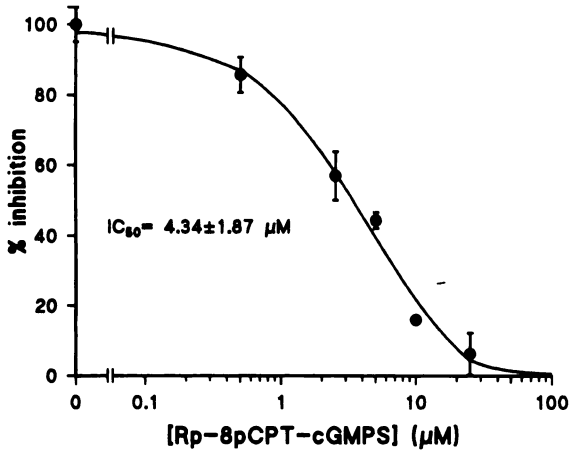

Fig. 9. Concentration-dependent reversion of CNP inhibitory effect by Rp-8-pCPT-cGMPS. Chromaffin cells were preincubated for 5 min with different concentrations of Rp-8-pCPT-cGMPS before 45 min treatment with 100 nM CNP. Cells were stimulated to secrete with 50 μM acetylcholine for 2 min. A 43.57 ± 1.63% of inhibition caused by CNP on catecholamine secretion is normalized to 100%. Experiments were performed in triplicate, and data are mean ± standard error from two different cellular preparations.

catecholamine release and on the inhibitory effects of 45-min preincubation of chromaffin cells with SNP or CNP. Pretreatment of chromaffin cells with PTX (150 ng/ml) over 24 hr produced an increase in acetylcholine-stimulated catecholamine release of ~53% compared with the control value (non-PTX-treated cells). The basal release was also increased in PTX-treated cells, reaching twice that of control cells (data not shown). Nevertheless, pretreatment of chromaffin cells with PTX did not prevent CNP or SNP from having an inhibitory effect on catecholamine release, and the level of inhibition obtained was similar to that obtained in non-PTX-treated cells, although the net catecholamine release was higher in PTX-pretreated cells. These results indicate that there are no PTX-sensitive G proteins involved in this cGMP increase-mediated inhibitory mechanism.

Discussion

The present study shows that agents that increase cGMP in bovine chromaffin cells caused an inhibition of catecholamine secretion and calcium influx elicited by high doses of the secretagogues acetylcholine and KCl in these same cells. This response occurred regardless of whether the cells were exposed to SNP, which releases NO, activating soluble guanylate cyclase, or to CNP, which stimulates the particulate guanylate cyclase.

In some tissues, cGMP can produce its biological effects by modifying cAMP levels (24). According to our results, the cAMP levels do not seem to be regulated by cGMP levels through the action of cGMP-stimulated or -inhibited cyclic nucleotide phosphodiesterases. Thus, cGMP is the agent responsible for the observed effects that cannot attributed to cAMP.

Although SNP could alter cellular function through NO- and cGMP-independent mechanisms and CNP has been shown to produce neuromodulatory effects independent of cGMP (28, 29), several results show that cGMP is the messenger involved in the inhibitory effects observed in chromaffin cells: (i) there is a close parallel between the concentrations of both compounds required to increase cGMP levels and those that induce inhibitory effects on calcium entry and catecholamine release, and (ii) the specific cGMP phosphodiesterase inhibitor zaprinast (30) was able to increase cGMP levels and inhibit both acetylcholine-stimulated $[Ca^{2+}]_i$ increases and catecholamine release. This indicates that the inhibitory effects can also be obtained not only by stimulating the biosynthetic pathway but also by inhibiting the catabolic one. In addition, (iii) we used two drugs that affect the cGMP pathway at different points. First, the soluble guanylate cyclase inhibitor methylene blue was able to attenuate the inhibition induced by SNP but not by CNP, indicating that a NO-activated guanylate cyclase is involved in the effects mediated by SNP. Second, the novel specific inhibitor of the PKG Rp-8-pCPT-cGMPS (26) reversed the inhibitory effects produced by preincubation with SNP or CNP in a concentration-dependent manner. These results indicate that these compounds modulate catecholamine secretion through a mechanism that gives rise to cGMP production and activation of PKG.

