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ACTIVATION OF NO:cGMP PATHWAY BY ACETYLCHOLINE IN BOVINE CHROMAFFIN CELLS

POSSIBLE ROLE OF Ca^{2+} IN THE DOWN-REGULATION OF cGMP SIGNALING

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Abstract—The production of cyclic GMP (cGMP) induced by acetylcholine and other stimuli was studied in bovine chromaffin cells. Acetylcholine increased intracellular cGMP in a transitory (peak at 2 min) and concentration-dependent manner (estimated half maximal increase, $EC_{50} = 61 \pm 5$ μM). N^G-nitro-L-arginine methyl ester (NAME) inhibited such a rise in cGMP with a half maximal inhibitory concentration (IC_{50}) of 231 ± 55 μM. The acetylcholine-induced increase in cGMP was also inhibited by a calmodulin antagonist (calmidazolium, 30 μM) and by the absence of extracellular calcium. Other agents that strongly increased cytosolic calcium concentration ($[Ca^{2+}]_i$) as acetylcholine did, such as the nicotinic-agonist, 1,1-dimethyl-4-phenylpiperazinium (DMPP), high-KCl (50 mM), and ionomycin, also caused a rise in cGMP in cultured bovine chromaffin cells. Veratridine, an activator of sodium channels, produced a slowly developing calcium increase and no significant cGMP production. The muscarinic-agonist, muscarine, failed to increase cytosolic calcium, and was the weakest stimulator of cGMP production. cGMP formation, induced by sodium nitroprusside (SNP, 100 μM) and by C-type natriuretic peptide (CNP, 100 nM), was inhibited by 30–40% by increasing $[Ca^{2+}]_i$ with ionomycin. This inhibition was abolished by calmidazolium (30 μM) and by the absence of calcium in the extracellular medium. In conclusion, bovine chromaffin cells synthesize nitric oxide (NO) to activate guanylate cyclase in response to several stimuli, which increase $[Ca^{2+}]_i$. Moreover, the increase in $[Ca^{2+}]_i$ also stimulates a Ca^{2+}/calmodulin phosphodiesterase, which could down-regulate the levels of cGMP in these cells.

Key words: acetylcholine; bovine chromaffin cells; cyclic GMP; nitric oxide; Ca^{2+}/calmodulin-phosphodiesterase

Stimulation of nicotinic acetylcholine receptors in chromaffin cells triggers secretion of catecholamines through a mechanism implies membrane depolarization and activation of voltage-dependent Ca^{2+}-channels [1, 2]. Acetylcholine also elevates cGMP† in bovine chromaffin cells, although this elevation is thought to be mediated by muscarinic receptor activation [3–5]. Some authors, conversely, have reported cGMP increases by nicotinic agonists, nicotine, and DMPP in bovine chromaffin cells and cat adrenal medulla [6, 7]. Cyclic GMP levels in chromaffin cells from different species are also increased by agents that spontaneously generate nitric oxide [8], by natriuretic peptides [8, 9], and by different stimuli that trigger secretion in chromaffin cells [7].

NO, which is regarded as an endothelium-derived relaxing factor (EDRF), plays its physiological functions as intra- and intercellular messenger molecule by activation of a soluble guanylate cyclase, resulting in formation of cGMP [10]. The formation of NO from L-arginine is catalyzed by the enzyme NO synthase (NOS) [11]. The presence of NOS has been demonstrated in whole adrenal glands and in cultured chromaffin cells by both histochemical and biochemical methods [7, 12]. The enzyme is Ca^{2+} and calmodulin (CaM)-dependent and is inhibited by arginine analogues, such as N-nitro-L-arginine methyl ester (L-NAME) [7, 12]. In cat adrenal medulla, all stimuli that increase cytosolic calcium activate NOS and produce an increase in cGMP [7]. In contrast, it has been reported that cGMP formation is inhibited by Ca^{2+} at concentrations required for the activation of NOS due to the presence of Ca^{2+}-sensitive guanylate cyclase activity [10, 13]. The direct inhibition of guanylate cyclase by Ca^{2+} contrasts, however, with results showing that purified soluble guanylate cyclase is not significantly affected by micromolar concentrations of free Ca^{2+} [14]. Alternatively, various investigators have reported the existence of a Ca^{2+}/calmodulin-stimulated cGMP-specific phosphodiesterase (PDE), which may be responsible for the apparent inhibition of cGMP formation by Ca^{2+} [14, 15].

