Regulation of Synthesis of Guanosine 3':5'-Cyclic Monophosphate in Neuroblastoma Cells

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(Received 16 January 1978)

The increase in intracellular cyclic GMP concentrations in response to muscarinic-receptor activation in N1E-115 neuroblastoma cells is dependent on extracellular Ca^{2+} ion. The calcium ionophore A23187 can also evoke an increase in cyclic GMP in the presence of Ca^{2+} ion. Most (about 85%) of the guanylate cyclase activity of broken-cell preparations is found in the soluble fraction. The soluble enzyme can utilize MnGTP ($K_m = 55 \mu\text{M}$), MgGTP ($K_m = 310 \mu\text{M}$) and CaGTP ($K_m > 500 \mu\text{M}$) as substrates. Free GTP is a strong competitive inhibitor ($K_i \approx 20 \mu\text{M}$). The enzyme possesses an allosteric binding site for free metal ions (Ca^{2+} , Mg^{2+} and Mn^{2+}). The membrane-bound guanylate cyclase is qualitatively similar to the soluble form, but has lower affinity for the metal-GTP substrates. Entry of Ca^{2+} into cells may increase cyclic GMP concentration by activating guanylate cyclase through an indirect mechanism.

Acetylcholine raises intracellular concentrations of cyclic GMP in several tissues (George *et al.*, 1970; for review see Goldberg & Haddox, 1977). This response is mediated through the muscarinic receptor (George *et al.*, 1970; Lee *et al.*, 1972; Schultz *et al.*, 1973; Matsuzawa & Nirenberg, 1975), which is located in the plasma membrane (Yamamura & Snyder, 1974; Alberts & Bartfai, 1976). In contrast with studies with adenylate cyclase (EC 4.6.1.1), where coupling to hormone or neurotransmitter receptors can be demonstrated in plasma-membrane fractions (for review see Nathanson, 1977), guanylate cyclase (EC 4.6.1.2) activity is not affected by acetylcholine in broken-cell preparations (Limbird & Lefkowitz, 1975; Bartfai *et al.*, 1977). A requirement for extracellular Ca^{2+} for guanylate cyclase activation in tissue slices (Schultz *et al.*, 1973) and whole cells (Haymovits & Scheele, 1976) was demonstrated.

Matsuzawa & Nirenberg (1975) demonstrated a 200-fold increase in cyclic GMP concentrations in mouse neuroblastoma clone N1E-115 (Amano *et al.*, 1972) in response to muscarinic stimulation. This is in contrast with the 2–5-fold increase observed in other cell systems (Bartfai *et al.*, 1977). (In all of these studies appropriate inhibitors were used to block 3':5'-cyclic nucleotide phosphodiesterase activity.) Neuroblastoma cells can be grown as homogeneous populations and possess many neuronal properties. In the present study, we have examined the role of Ca^{2+} in activation of guanylate

cyclase in neuroblastoma cells, by using whole cells and subcellular fractions prepared from them.

Experimental

Materials

Cyclic [G- ^3H]GMP (sp. radioactivity 5–10 Ci/mmol) and [γ - ^{32}P]GTP (sp. radioactivity 2–10 Ci/mmol) were purchased from New England Nuclear, Boston, Ma, U.S.A.

C-Carboxypropionyl[^{125}I]iodotyrosine methyl ester of cyclic GMP was synthesized as described by Steiner *et al.* (1972) by using carrier-free Na^{125}I (350 mCi/ml; New England Nuclear). The phosphodiesterase inhibitor SQ 20009 was a gift from Dr. S. J. Lucania, Squibb Co., Princeton, NJ, U.S.A. All other reagents were of analytical grade.

Cell culture

Noradrenergic neuroblastoma clone N1E-115 was used between 20 and 34 subcultures after isolation as a single cell (Amano *et al.*, 1972). Stock cultures were grown as monolayers on 75 cm² tissue-culture flasks (Corning, Corning, NY, U.S.A.) at 37°C in a humidified atmosphere (air/CO₂, 19:1) by using the Dulbecco-Vogt modification of Eagle's medium H-el (Grand Island Biological Co., Grand Island, NY, U.S.A.) supplemented with 3% (v/v) foetal calf serum (Flow, Rockville, MD, U.S.A.). Cells were fed at 1 to 5 day intervals and subcultured weekly at a 1:10 ratio after re-suspension by trituration. For

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experiments, parallel sets of 60mm- or 150mm-diam. tissue-culture dishes (Falcon, Div. of Becton-Dickinson, Oxnard, CA, U.S.A.) were seeded with 5×10^4 or 3×10^5 cells per dish respectively, in 5ml or 25ml of medium respectively. Cultures were fed on day 5 or 6 after plating by adding 0.5vol. of fresh medium to the medium conditioned by cell growth and used on the following day. At this time cultures were 60–70% confluent and most of the cells had formed processes greater than 50 μ m in length.