In previous studies with the permeable analogue of cGMP, 8-Br-cGMP, we provided evidence that in bovine chromaffin cells the NO/cGMP pathway is involved in inhibition of secretagogue-stimulated catecholamine release and calcium influx (16, 20). We proposed a mechanism based on the modulation of voltage-dependent calcium channels, in particular, the dihydropyridine-insensitive type, which exists and actively participates in catecholamine secretion from bovine chromaffin cells. Here, we show that the natriuretic peptide and the NO generator also produce inhibition of secretagogue-induced calcium influx but only in conjunction with high doses of acetylcholine or KCl. When low doses of the secretagogue were used to elicit the calcium and secretory response, neither inhibition nor stimulation occurred. The depolarizations induced by low doses of secretagogues are small, and therefore the activation of voltage-dependent calcium channels is also very low. Under these conditions, both calcium response and catecholamine secretion from control cells were diminished, and, interestingly, inhibition by SNP and CNP was not observed. It must be taken into consideration that chromaffin cells possess only high voltage-activated calcium channels (31). Thus, according to these results, SNP and CNP may be affecting the calcium entry through these channels that would participate to a great extent at high depolarizations induced by high doses of secretagogue. Also, when the inhibition of calcium peak was observed (at 50 μM acetylcholine and 30 mM KCl), no change was found in the rate of recovery of $[Ca^{2+}]_i$ after the Ca^{2+} peak evoked by both secretagogue stimuli. These $[Ca^{2+}]_i$ transients exhibit a similar shape in the presence or absence of either SNP or CNP. This means that secondary calcium processes such as the buffering/Ca^{2+} extrusion capacity or other components of calcium entry of the chromaffin cells were not modified by treatment with SNP or CNP. Moreover, neither drug was able to modify the calcium response to histamine in Ca^{2+}-free medium, a response that is exclusively due to mobilization from internal stores. Although the purpose of this work was not to study this type of calcium response, this latter result suggests that, at least in chromaffin cells, it is not modulated by the NO/cGMP pathway. Taken together, these results indicate that the reduction in $[Ca^{2+}]_i$ increase seems to be due to an inhibition of calcium influx through voltage-sensitive cal-