Data from the literature support a dual role of cGMP in chromaffin cell function. Cyclic GMP appears to stimulate catecholamine synthesis through activation of tyrosine hydroxylase [16, 17], the rate-limiting step in catecholamine biosynthesis, whereas it plays an inhibitory role in the regulation of secretion from chromaffin cells [5, 18–20]. Recently, we have shown that the membrane-permeable cGMP analogue, 8-bromo-cGMP, inhibits catecholamine secretion and calcium influx in cultured bovine chromaffin cells, and that the cGMP-dependent protein kinase (PKG) appears to be involved in these effects [21, 22].

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† Abbreviations: $[Ca^{2+}]_i$, intracellular calcium concentration; CaM, calmodulin; Ca^{2+}/calmodulin PDE, calcium and calmodulin-dependent phosphodiesterase; cGMP, cyclic GMP; CNP, C-type natriuretic peptide; DMPP, 1,1-dimethyl-4-phenylpiperazinium; EC_{50}, half maximal stimulatory concentration; IBMX, isobutyl-1-methylxanthine; IC_{50}, half maximal inhibitory concentration; NAME, N^G-nitro-L-arginine-methyl-ester; NO, nitric oxide; NOS, L-arginine:NO synthase; SNP, sodium nitroprusside.

The aim of the present experimental work was to study the increase in cGMP in bovine chromaffin cells in response to acetylcholine and other stimuli known to induce catecholamine secretion. Moreover, we have investigated the requirement of extracellular calcium for cGMP production by acetylcholine and the involvement of NOS in this effect. Our results demonstrate that acetylcholine elevates cGMP levels in bovine cultured chromaffin cells through a cascade of events which are:

1. stimulation of nicotinic acetylcholine receptors;
2. membrane depolarization and activation of voltage-dependent Ca^{2+} channels;
3. elevation of $[Ca^{2+}]_i$;
4. activation by Ca^{2+}/CaM of NOS and subsequent production of NO; and
5. activation by NO of soluble guanylate cyclase, resulting in formation of cGMP.

We also propose that a feedback mechanism to down-regulate the cGMP formation exists. Such a mechanism could involve the activation of a Ca^{2+}/CaM-dependent cGMP-specific PDE upon elevation of $[Ca^{2+}]_i$.

MATERIALS AND METHODS

Materials

Inorganic salts were from Merck (Germany). Acetylcholine chloride, L-NAME, 1,1-dimethyl-4-phenylpiperazinium (DMPP), (±) muscarine chloride, sodium nitroprusside (SNP), veratridine, and IBMX were from Sigma (St. Louis, MO, U.S.A.). Ionomycin was from Calbiochem (San Diego, CA, U.S.A.). Calmidazolium (R 24571) was from Boehringer Mannheim (Germany). C-type natriuretic peptide (CNP) was from Peninsula Laboratories (Belmont, CA, U.S.A.). Fura-2 AM was from Molecular Probes (Eugene, OR, U.S.A.). Radioimmunoassay cyclic GMP kit was from Amersham (U.K.). Culture media and fetal calf serum were from GIBCO BRL (U.K.).

Preparation of chromaffin cells

Chromaffin cells were obtained after digestion of bovine adrenal glands with collagenase (EC 3.4.24.3) in retrograde perfusion, as described by Miras-Portugal *et al.* [23]. 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 (in mM) NaCl 154, KCl 5.6, $NaHCO_3$ 3.6, glucose 5, and HEPES 5, 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 according to the method described by Wilson [24]. Over 90–95% of the collected cells were chromaffin cells, as they were massively and clearly stained by neutral red [25]. Occasionally, the purity of the cultures was lower than this range; in this event, cells were then purified by differential plating according to the method of Banerjee *et al.* [26]. Purified chromaffin cells were suspended at a density of 10^6 cells ml^{-1} in Dulbecco's modified Eagle's medium (DMEM), containing 10% heat-inactivated fetal calf serum and standard antibiotics. For cyclic GMP measurements, cells were plated in collagen-treated 24-well Costar cluster dishes at a density of 10^6 cells/well in culture medium supplemented with 10 μM cytosinearabinofuranoside and 10 μM fluorodeoxyuridine, maintained at 37°C in 5% CO_2/95% air; these cells were used for 3–5 days following cell isolation. For cytosolic calcium measurements, cells were maintained in suspension and kept at 4°C. These cells were used 2–3 days following cell isolation.