Cellular concentrations of cyclic GMP

Cultures grown on 60mm dishes were removed from the incubator and washed with 3×1 ml of an iso-osmotic phosphate-buffered saline containing 129mM-NaCl, 5mM-KCl, 7.4mM- Na_2HPO_4 , 1.2mM- KH_2PO_4 , 0.74mM- MgSO_4 , 0.5mM-1-isobutyl-3-methylxanthine (a phosphodiesterase inhibitor), 5.3mM-glucose and 46mM-sucrose (pH 7.2), 330mosm. Cells were preincubated at 37°C for 15min in 2ml of the same buffer containing 0–2.5mM- CaCl_2 with or without other test reagents or ions. The cells were then incubated with carbachol (1mM) or other agents for 1min at 37°C. The incubation was terminated by removing buffer and adding 0.7ml of 7% (w/v) trichloroacetic acid to each dish. Cells were kept on ice for 30min, the trichloroacetic acid extract was removed to a centrifuge tube, the dish rinsed once with 0.7ml of 7% (w/v) trichloroacetic acid and the pooled extracts were centrifuged at 2500g for 30min. The supernatant was extracted with 5×10 ml of water-saturated ether and freeze-dried. The residue was dissolved in 0.5ml of 50mM-sodium acetate buffer (pH 6.2) containing 2mM-EDTA and assayed for cyclic GMP as described below. Blanks for the cyclic GMP assay consisted of 50mm dishes without cells treated in the same manner. All incubations were run in duplicate or triplicate and assayed for cyclic GMP in triplicate. Values are expressed as the mean $\pm$ S.E.M. for the six to nine determinations. The trichloroacetic acid pellet and the precipitate remaining on each dish were dissolved and pooled in 1ml of 0.4M-NaOH; protein was determined by the method of Lowry *et al.* (1951) with bovine serum albumin as standard.

Preparation of subcellular fractions

Cultures grown on 150mm dishes were rinsed twice with iso-osmotic phosphate-buffered saline and once with a homogenate buffer containing 1mM-potassium phosphate (pH 7.6) and 10% (w/v) sucrose. Dishes were drained for 45s and the cells were scraped off into a small volume of homogenate buffer (approx. 0.3ml/dish) by using a Teflon-coated straight edge. Homogenates were prepared on ice by

using a power-driven Teflon/glass Dounce homogenizer. Nuclei and unbroken cells were removed by centrifugation at 1000g for 10min. The supernatant was further centrifuged at 100000g for 1h, yielding a membrane pellet and a soluble cell fraction. The membrane pellet was resuspended in 50mM-triethanolamine/HCl buffer (pH 7.6) to give a protein concentration of 1.0–1.5mg/ml; this suspension is referred to as the membrane-bound enzyme. In all experiments, the soluble cell fraction was passed through a Sephadex G-25 column (1cm $\times$ 35cm, pre-equilibrated and eluted with 50mM-triethanolamine/HCl, pH 7.6) to separate guanylate cyclase from endogenous cyclic GMP, GTP, ATP, Ca^{2+} and other low-molecular-weight compounds. The pooled protein fraction from this column was used as the soluble enzyme. Samples were assayed directly for guanylate cyclase without freezing.

Guanylate cyclase assay

The incubation mixture contained, in a final volume of 200 μ l, 50mM-triethanolamine/HCl (pH 7.6), 1mM-SQ 20009 (a phosphodiesterase inhibitor), various amounts of MnGTP, MgGTP or other cation-GTP complexes in the concentration range 0.02–1.5mM, and either 0.02–0.4mg of protein from the soluble enzyme fraction or 0.05–0.1mg of protein from the membrane fraction. The reaction was initiated by adding protein, and incubation was carried out at 37°C for 3min. The reaction was terminated by the rapid addition of 200 μ l of 0.4M-zinc acetate (pH 6.5) and 200 μ l of 0.4M- Na_2CO_3 (pH 11.5). Samples were mixed vigorously, frozen on solid CO_2 and then allowed to thaw during centrifugation at 2000g for 15min at 4°C. A 100 μ l sample of the supernatant was assayed for cyclic GMP by the radioimmunoassay of Steiner *et al.* (1972). [The ZnCO_3 precipitates 85–90% of the GTP, but only 5–7% of the cyclic GMP (Böhme *et al.*, 1974).]

The cyclic GMP content of each tube was calculated with reference to a calibration curve included in each radioimmunoassay. This curve was constructed by using various amounts (0–2pmol) of authentic cyclic GMP in the presence of a 100 μ l portion of 'reagent mixture'. This reagent mixture was the supernatant prepared by running the guanylate cyclase reaction with heat-denatured enzyme, and terminating the reaction by the ZnCO_3 -precipitation step in the same manner as for the experimental samples. Enzyme activity was proportional to incubation time and protein concentration. Values for guanylate cyclase activity represent the average of at least two independent assays, in each of which cyclic GMP formation was determined by radioimmunoassay in triplicate.