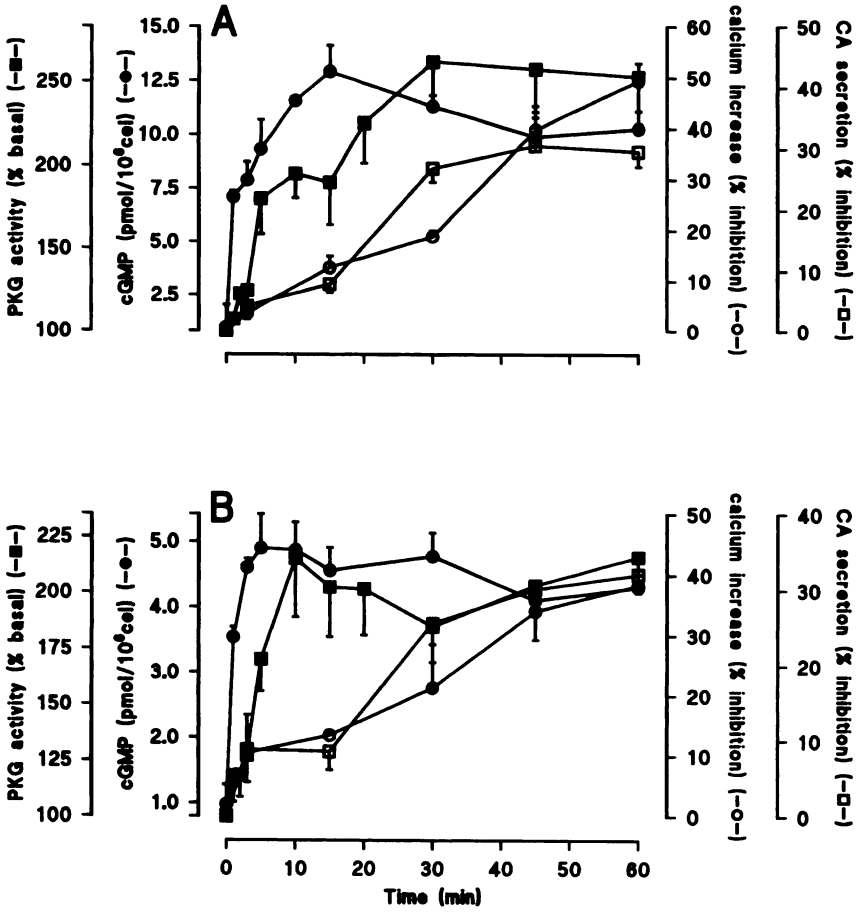

Fig. 10. Time-dependent effect of SNP and CNP on PKG activity from bovine chromaffin cells. Cells were preincubated for the indicated times with 100 μM SNP (A) or 100 nM CNP (B), and then the PKG activity (■) was measured by with an *in vitro* phosphorylation assay as described in Experimental Procedures. PKG is expressed as a percentage of basal activity; 100% corresponds to 441.19 ± 89.43 units/mg protein (1 unit is defined as the enzymatic activity able to incorporate 1 pmol phosphate/min into the heptapeptide substrate at 30°). For comparison, cGMP levels (●), calcium increases (○), and catecholamine (*CA*) secretion (□) from Figs. 1, 3, and 5, respectively, are also shown. Experiments were performed in triplicate, and data are mean ± standard error from two different cellular preparations.

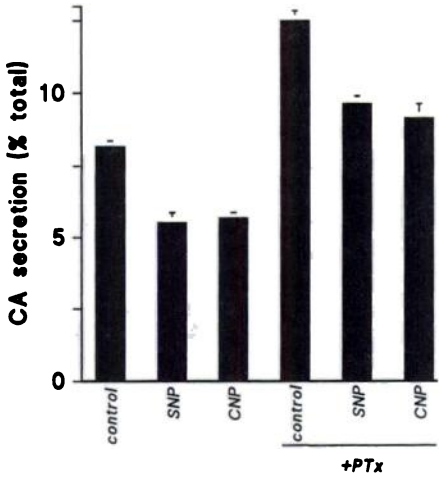

Fig. 11. Effect of PTX (*PTx*) pretreatment on the inhibition by SNP and CNP of the acetylcholine-stimulated catecholamine (*CA*) secretion. Chromaffin cells in culture were treated with 150 ng/ml PTX for 24 hr. After this pretreatment, cells were preincubated with 100 μM SNP or 100 nM CNP for 45 min, and the 50 μM acetylcholine-evoked catecholamine secretion during a period of 2 min was measured. Experiments were performed in triplicate, and data are mean ± standard error from two different cellular preparations.

cium channels, as we previously observed with 8-Br-cGMP (20).

Because the inhibition of both the epinephrine and the norepinephrine release was observed, the mechanism by which cGMP acts seems to be present in both secretory cell types (i.e., adrenergic and noradrenergic cells). On the other hand, in agreement with the work of other authors (17), we also observed a modest increase in the total content of catecholamines after long pretreatment with SNP or CNP. This effect has been account for by increased catecholamine synthesis by activation of the cGMP pathway, presumably through phosphorylation of regulatory residues of tyrosine hydroxylase by PKG (17).

The results of the current demonstrated that this neuromodulatory pathway based on cGMP has physiological importance because its natural ligands (i.e., NO and CNP), can arise from different sources within the adrenal gland.

We questioned the sources of NO and CNP in the adrenal medulla. The anatomic characteristics of the gland make it necessary to consider the types of cells present in the medulla. First, endothelial cells that surround the clusters in which chromaffin cells are grouped can synthesize and release several substances, including CNP and NO (5, 15). In experiments with bovine chromaffin cells and aortic endothelial cells, it was suggested that factor or factors released by endothelial cells might have an inhibitory role on the chromaffin cell secretory response (7). This inhibition was sensitive to NOS and soluble guanylate cyclase inhibitors. These results indicate that this factor could be NO. Another source of NO is innervation of chromaffin cells. From immunohistochemical studies, strongly labeled NOS-immunoreactive fibers have been found entering the medulla and coming into contact with chromaffin cells (8). Therefore, the NO pathway is of special interest because it may be regulated by synaptic inputs. Finally, the chromaffin cells themselves can be the source (and, of course, the destination) of both CNP and NO.