Intracellular cyclic GMP measurements

Cells were serum-deprived for 24 hours before cGMP measurement, then washed twice with Locke's solution (composition in mM: NaCl 140, KCl 4.4, $CaCl_2$ 2.5, $MgSO_4$ 1.2, KH_2PO_4 1.2, $NaHCO_3$ 4, glucose 5.6, and HEPES 10, pH 7.4). The cells were preincubated with 0.5 mM 3-isobutyl-1-methylxanthine (IBMX) in Locke's solution (with N-nitro-L-arginine methyl ester (L-NAME) when used) at 37°C for 30 min, and then stimulated with agents in 0.5 ml of Locke's solution containing 0.5 mM IBMX for the indicated times. Incubations were terminated by aspirating the medium and adding 400 μl of 6% trichloroacetic acid. Cells were then scraped out of the wells and centrifuged. The supernatant fraction was neutralized with 3M KOH and evaporated on a Speed-vac (Savant) [27]. The samples were resuspended in 4 mM EDTA, 50 mM Tris, pH 7.5, and cyclic GMP content was determined using a commercial [^{3}H]cyclic GMP radioimmunoassay kit (Amersham).

Measurement of cytosolic [Ca^{2+}]

Cytosolic Ca^{2+} concentration 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 by centrifugation. The recordings were performed in 1 ml samples containing 10^6 cells in thermostated and stirred cuvettes in a Perkin-Elmer LS-50 fluorimeter. The excitation and emission wavelengths were set to 340 and 510 nm, respectively. At the end of each experiment, the cells were lysed and the dye content calibrated according to the method described by Castro *et al.* [28]. The cytosolic Ca^{2+} concentration ($[Ca^{2+}]_i$) was derived from fluorescence traces using the equation of Grynkiewicz *et al.* [29].

Analysis of data

Cyclic GMP experiments were performed in triplicate, and results expressed as means ± SEM. Concentration-response relationships were fitted to a sigmoidal model of the form log-concentration versus response using the Parameter Fitter program (Biosoft).

The data from fluorimeter were interfaced to a computer to allow offline analysis, and the presented numerical values are means ± SEM of the magnitude of the increase in the calcium peak.

RESULTS

cGMP increases elicited by acetylcholine in cultured bovine chromaffin cells

The basal levels of cGMP in cultured bovine chromaffin cells upon preincubation for 30 min with the nonselective phosphodiesterase inhibitor IBMX (0.5 mM) were 1.840 ± 0.107 pmol/10^6 cells. As shown in Fig. 1A, acetylcholine (50 μM) produced a transitory 2.5-fold increase in cGMP levels, reaching a peak at 2 min and decreasing towards basal levels at longer times of stimulation. In Fig. 1B (insert), the production of