Concentrations of MnGTP and MgGTP were calculated by using stability constants of 353000M^{-1}

and 65000M^{-1} respectively (Garbers *et al.*, 1975). Free Ca^{2+} concentrations were adjusted with Ca^{2+} -EGTA buffers, assuming a stability constant of 4400000M^{-1} determined by Rodan & Feinstein (1976). The amount of MnEGTA and MgEGTA chelates were calculated by using the stability constants $56 \times 10^6\text{M}^{-1}$ (estimated from Sillén & Martell, 1971) and 10^2M^{-1} (Rodan & Feinstein, 1976) respectively. Hydrolysis of GTP was determined by measuring release of radioactivity from $[\gamma\text{-}^{32}\text{P}]\text{GTP}$ under the conditions of the guanylate cyclase assay. Less than 5% of the total GTP was hydrolysed during the 3 min incubation, except at very low substrate concentrations, such as $20\text{ }\mu\text{M}$ -MnGTP, where 15–20% of the GTP was hydrolysed.

Phosphodiesterase assay

Phosphodiesterase activity was determined by measuring the formation of $[\text{H}^3]\text{guanosine}$ from cyclic $[\text{G-}^3\text{H}]\text{GMP}$ in a two-step reaction modified from Brooker *et al.* (1968). The incubation mixture contained 20mM -Tris/HCl (pH 8.0), 40mM - MgCl_2 , $1\text{ }\mu\text{M}$ -cyclic $[\text{H}^3]\text{GMP}$ ($0.5\text{ }\mu\text{Ci}$) and $0\text{--}70\text{ }\mu\text{l}$ of homogenate or homogenate buffer in a final volume of $100\text{ }\mu\text{l}$. The reaction was initiated by the addition of protein, and incubation was carried out at 30°C for 10 min. The reaction was terminated by boiling for 1 min. The radioactive $5'\text{-GMP}$ formed was then converted quantitatively into guanosine and P_i by the addition of $20\text{ }\mu\text{l}$ of bacterial alkaline phosphatase (Worthington, Freehold, NJ, U.S.A.) (BAPF 5SN317, 27.5 units/ml) and incubation at 30°C for 15 min. After completion of this reaction, $600\text{ }\mu\text{l}$ of a resin slurry (Dowex AG1-X8, 200–400 mesh, Cl^- form; Bio-Rad, Richmond, CA, U.S.A.) in water (2:1, v/v) was added. Radioactivity was determined by addition of 4 ml of scintillation fluid [116ml of Liquifluor (New England Nuclear), 1834ml of toluene and 1000ml of Triton X-100] directly to the reaction vial. Reactions were run in triplicate under conditions that were linear with respect to incubation time and protein concentration; values were corrected for quenching. No-protein samples and boiled protein samples were used for estimating the blank values, which were then subtracted from each experimental value. Assays were performed by Dr. Maria Rosa Castro de Costa. Enzyme activity *in vitro* was completely blocked by 1mM -SQ 20009 or by 1mM -1-isobutyl-3-methylxanthine. In whole cells the latter inhibitor was more effective than SQ 20009 in blocking phosphodiesterase activity.

Results

Experiments with whole cells

In agreement with the report of Matsuzawa & Nirenberg (1975), we have found (results not shown)

that in the presence of 0.5mM -1-isobutyl-3-methylxanthine: (i) the largest increase in intracellular concentration of cyclic GMP occurred after 30–60 s of exposure to 1mM -carbachol; (ii) maximal increases in cyclic GMP were obtained with 1mM -carbachol; (iii) $1\text{ }\mu\text{M}$ -atropine completely blocked these increases in cyclic GMP. The accumulation of cyclic GMP in response to carbachol represented an increase of between 1.8- and 82-fold above resting values and decreased with increasing subculture number (Table 1). This fall in responsiveness to muscarinic stimulation was accompanied by a decrease in the specific activity of guanylate cyclase measured in homogenates. However, there was no change in the distribution of guanylate cyclase activity between soluble and membrane fractions or in the K_m values for MnGTP of the two forms of the enzyme (results not shown). Cyclic nucleotide phosphodiesterase activity did not vary with subculture number.

Intracellular cyclic GMP concentrations were dependent on extracellular concentrations of Ca^{2+} . Resting concentration of cyclic GMP ranged from 0.04 to 2.9 pmol/mg of protein after 15 min of incubation in the standard assay buffer with 1mM - Ca^{2+} and 0.5mM -1-isobutyl-3-methylxanthine.

We have no explanation for the marked variation in cyclic GMP concentration of cells that were grown under identical conditions and showed the same degree of morphological differentiation upon microscopic examination. The resting concentration of cyclic GMP was directly proportional to the concentration of extracellular Ca^{2+} in the range $0.05\text{--}2.5\text{mM}$ (Fig. 1). Stimulation of cyclic GMP synthesis by carbachol was also dependent on extracellular Ca^{2+} ; a maximal increase above basal values occurred at about 0.5mM - Ca^{2+} .