CNP exists in these cells and can be secreted from them during the secretory response, as has been described previously (14). The presence in chromaffin cells of the neuronal isoform of the constitutive NOS has been demonstrated by histochemical and biochemical studies (8–10). We recently showed that the agonists used in this study to elicit calcium and secretory responses, acetylcholine and KCl, were able to increase chromaffin cGMP levels (11). Such increases were mediated by NO production from NOS and required calcium entry and activation by Ca^{2+} of calmodulin. However, it is noteworthy that these increases in cGMP levels were transitory and modest due to the simultaneous activation by Ca^{2+}/calmodulin of a cyclic nucleotide phosphodiesterase activity. The presence of this enzymatic activity would allow chromaffin cells to respond to exogenous NO with a cGMP elevation but would limit such a response when the NO is produced endogenously through activation of NOS with secretagogue compounds via $[Ca^{2+}]_i$ increase. Because of this phosphodiesterase activity, we decided to determine the role of cGMP on chromaffin cell function by using compounds able to stimulate the cGMP signaling but in a way that is independent of calcium signaling; we used the NO donor and the natriuretic peptide.

Results obtained with other neural tissues correlate well with the present observations. For example, NO reduces depolarization-induced calcium influx in the pheochromocytoma cell lineage, PC12 cells, via a cGMP-mediated mechanism (32). Furthermore, it has been demonstrated that NO may be involved in transient presynaptic depression in the CA1 region of the hippocampus through activation of soluble guanylate cyclase (33) and that nitroprusside inhibits neurotransmitter release at the frog neuromuscular junction (34). Also, a mechanism based on inhibition of calcium entry has been reported in the hippocampus, in which cGMP depresses a high voltage-activated Ca^{2+} current (35).

Although little work has been done on the role of cGMP in the secretory response of chromaffin cells, there are some reports indicating a modulatory role. Previous studies showed inhibitory effects of NO, although no possible mechanism of action was studied (10, 18). The work of O'Sullivan and Burgoyne (19) deserves special mention. These authors described a biphasic effect of cGMP on catecholamine secretion from chromaffin cells; i.e., it was stimulatory when low doses of the secretagogue nicotine were used and inhibitory at higher concentrations. Several explanations can be provided to account for the divergent results obtained at low doses of secretagogue. First, the experimental protocols followed by these authors were very different from ours. They added simultaneously the secretagogue and the cGMP-increasing compound (SNP or atrial natriuretic peptide). According to our results, both the increase in cGMP production and the PKG activity require time to develop. Therefore, the immediate stimulatory effect observed by these authors can hardly come from a cGMP-based mechanism. We analyzed the effect of SNP and CNP on catecholamine secretion and calcium influx elicited by low doses of secretagogue after preincubation for 45 min with SNP and CNP. These conditions ensured that when the secretion and calcium responses were measured, increased cGMP production and PKG activity really existed. Second, there are differences in the methods used to elicit catecholamine release. In the work carried out by these authors, the duration of exposure to the secretagogue was 10 min, whereas we measured secretion over a 2-min period. Short periods of time better reflect the rapid response of the pool of granules ready to be secreted and minimize the influences of other processes such as transport of vesicles to the plasma membrane or the accumulation of the released endogenous substances in the extracellular environment, including catecholamines, which may affect the secretory function.