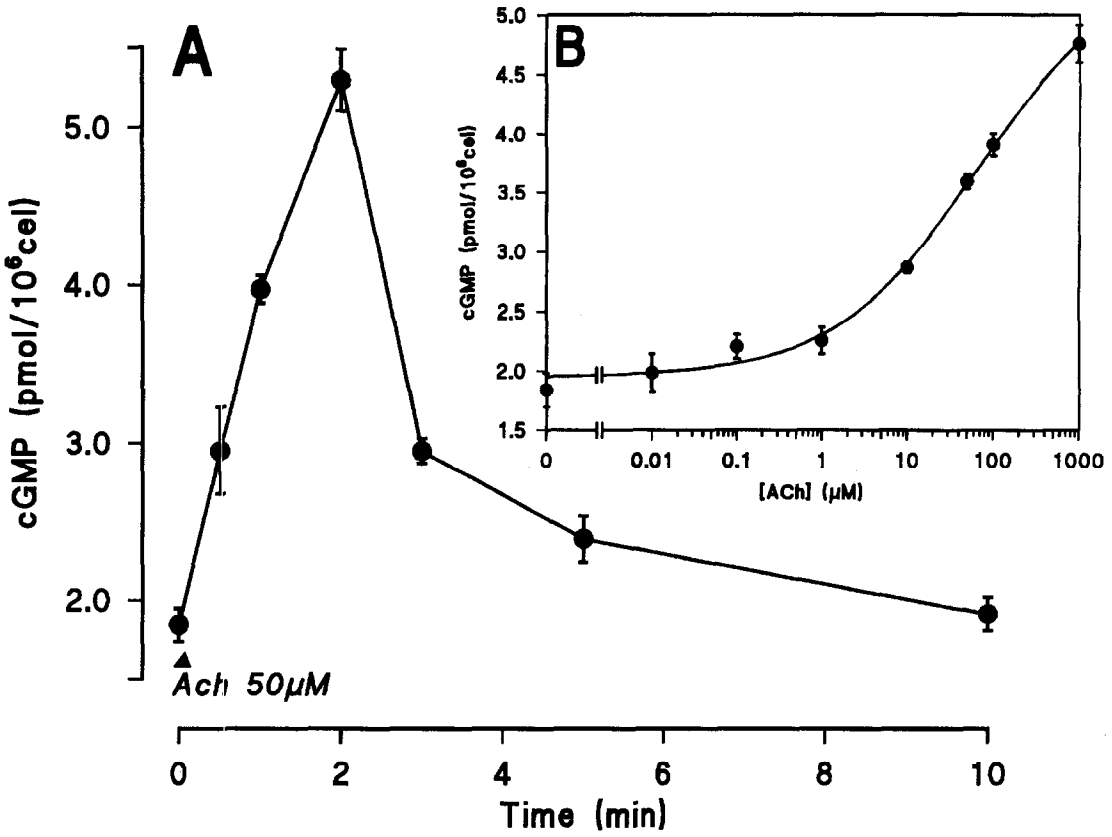

Fig. 1. Acetylcholine-stimulated cyclic GMP formation in bovine chromaffin cells. (A) Time course of cGMP production evoked by acetylcholine (50 μM). (B) Concentration-response curve for cGMP production induced by 1 min stimulation with acetylcholine. Cultured bovine chromaffin cells (10^6 cells/dish) were preincubated with 0.5 mM IBMX at 37°C for 30 min. After preincubation, they were incubated for the indicated time with acetylcholine at the required concentration in the presence of 0.5 mM IBMX. Cyclic GMP in the cells was assayed as described under Materials and Methods. Experiments were performed in triplicate and data, expressed in pmol/10^6 cells, are the mean ± SEM from two different preparations of chromaffin cells.

cGMP at 1 min stimulation with increasing acetylcholine concentrations from 10 nM to 1 mM is represented. The cGMP formation started to differ significantly from the basal value at 10 μM, and a plateau level was not reached even at the highest concentration employed. The estimated half maximal concentration (EC_{50}) was 61.05 ± 5.24 μM.

The rise in cGMP levels induced by acetylcholine was almost fully inhibited by the arginine analogue, L-NAME, having an IC_{50} value of 231.86 ± 55.95 μM (Fig. 2). This molecule is a specific inhibitor of NOS activity, indicating that in acetylcholine-stimulated cGMP production, the activation of NOS is involved.

Requirement of calcium for cGMP regulation

Table 1 shows the production of cGMP, measured at 1 min, stimulated by acetylcholine and other stimuli known to induce catecholamine secretion in chromaffin cells. The cytosolic variations in $[Ca^{2+}]_i$ elicited by the secretagogues were also studied using the calcium-sensitive dye fura-2. Acetylcholine, DMPP, KCl (50 mM), and ionomycin evoked a rapid increase in $[Ca^{2+}]_i$, after which the level decreased slowly to a plateau level. The values of the increase in the calcium peak are also shown in this table.