Table 1. Carbachol stimulation of cyclic GMP synthesis and specific activity of guanylate cyclase at different subcultures

Cyclic GMP concentrations of cells incubated in the presence of 1mM - Ca^{2+} were determined under standard conditions. Guanylate cyclase activities of whole homogenates were measured under standard conditions by using 2mM -Mn $^{2+}$ and 1mM -GTP.

Number of subculture	Effect of 1mM -carbachol on cyclic GMP concentrations (-fold increase)	Specific activity of guanylate cyclase (pmol/min per mg of protein)
22	51, 82	36, 41
23	16, 17, 18, 22	38
24	2.5, 4.2, 7.5, 8.1, 10.4, 11	36
25	4.2, 7.2	8.0, 23
29	2.2, 2.8, 5.5	3, 8.4
34	1.8

Fig. 1. Effect of extracellular Ca^{2+} concentration on the basal and carbachol-stimulated intracellular concentration of cyclic GMP

The monolayers of cells were rinsed three times with Ca^{2+} -free buffer and preincubated with 2 ml of buffer containing the indicated concentrations of Ca^{2+} (or 0.1 mM-EGTA at $[\text{Ca}^{2+}]=0$ mM) for 15 min at 37°C . The cells were then incubated for 30 s in the absence (Δ) or presence of 1 mM-carbachol ($\blacktriangle$). The experiment shown is one of three similar experiments and was carried out with cells of subculture no. 24. The bars indicate S.E.M. for $n=4$.

The importance of Ca^{2+} in regulating intracellular cyclic GMP concentration of neuroblastoma cells is further illustrated in Table 2. Removal of the extracellular Ca^{2+} by incubating cells in Ca^{2+} -free buffer with 0.1 mM-EGTA nearly abolished carbachol-induced increases in cyclic GMP. When cells were exposed to the Ca^{2+} -ionophore A23187 in buffer containing 1 mM- Ca^{2+} , cyclic GMP increased 10–20-fold above resting concentrations within 30 s. Carbachol was not able to increase cyclic GMP further, and ionophore A23187-induced increases were not blocked by atropine. In the absence of extracellular Ca^{2+} the ionophore had no effect on intracellular cyclic GMP concentrations within 30 s (Table 2), but did increase them after 2–3 min (results not shown), probably owing to its ability to release Ca^{2+} from intracellular stores. Ethanol, the vehicle used to dissolve ionophore A23187, caused a 2-fold increase in cyclic GMP.

Sr^{2+} partially replaced Ca^{2+} , whereas Mg^{2+} was unable to replace Ca^{2+} in supporting the increase in cyclic GMP evoked by carbachol.

NaN_3 (1 mM), a persistent activator of guanylate cyclase in many tissues (Kimura & Murad, 1974; De Rubertis & Craven, 1976), caused a 10–80-fold increase in cyclic GMP in neuroblastoma cells during a 1 min incubation period. The response to NaN_3 was decreased by 40–50% by removing Ca^{2+} from the buffer with EGTA, and potentiated by the Ca^{2+} -ionophore A23187. Atropine (1 μM) had no effect on the response to NaN_3 (results not shown).

Table 2. Effect of Ca^{2+} -ionophore A23187 on cyclic GMP concentrations

Cells were preincubated for 15 min in buffer containing 1 mM- Ca^{2+} , or in Ca^{2+} -free buffer containing 0.1 mM-EGTA. Carbachol, ionophore A23187 and atropine were then added and the incubation was continued for 30 s. Expts. 1, 2 and 3 were carried out with subculture nos. 29, 24 and 24 respectively. The results are expressed as mean $\pm$ S.E.M. for quadruplicate determinations of cyclic GMP. *, Not significantly different from control; **, significantly different from control ($P<0.05$).

Expt.	Conditions	Cyclic GMP (pmol/mg of protein)	
		Control	Carbachol
1	No addition	1.0 $\pm$ 0.3	4.5 $\pm$ 0.6**
	EGTA (0.1 mM) in Ca^{2+} -free medium	0.7 $\pm$ 0.2*	1.1 $\pm$ 0.3*
	A23187 (10 $\mu\text{g}/\text{ml}$)	16.9 $\pm$ 0.3**	12.5 $\pm$ 0.3**
2	No addition	1.15 $\pm$ 0.15	2.6 $\pm$ 0.1**
	EGTA (0.1 mM) in Ca^{2+} -free medium	1.50 $\pm$ 0.25*	1.35 $\pm$ 0.45*
	A23187 (10 $\mu\text{g}/\text{ml}$)	10.45 $\pm$ 0.80**	8.8 $\pm$ 0.9**
	A23187 (10 $\mu\text{g}/\text{ml}$) + EGTA (0.1 mM) in Ca^{2+} -free medium	1.0 $\pm$ 0.55*	1.60 $\pm$ 0.25*
	Ethanol (0.5% v/v)	2.50 $\pm$ 0.40**	2.75 $\pm$ 0.55**
3	No addition	0.40 $\pm$ 0.24	3.05 $\pm$ 0.65**
	A23187 (10 $\mu\text{g}/\text{ml}$)	10.2 $\pm$ 1.5**	
	A23187 (10 $\mu\text{g}/\text{ml}$) + atropine (1 μM)	10.75 $\pm$ 1.70**	
	Ethanol (0.5% v/v)	1.10 $\pm$ 0.54*	