Furthermore, a biphasic effect of different concentrations of cGMP on calcium entry has been described in other tissues. For example, Xu *et al.* (36) showed a stimulatory effect at low concentrations of cGMP and an inhibitory effect when the second messenger pathway was stimulated to produce high amounts of cGMP. Using the pancreatic acini as a model in which calcium entry is evoked by depletion of internal stores, these authors showed that cGMP may function as an intracellular messenger between the internal stores and the plasmalemma calcium channels. The Ca^{2+} released from internal stores would increase the cGMP levels via production of NO. Subsequently, the cGMP generated may positively or negatively modulate the capacitative calcium entry in the plasma membrane (depending on the loading of the stores). However, it must not be forgotten that these authors studied very different phenomena than those investigated here. Thus, under our experimental conditions, we did not observe any biphasic cGMP effect but only an inhibition of calcium entry and secretory response at the highest acetylcholine and KCl concentrations used, presumably due to an effect on voltage-dependent calcium channels.

Regarding the effect of CNP, Babinski *et al.* (13) described the inhibitory effects of CNP on catecholamine secretion from chromaffin cells. However, they proposed an action independent of cGMP for such an inhibitory role. These authors reported that 1-min incubation with 10 pM CNP produced an inhibition of nicotinic-evoked, but not of depolarization-evoked, calcium currents and catecholamine secretion. In our study, on the other hand, CNP was able to inhibit both acetylcholine- and KCl-evoked catecholamine release and $[Ca^{2+}]_i$ increase. Moreover, no inhibition was found either at a time as short as 1 min or at concentrations as low as 10 pM. We found that these effects are mediated by cGMP increases, and thus the receptor involved would be the R-1C subtype, which is activated by CNP at a nanomolar range and coupled to cGMP synthesis, and not ANF-R_2, which is activated at a picomolar and not coupled to cGMP increases (37). It is not possible to explain these discrepancies at present.

An aspect of our results that deserves special attention is the time course of the signaling. We measured four different parameters: cGMP increase, PKG activity, catecholamine release, and $[Ca^{2+}]_i$ increase. The results show that the increases in cGMP levels are accompanied by the activation of PKG. However, to observe an inhibition of both catecholamine release and calcium influx, longer incubation times with SNP and CNP are required. As the inhibitory effect persisted even when cells were stimulated for 5 min with either SNP or CNP followed by a 55-min wash period, it seems that only short periods of stimulation are needed to activate the PKG enzyme, but the mechanism linking PKG activation and calcium influx inhibition, and therefore catecholamine release, is rather slow. The question that remains and requires thorough investigation is whether the response to phosphorylating agents reflects a direct action of the ki-

nase on calcium channels or an indirect action mediated by other regulatory molecules whose own activity depends on phosphorylation.

In addition, it is worth pointing out that although SNP was more potent than CNP in increasing cGMP levels and PKG activity, the inhibitory effect on catecholamine release and calcium influx was similar to that produced by CNP. These results could indicate that full activation of PKG is not necessary for maximal inhibitory effects to be produced.

As discussed above, the secretagogue-evoked $[Ca^{2+}]_i$ transient inhibition caused by SNP or CNP seems to be due to an inhibition of voltage-sensitive calcium channels as is seen in other tissues, such as heart and smooth muscle, in which cGMP inhibits calcium currents by stimulating PKG (38). PTX-sensitive G proteins of the G_i and G_o families are involved in stimulation and inhibition of voltage-dependent Ca^{2+} channels (39). There is evidence that PKG modulates calcium and cation channels in different cellular preparations through a membrane-delimited pathway triggered by a G protein (27). We therefore examined whether PTX pretreatment of chromaffin cells could prevent SNP or CNP inhibitory effects on catecholamine secretion. As has been shown, pretreatment of chromaffin cells with PTX effectively increases catecholamine release, as has been described (40), but does not have any effect on the inhibition caused by SNP or CNP. Thus, SNP and CNP inhibit catecholamine release and calcium influx via a mechanism that involves PKG activation but not a G protein.

In conclusion, the data reported here show that agents that strongly increase cGMP levels in chromaffin cells, such as NO and CNP, produce significant inhibition of the secretory response. This modulation seems to be due to an inhibition of voltage-dependent calcium channels through a mechanism mediated by PKG. From these results, several key issues arise and should stimulate future research regarding, for example, an understanding of the role of cGMP within the complex context of the adrenal medulla in which different sources of NO and CNP have been described. Also, it is necessary to study in depth the precise molecular mechanism linking PKG activation and the action on calcium channels, as this remains unknown.

Acknowledgments

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