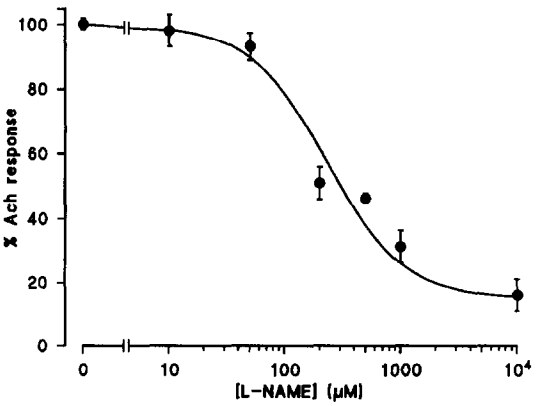

Fig. 2. Effect of L-NAME preincubation on cGMP formation evoked by acetylcholine. Cultured bovine chromaffin cells were preincubated with 0.5 mM IBMX plus increasing concentrations of L-NAME for 30 min at 37°C. After preincubation, they were incubated with acetylcholine (100 μM) for 1 min in the presence of 0.5 mM IBMX, and cGMP in the cells was assayed. Data, expressed as a percentage of acetylcholine response without preincubation with the arginine analogue, are means ± SEM of two experiments carried out in triplicate. The value of 100% is equal to an increase of 2.06 ± 0.1 pmol/10^6 cells over the basal level.

Table 1. cGMP formation and calcium increases elicited by different stimuli

	cyclic GMP		$[Ca^{2+}]_i$
	pmol/10^6cel	% basal	Net increase (nM)
Acetylcholine 100 μM	3.906 ± 0.094*	212.32	833.98 ± 31.83
Ach 100 μM in Ca^{2+}-free medium	1.871 ± 0.031 NS	101.71	32.91 ± 7.31
DMPP 50 μM	4.121 ± 0.113*	223.97	784.21 ± 14.05
Muscarine 100 μM	2.155 ± 0.213 NS	117.12	19.30 ± 3.86
KCl 50 mM	3.459 ± 0.239*	188.01	792.65 ± 37.48
Ionomycin 10 μM	3.598 ± 0.182*	195.54	1134.84 ± 24.66
Veratridine 30 μM	2.085 ± 0.151 NS	113.31	160.17 ± 12.76
SNP 100 μM	15.100 ± 0.507*	820.65	no change
CNP 100 nM	10.500 ± 0.514*	570.65	no change

For comparison, the effects of the NO-generator, SNP (100 μM), and the natriuretic peptide, CNP (100 nM) are also shown. cGMP formation is expressed as content in pmol/10^6cel and percentage of basal level upon 1 min stimulation with the different agents. Calcium increases are expressed in nM as the net increase in the calcium peak. The chromaffin basal levels of cGMP and $[Ca^{2+}]_i$ were 1.840 ± 0.107 pmol/10^6cel and 130 ± 15 nM, respectively. cGMP and $[Ca^{2+}]_i$ levels were assayed as described under Materials and Methods. Data are means ± SEM of two experiments performed in triplicate.

* For cGMP results, $p < 0.001$ indicates a significant difference from the control response according to Student's t-test.

† NS, not significant.

The acetylcholine-stimulated cGMP increase in chromaffin cells was abolished when the stimulation was performed in an EGTA-based buffer without free calcium. Under this experimental condition, the increase in $[Ca^{2+}]_i$ evoked by acetylcholine was also almost completely abolished in comparison with the increase evoked in the presence of 2.5 mM Cl_2Ca in the extracellular medium. DMPP and muscarine were employed as specific agonists for nicotinic and muscarinic receptors, respectively, in order to stimulate chromaffin cells. DMPP (50 μM) was a more potent stimulator of cGMP production than muscarine (100 μM), and similar to acetylcholine (100 μM). Muscarine, the poorest stimulator of cGMP production, also failed to increase cytosolic calcium concentration. The activation of sodium channels with veratridine (30 μM) produced a slowly developing calcium increase, and was not able to raise cGMP levels. Depolarization of chromaffin cells with 50 mM KCl, which raised $[Ca^{2+}]_i$ through activation of voltage-dependent calcium channels, and treatment with ionomycin (10 μM), a calcium ionophore, also produced an increase in cGMP levels similar to that obtained with acetylcholine or DMPP.