Experiments with soluble and membrane-bound cyclase

The distribution of guanylate cyclase and cyclic nucleotide phosphodiesterase activities was examined in soluble and membrane fractions prepared from N1E-115 cells. For guanylate cyclase 83% of the total activity was recovered in the soluble supernatant after centrifugation at 100000g for 1 h, and only 4% in the membrane pellet. The specific activities of the soluble and membrane-bound enzyme were 101.0 and 3.2 pmol of cyclic GMP formed/min per mg of protein respectively. For phosphodiesterase, 61% of the activity was recovered in the supernatant and 18% in the pellet. This distribution pattern was not altered when the cell suspension was sonicated instead of homogenized in a glass/Teflon homogenizer. The only difference observed was a slight increase in the recovery of the activities in the 1000g supernatant.

Several bivalent cations and La^{3+} at 2 mM concentration at 1 mM-GTP supported the activity of the soluble and membrane-bound forms of guanylate cyclase from neuroblastoma cells. Mn^{2+} and Mg^{2+}

were the most effective of the metal ions tested, yielding activities of 118 and 66.6pmol/min per mg of protein respectively; La^{3+} and Ca^{2+} were partially effective. Several other bivalent cations, including Fe^{2+} , Co^{2+} , Ni^{2+} , Zn^{2+} and Cu^{2+} (2mM), gave activities of less than 0.1pmol/min per mg of protein with the soluble enzyme and less than 0.003pmol/min per mg of protein with the membrane-bound enzyme. The ability of several metal ions to support guanylate cyclase activity, as measured by the K_m and V_{max} values, paralleled the stability with which they form complexes with GTP (Table 3). Gel filtra-

tion on Sephadex G-25 of the soluble fraction increased the activity by 20–30%, but did not change the relative affinity of the enzyme for the metals examined.

Free GTP (uncomplexed with metal ions) acted as a competitive inhibitor of guanylate cyclase activity when either MnGTP or MgGTP was used as the substrate (Figs. 2a and 2b). In these experiments the concentration of free GTP was kept constant (10 and 100, 20 and 200 μM), whereas the amount of metal-GTP was varied. Free GTP had an inhibition constant of $17 \pm 6 \mu\text{M}$ (mean $\pm$ S.E.M.) ($n=4$) with MnGTP as the substrate, as determined from linear Dixon plots. In similar experiments with MgGTP as the substrate an inhibition constant for free GTP of $25 \pm 11 \mu\text{M}$ (mean $\pm$ S.E.M.) ($n=3$) was obtained. The initial velocity of guanylate cyclase measured with CaGTP as substrate was too low for a detailed kinetic analysis (the K_m value for CaGTP was higher than 500 μM). By using double-reciprocal plots and the K_i value for GTP, the K_m value for MnGTP was calculated to be $55 \pm 16 \mu\text{M}$ ($n=3$) at a free GTP concentration of zero. The K_m values for all substrates were constant with changing subculture number. The V_{max} values changed with subculture number, but were highest for MnGTP, followed by MgGTP and CaGTP in all experiments.

The effect of various concentrations of free Ca^{2+} on soluble guanylate cyclase activity was examined by using both MnGTP and MgGTP as substrates. Concentrations of free Ca^{2+} were maintained with Ca^{2+} -EGTA buffers; free GTP was kept constant at 20 μM . For both MnGTP and MgGTP, the experiments were carried out at two substrate concentrations, the lower of which gave approximately half-

Table 3. Stability constants of metal-GTP and steady-state kinetic constants of soluble guanylate cyclase

The stability constant K is defined as: $K = [\text{metal-GTP}]/[\text{metal}][\text{GTP}]$. K_m is the Michaelis constant for the various metal-GTP complexes, extrapolated to a free GTP concentration of zero. V_{max} is the maximal velocity calculated for a free GTP concentration of zero by using the various metal-GTP complexes as substrate. K_a denotes the concentration of free metal that gives a 100% increase in the velocity measured with 0.1mM-MnGTP as substrate and 20 μM -free GTP. Steady-state kinetic constants were obtained by using the soluble enzyme from subculture no. 24. K_m and K_a values were similar for subculture nos. 27 and 29.