The production of cGMP in bovine chromaffin cells is also stimulated independently of calcium signaling by sodium nitroprusside (SNP) and C-type natriuretic peptide (CNP). SNP spontaneously releases NO and stimulates soluble guanylate cyclase, and the CNP peptide stimulates particulate guanylate cyclase [30].

In Fig. 3A and B, the time courses of SNP- and CNP-stimulated cGMP production are shown. SNP (100 μM) produced a continuously increasing cGMP formation while CNP (100 nM) invoked a high level of cGMP throughout stimulation, although it did reach an equilibrium. Both SNP and CNP were more potent in producing cGMP increases than the secretagogue stimuli employed in this work. To determine whether cGMP production could be down-regulated by Ca^{2+}, the SNP- and CNP-stimulated cGMP were measured in the presence of ionomycin (10 μM). As has also been shown in Fig. 3A and B, ionomycin inhibited the cGMP production elicited by SNP and CNP to a similar extent (30–40%). When cells were preincubated for 10 min with ionomycin prior to treatment with SNP or CNP, a greater inhibition (55–60%) was observed (data not shown). The presence of calmidazolium (30 μM), a calmodulin antagonist, as well as the absence of extracellular calcium, prevented the inhibition by ionomycin of SNP- and CNP-induced cGMP production (Fig. 4), indicating that the activation of a Ca^{2+}/CaM-activated cGMP-specific phosphodiesterase could be involved in the Ca^{2+} inhibitory mechanism. On the other hand, calmidazolium abolished the cGMP increase elicited by acetylcholine (Fig. 4). Although it has been described that anti-calmodulin drugs inhibit Ca^{2+} entry [31], and could, subsequently, inhibit acetylcholine-stimulated cGMP production, calmidazolium also blocked ionomycin-stimulated cGMP production. These results show that the inhibition of Ca^{2+}/calmodulin inhibited NOS activation and thus cGMP production induced by acetylcholine or ionomycin.

DISCUSSION

Nitric oxide, known to be generated in many different cell systems, produces some of its actions through acti-

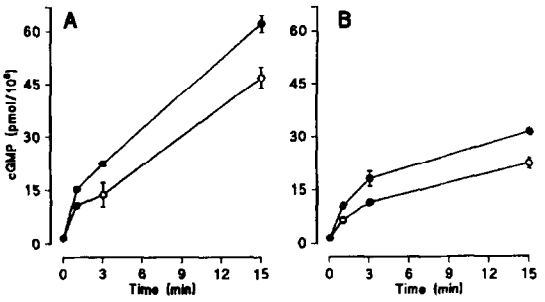

Fig. 3. Effect of ionomycin on cGMP production induced by SNP and CNP. Time courses of cGMP production evoked by (A) SNP 100 μM and (B) CNP 100 nM in the presence (○) and absence (●) of ionomycin 10 μM. Cultured bovine chromaffin cells were preincubated with 0.5 mM IBMX at 37°C for 30 min. After preincubation, they were incubated for the indicated time with SNP or CNP in the presence of 0.5 mM IBMX. When ionomycin was used, it was added simultaneously to the stimulus. Experiments were performed in triplicate, and data are means ± SEM from two different preparations of chromaffin cells.

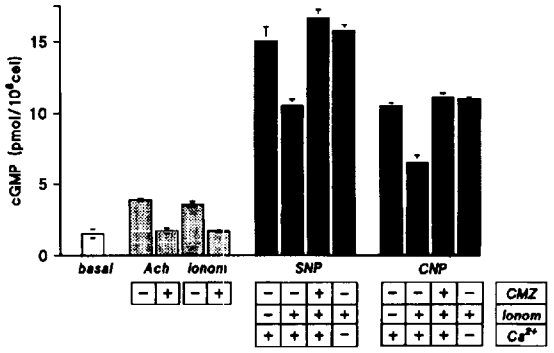

Fig. 4. Effect of the calmodulin antagonist calmidazolium on cGMP formation elicited by acetylcholine and ionomycin, and on reduction by ionomycin of SNP- and CNP-induced cGMP formation. Cultured bovine chromaffin cells were preincubated with 0.5 mM IBMX at 37°C for 30 min. When calmidazolium (CMZ, 30 μM) was used, it was added for the last ten minutes of preincubation with IBMX. After preincubation, cells were incubated in the presence of 0.5 mM IBMX for 1 min with acetylcholine (Ach, 100 μM) or ionomycin (Ionom, 10 μM), or with SNP (100 μM) or CNP (100 mM) in the presence and absence of ionomycin (10 μM) with or without extracellular calcium. When experiments were performed in the absence of extracellular calcium, the medium contained a mixture of Ca^{2+}/EGTA, calculated to give a final $[Ca^{2+}]_i$ ~100 nM. Data are means ± SEM of two experiments carried out in triplicate.

vation of a soluble guanylate cyclase [32]. In recent findings by Moro *et al.* [7] and Oset *et al.* [12], the presence of a constitutive NO synthase has been reported in bovine adrenal medulla cytosol. This enzyme is L-arginine- and Ca^{2+}/CaM-dependent, and synthesizes NO in response to a variety of stimuli. The released NO subsequently increases cGMP production [7]. In this experimental work, we show that cGMP levels in cultured bovine chromaffin cells are increased by several secretagogues, such as acetylcholine, DMPP, high-potassium, and ionomycin.

Acetylcholine stimulation resulted in a 2.5-fold increase in cellular cGMP, with an estimated half maximal increase at 60 μM. The acetylcholine-induced accumulation of cGMP in chromaffin cells was effectively blocked by L-NAME (IC_{50} = 230 μM), a compound that inhibits nitric oxide synthesis, by the Ca^{2+}/calmodulin antagonist calmidazolium, and by the absence of calcium in the extracellular medium. These results indicate that most, if not all, of the cGMP produced was mediated by NO release, which can be generated in the catalysis by NOS from its intrinsic substrate, L-arginine, as shown in a previous report [7]. Challenging chromaffin cells with acetylcholine resulted in an immediate increase in $[Ca^{2+}]_i$, after which the level decreased slowly to a plateau level. In the present paper, we show that acetylcholine produced a transient increase in cGMP, being maximal at 2 min. Thus, although a delay between the initial $[Ca^{2+}]_i$ increase and cGMP production exists, the time course of cGMP production followed the changes in $[Ca^{2+}]_i$. These results correlate well with the activation of NO synthase by increases in $[Ca^{2+}]_i$ and the subsequent activation of guanylate cyclase by released NO. As previously reported [7, 12], the NOS present in these cells was entirely Ca^{2+}-dependent, being inactive below 100 nM and fully active at 1 μM. The basal calcium level was approximately 130 nM, and the increase elicited by acetylcholine was 800 nM. Although it would not be strictly correct to compare results of NOS assays obtained *in vitro* with calcium increases measured by fura-2 technique, which represent a calcium average concentration, the $[Ca^{2+}]_i$ reached with acetylcholine was sufficient to almost fully activate the NOS in these cells. Additionally, other stimuli that strongly increase $[Ca^{2+}]_i$, such as high-potassium, which activates voltage-dependent Ca^{2+} channels and the calcium ionophore ionomycin, also increased cGMP levels in these cells. Nevertheless, veratridine, which produced a rather small calcium increase, was not able to raise cGMP levels. From this process, a clear relationship between calcium increases and cGMP formation exists. Since the effects of agonists on cGMP were almost identical and followed the $[Ca^{2+}]_i$ changes, it seems that the agonists did not have any effect on NOS activity beyond that induced by the $[Ca^{2+}]_i$ changes.