Bivalent metal ion	K (M^{-1})	K_m (μM)	V_{max} (pmol of cyclic GMP/min per mg of protein)	K_a (mM)
Mn^{2+}	353 000	55	57	0.32
Mg^{2+}	65 000	310	16.6	0.09
Ca^{2+}	25 000	500	1	0.05

Fig. 2. Double-reciprocal plots of soluble guanylate cyclase activity at various free GTP concentrations (a) MnGTP as substrate at two constant concentrations of free GTP (Δ , 100 μM ; $\bullet$, 10 μM). The concentrations of MnGTP and free GTP were calculated by using a stability constant of $353\,000 \text{ M}^{-1}$ for MnGTP. The enzyme was derived from cells of subculture no. 24. (b) MgGTP as substrate at two constant concentrations of free GTP (Δ , 200 μM ; $\bullet$, 20 μM). The concentrations of MgGTP and free GTP were calculated by using a stability constant of $65\,000 \text{ M}^{-1}$ for MgGTP. The enzyme was derived from cells of subculture no. 23. The velocities were measured in triplicate or quadruplicate and the lines were fitted with linear regression.

maximal activity and the higher of which gave maximal activity in the presence of $20\text{ }\mu\text{M}$ -GTP in the free form. For MnGTP these values were 0.1 and 1 mM, and for MgGTP they were 0.3 and 1 mM. Concentrations of Ca^{2+} greater than 0.05 mM stimulated soluble guanylate cyclase activity at both MnGTP concentrations (Fig. 3a). Free Mn^{2+} and Mg^{2+} also stimulated guanylate cyclase activity with MnGTP as substrate [but these cations were less effective than Ca^{2+} in similar concentration (Table

3)]. In contrast, in similar experiments with MgGTP as the substrate, Ca^{2+} stimulated enzyme activity at concentrations of $0.01\text{--}0.5\text{ }\mu\text{M}$ and inhibited enzyme activity at concentrations greater than $1\text{ }\mu\text{M}$ (Fig. 3b).

The effect of Ca^{2+} on soluble guanylate cyclase activity was further investigated by varying substrate concentrations in the presence of constant concentrations of free Ca^{2+} . Formation of cyclic GMP from MnGTP ($0.05\text{--}1\text{ mM}$) was stimulated by increasing concentrations of Ca^{2+} (Fig. 4a). In

Fig. 3. Activation of soluble guanylate cyclase by Ca^{2+} ions

The Ca^{2+} concentrations were maintained by Ca^{2+} -EGTA buffers, and were calculated on the basis of a Ca^{2+} -EGTA stability constant of $4.4 \times 10^6\text{ M}^{-1}$. The concentration of the Ca^{2+} -EGTA complex was kept constant at 2.5 mM . The open symbols correspond to 2.5 mM -EGTA with no Ca^{2+} . Addition of Ca^{2+} did not change the substrate concentration by more than 3% for MnGTP and 13% for MgGTP, even at the highest concentrations of Ca^{2+} tested. (a) Effect of Ca^{2+} on activity measured at two constant MnGTP concentrations ($\bullet$, 0.1 mM ; $\blacktriangle$, 1 mM), with free GTP maintained at $20\text{ }\mu\text{M}$. The enzyme was derived from cells of subculture no. 28. (b) Effect of Ca^{2+} on activity measured at two constant MgGTP concentrations ($\bullet$, 0.3 mM ; $\blacktriangle$, 1 mM), with free GTP maintained at $20\text{ }\mu\text{M}$. The enzyme was derived from cells of subculture no. 24.

Fig. 4. Effect of Ca^{2+} on the activity of soluble guanylate cyclase at various substrate concentrations

Ca^{2+} concentrations (μM) (indicated in the Figure) were maintained as described in the legend to Fig. 3. (a) Effect of Ca^{2+} on activity measured with MnGTP as substrate at $20\text{ }\mu\text{M}$ -free GTP. The enzyme was derived from cells of subculture no. 28. (b) Effect of Ca^{2+} on activity measured with MgGTP as substrate at $20\text{ }\mu\text{M}$ -free GTP. The enzyme was derived from cells of subculture no. 27.

Fig. 5. Effect of Ca^{2+} concentration on the activity of membrane-bound guanylate cyclase. Ca^{2+} concentrations were calculated and maintained as described in the legend to Fig. 3. (a) Effect of Ca^{2+} on activity measured with MnGTP (●, 0.5 mM; ▲, 1 mM) as substrate at $20 \mu\text{M}$ -free GTP. The enzyme was derived from cells of subculture no. 29. (b) Effect of Ca^{2+} on activity measured with MgGTP (●, 0.3 mM; ▲, 1 mM) as substrate at $20 \mu\text{M}$ -free GTP. The enzyme was derived from cells of subculture no. 24.

parallel experiments, increasing concentrations of Ca^{2+} (5–500 μM) progressively inhibited guanylate cyclase activity with MgGTP (0.05–1.5 mM) as the substrate (Fig. 4b).