Furthermore, we have studied which type of acetylcholine receptor is involved in cGMP elevation. The selective nicotinic receptor agonist DMPP increased cGMP to the same level as that of acetylcholine, whereas the muscarinic receptor agonist, muscarine, proved to be the weakest agonist employed. These results correlate well with the small increase in $[Ca^{2+}]_i$ elicited by muscarine compared with that produced by DMPP or acetylcholine. Thus, in our preparations, the acetylcholine effect on cGMP production was mostly mediated through nicotinic receptor activation, subsequent depolarization, activation of voltage-dependent Ca^{2+} channels, and elevation of $[Ca^{2+}]_i$. Additionally, the acetylcholine concentration required to increase cGMP (EC_{50} = 60 μM) favours nicotinic receptor involvement in this action with respect to the muscarinic type activated by lower concentrations of acetylcholine. These results clearly contrast with those reported by other authors [3–5], who postulate the involvement of muscarinic receptor in acetylcholine-stimulated cGMP increases in bovine chromaffin cells. Although it is difficult to explain this discrepancy, these investigators used chromaffin cells in suspension the day following cell isolation, whereas in our study, chromaffin cells attached to plastic dishes were employed during the 3rd to 5th days of culture. In addition, in another study, a functional shift from muscarinic to nicotinic receptor involved in cGMP accumulation was described to occur during the primary culture of adrenal chromaffin cells [6]. Moreover, nicotinic as well as muscarinic agonists were able to increase cGMP levels in cat adrenal glands [7]. It is likely that the efficacy of nicotinic or muscarinic agonists in increasing cGMP is a function of the relative density of each type of cholinergic receptor and of its capacity for increasing $[Ca^{2+}]_i$ in chromaffin cells from different species.

The cGMP increases elicited by secretagogue stimuli have been shown in all cases to be less than those produced by SNP or CNP. Whereas acetylcholine and the other secretagogues increased cGMP levels 2.5-fold, SNP and CNP produced increases of 8- and 6-fold, respectively. Moreover, the time courses of cGMP production elicited by these compounds are also different. The secretagogue stimuli produced a transient increase in cGMP (in the case of acetylcholine), while SNP and CNP produced a sustained increase in cGMP. An inhibition of soluble guanylate cyclase at Ca^{2+} concentrations required for the activation of NO synthase in rat brain and adrenal medulla has been described [10, 13].

This indicates a down-regulation of the signal in NO-producing cells. This fact would explain the higher potency of SNP on cGMP production as compared to the secretagogues employed. However, results from other authors have shown that purified soluble guanylate cyclase is not inhibited by micromolar concentrations of Ca^{2+} [14, 15], and that a highly active Ca^{2+}/CaM-activated cGMP-specific phosphodiesterase is present in synaptic areas of the brain [14, 15]. The production of cGMP by SNP or CNP was inhibited by the presence of ionomycin, which increases cytosolic calcium concentration. Therefore, as CNP activates a particulate guanylate cyclase whose regulation by Ca^{2+} has not been demonstrated, it is more likely that the increase in $[Ca^{2+}]_i$ could activate a Ca^{2+}/CaM PDE rather than inhibit soluble guanylate cyclase. Additionally, the calmodulin antagonist, calmidazolium, and the lack of extracellular calcium were able to prevent the inhibitory effect of ionomycin on SNP- and CNP-produced cGMP. The presence of this type of phosphodiesterase, one not inhibited by IBMX at concentrations up to 1 mM [14], could also account for the transitory increase in cGMP produced by acetylcholine, even in the presence of IBMX 0.5 mM.

In a previous work, we have shown that the NO:cGMP pathway inhibits both catecholamine secretion and calcium influx in bovine chromaffin cells. This effect appears to be mediated by activation of the cyclic GMP-dependent protein kinase through inhibition of the dihidropyridine-insensitive calcium channels [20–22]. Thus, a regulatory role of cGMP on chromaffin cell function exists. It is important to note what kind of stimuli increases cGMP in these cells, as well as the mechanisms that tightly regulate the intracellular levels of this cyclic nucleotide.

In this work, we show that acetylcholine, through nicotinic receptor stimulation and other stimuli that strongly increase $[Ca^{2+}]_i$, activate the NOS present in these cells, and that the released NO activates soluble guanylate cyclase. The $[Ca^{2+}]_i$ increases evoked by these agents also cause activation of a Ca^{2+}/CaM-stimulated cGMP-specific phosphodiesterase, which might account for the apparent inhibition by Ca^{2+} of cGMP production. This phosphodiesterase activity may effectively down-regulate the NO signaling in these cells, and could serve to tightly regulate the cGMP levels in these cells in response to several stimuli.

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