Membrane-bound guanylate cyclase from these cells was also examined by using MnGTP and MgGTP as substrates. Enzyme activity did not obey Michaelis–Menten kinetics. The activity of the enzyme was half-maximal with about $250 \mu\text{M}$ -MnGTP and $500 \mu\text{M}$ -MgGTP in the presence of $20 \mu\text{M}$ -GTP in the free form. Free GTP also inhibited membrane-bound enzyme activity, but to a lesser extent than soluble activity (results not shown). With MnGTP (0.5 and 1 mM) as the substrate, free Ca^{2+} activated the membrane-bound enzyme at concentrations greater than $1 \mu\text{M}$ (Fig. 5a). In contrast, with MgGTP as substrate, maximal activity was observed with $0.1 \mu\text{M}$ - Ca^{2+} , and concentrations greater than $1 \mu\text{M}$ were inhibitory (Fig. 5b). The increase in velocity at Ca^{2+} concentrations higher than $100 \mu\text{M}$ can be explained partly by formation of the alternative substrate CaGTP and more significantly by removal of inhibitory free GTP. The Ca^{2+} -binding protein purified from bovine brain (Lin *et al.*, 1974), at concentrations of 0.4 or $1.0 \mu\text{g}/\text{ml}$, had no effect on soluble or membrane-bound guanylate cyclase activity with either MnGTP or MgGTP as substrates and 10 or $50 \mu\text{M}$ -free Ca^{2+} .

The effect of the muscarinic agonists carbachol (1 mM) and acetyl- β -methylcholine (1 mM) on soluble and membrane-bound enzyme activity was tested with both MnGTP and MgGTP as substrates. Under a variety of experimental conditions these agonists

had no effect on guanylate cyclase activity (results not shown). Carbachol did interfere with the radioimmunoassay for cyclic GMP, but this was corrected for by using internal standards in the calibration curves.

Discussion

Neuroblastoma cells of clone N1E-115 provide an excellent system for studies of muscarinic activation of guanylate cyclase activity because these cells have the largest increase in cyclic GMP concentration yet reported (Matsuzawa & Nirenberg, 1975), and activation occurs in a homogeneous population of cells. The presence of muscarinic receptors (Strange *et al.*, 1977) and stimulation of cyclic GMP synthesis through these receptors has been shown (Richelson, 1977; Strange *et al.*, 1977) by other laboratories.

The present experiments indicate that in neuroblastoma cells, as in other intact cell systems (Haymovits & Scheele, 1976; De Rubertis & Craven, 1976; Christophe *et al.*, 1976) and in tissue slices, extracellular Ca^{2+} is an obligatory component of the increase in cyclic GMP seen in response to stimulation of muscarinic acetylcholine receptors (Schultz *et al.*, 1973; Van Sande *et al.*, 1975; De Rubertis & Craven, 1976). [All experiments were carried out at supramaximal carbachol concentrations (1 mM) to examine whether other agents (ionophore A23187, NaN_3) can further activate synthesis of cyclic GMP.] Half-maximal increase in cellular cyclic GMP concentrations could be observed between 10 and $50 \mu\text{M}$ -carbachol concentration with most passages of the

cells tested, in agreement with the findings by Strange *et al.* (1977).

Since our experiments were carried out in the presence of an inhibitor of cyclic nucleotide phosphodiesterase, increases in intracellular cyclic GMP concentrations probably reflect increased synthesis, rather than decreased degradation, of cyclic GMP. The requirement for Ca^{2+} ions appears to be rather specific. Extracellular Ca^{2+} might act at the membrane by increasing the binding affinity of muscarinic agonists for the receptor or within the cells by directly or indirectly activating guanylate cyclase. Evidence from other systems indicates that Ca^{2+} is not required for binding of muscarinic ligands to the receptor (Burgen *et al.*, 1974; Yamamura & Snyder, 1974; Alberts & Bartfai, 1976), and therefore supports the suggestion by Schultz *et al.* (1973) that Ca^{2+} entry into cells is required to increase cyclic GMP. In fact, in neuroblastoma cells, as in other types of cells, one can by-pass the muscarinic receptor by exposing the cells to the Ca^{2+} ionophore A23187. Increases in cyclic GMP mediated by the ionophore depend on extracellular Ca^{2+} and are insensitive to the muscarinic antagonist atropine. Homogenization of the cells led to loss of the effect of muscarinic agonists on cyclic GMP production, as observed in other systems (Limbird & Lefkowitz, 1975; De Rubertis & Craven, 1976).

We have attempted to learn more about the molecular events underlying the muscarinic induction of cyclic GMP synthesis in neuroblastoma cells by studying guanylate cyclase activity in broken-cell preparations with emphasis on the role of Ca^{2+} in regulation of the enzyme. It was shown by several investigators that Ca^{2+} in high concentrations (mM range) activates guanylate cyclase at suboptimal Mn^{2+} concentrations (Böhme, 1970; Kimura & Murad, 1974; Chrisman *et al.*, 1975; Garbers *et al.*, 1975; Nakazawa & Sano, 1976; Sulakhe *et al.*, 1976). These Ca^{2+} concentrations are far above the estimates of the cytoplasmic free Ca^{2+} concentration of $0.1 \mu\text{M}$ (Baker, 1976; Brinley, 1976). In the present study both MnGTP (the substrate used in most studies on guanylate cyclase) and MgGTP (which is thought to be the natural substrate) were utilized to examine the effects of Ca^{2+} on the activity. The behaviour of guanylate cyclase distinctly differs with these two substrates and it was noted that treatment with Triton X-100 (Frey *et al.*, 1977) or with NaN_3 (Kimura *et al.*, 1975; De Rubertis & Craven, 1976) can change the relative affinities of the enzyme towards MnGTP and MgGTP . These findings indicated the need for studies on guanylate cyclase- Ca^{2+} interactions in which both substrates are tested under identical conditions.

Most of the enzyme activity is found in the soluble cell fraction. Clearly, there can be only a 'loose'

association between this enzyme and the muscarinic receptor located in the plasma membrane. Examination of the soluble and membrane-bound guanylate cyclases shows that, in decreasing order, the Mn^{2+} , Mg^{2+} and Ca^{2+} complexes of GTP are the most active substrates for both enzymes. The low concentrations of free Ca^{2+} ($0.1\text{--}1 \mu\text{M}$) thought to be present in cells (Baker, 1976; Brinley, 1976) would appear to exclude the possibility of CaGTP serving as a natural substrate.

GTP was found to be a competitive inhibitor with respect to metal-GTP complex. The fact that the inhibition constants for free GTP were very similar when MnGTP or MgGTP was utilized as a substrate indicates that GTP is acting as an inhibitor probably through direct competition with metal-GTP complex for the catalytic site rather than by chelating an activatory free metal ion (Mn^{2+} or Mg^{2+}).

When steady-state kinetic experiments were carried out at various free metal ion and MnGTP concentrations, free metal ions were found to increase guanylate cyclase activity. This result was expected qualitatively in view of the fact that free metal ions can complex with free GTP, which acts as an inhibitor. However, the magnitude of activation afforded by these metal ions was not proportional to their ability to complex free GTP (cf. Table 3). In fact, Ca^{2+} , which gave the greatest activation, forms the least stable complexes with GTP. Thus the activation by Ca^{2+} cannot be explained by the 'inert-cation effect', i.e. that any metal ion that binds the inhibitory nucleotide GTP would exert. The Ca^{2+} activation of cyclic GMP formation from MnGTP can best be explained by assuming the existence of an allosteric metal-ion-binding site on the enzyme. Such an allosteric site has been described for guanylate cyclase from sea-urchin sperm (Garbers *et al.*, 1974, 1975).

With MgGTP as the substrate, Ca^{2+} in low concentrations ($0.01\text{--}0.1 \mu\text{M}$) caused a slight activation of soluble and membrane-bound forms of guanylate cyclase. At higher concentrations ($1 \mu\text{M}\text{--}1 \text{mM}$) Ca^{2+} inhibited enzyme activity. It is difficult to evaluate the possible physiological significance of these effects of Ca^{2+} on guanylate cyclase activity in neuroblastoma cells, because we do not know the range of free Ca^{2+} concentrations in these cells.

The molecular mechanism by which Ca^{2+} might activate guanylate cyclase in intact cells remains unresolved. Free Ca^{2+} might relieve inhibition of guanylate cyclase by ATP or PP_i , but free Mg^{2+} , which occurs at much higher concentrations (approx. 3mM) in cells (Brinley, 1976), is an equally potent chelator of these anions. A lipid factor may participate in the Ca^{2+} -mediated activation of guanylate cyclase, as observed in fibroblasts (Wallach &

Pastan, 1976) and in platelets (Glass *et al.*, 1977). Alternatively, this enzyme might be regulated by a Ca^{2+} -binding protein similar to the one that activates cyclic nucleotide phosphodiesterase and adenylate cyclase (Kakiuchik & Yamazaki, 1970; Lin *et al.*, 1974; Cheung *et al.*, 1975; Brostrom *et al.*, 1975), although this very protein was unable to stimulate guanylate cyclase activity from mammalian brain (Olson *et al.*, 1976) or neuroblastoma cells (the present experiments). Our studies are compatible with a role for an intermediary factor that would link Ca^{2+} entry to guanylate cyclase activation.

This study was supported by U.S. Public Health Service Grants NS-12105 to X.O.B. and DA-01627, MH-17387 and NS-08440 to P.G., and by grants from the Dysautonomia Foundation to X.O.B. and P.G. T.B. was a recipient of a EMBO fellowship, on leave of absence from the Department of Biochemistry, University of Stockholm, Sweden. We thank Dr. Robert Study and Dr. Maria Rosa Costa for advice and help, and Frank Wilson, Carmela Castiglione, Tim Liang and Stephen Grant for skilled technical assistance.

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