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MUSCARINIC RECEPTOR-MEDIATED CYCLIC GMP FORMATION BY CULTURED NERVE CELLS— IONIC DEPENDENCE AND EFFECTS OF LOCAL ANESTHETICS*

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Abstract—A new assay technique for measuring receptor-mediated cyclic GMP formation by cultured mouse neutroblastoma cells was used to study the role of ions in, and the effects of local anesthetics on, the function of the muscarinic receptor. The technique involved radioactively labeling intracellular stores of GTP by incubating cells with [^{3}H]guanine and isolating [^{3}H]cyclic GMP with a cation exchange resin (Dowex-50$^+$) column. High-pressure liquid chromatography of cell extracts and of eluates from the Dowex column showed that after 45 min the majority of the radioactivity in the cell extracts was [^{3}H]GTP and that, for carbamylcholine-stimulated cells, greater than 90 per cent of the radioactivity in the eluates was [^{3}H]cyclic GMP. In the absence of external Na^+ ($[Na^+]_e$, with cesium chloride as osmotic filler) or external ($[Ca^{2+}]_e$), the carbamylcholine-stimulated formation of [^{3}H]cyclic GMP was about 60 and 10 per cent of control, respectively, while removal of other ions had no significant effect. There was little difference in the responses at 10 mM vs 110 mM-$[Na^+]_e$, whereas the optimal $[Ca^{2+}]_e$ was around 5 mM. Ca^{2+} increased [^{3}H]cyclic GMP formation in response to carbamylcholine without affecting the apparent affinity of this agonist for the receptor. Local anesthetics were apparently competitive inhibitors of carbamylcholine with equilibrium dissociation constants (K_B) in the range of 6–250μM. The rank order for the apparent affinity of local anesthetics for the muscarinic receptor was tetracaine = butacaine = procaine > dibucaine = lidocaine ≫ ethyl aminobenzoate.

The muscarinic acetylcholine receptor mediates the formation of guanosine 3′,5′-cyclic phosphate (cyclic GMP) in many different tissues[1] including those derived from the nervous system (e.g. brain, sympathetic ganglia and neuroblastoma cells)[2–7] where this receptor-mediated formation of cyclic GMP may play a role in the postsynaptic transfer of information between cells[2, 7]. Many psychotherapeutic drugs (e.g. tricyclic anti-depressants and neuroleptics) competitively antagonize the muscarinic receptor[8–10], the predominant type of acetylcholine receptor in brain[11]. Our interest in the biochemical actions of these drugs has led us to study their effects on the muscarinic receptors of a clone of cultured mouse neuroblastoma cells[12]. These cultures provide a readily available, homogeneous population of cells, which simplifies experimentation and interpretation of results. For our assay of muscarinic receptor effects, we utilized the muscarinic receptor-mediated formation of cyclic GMP, which is rapidly assayed in these cultured cells by radioactively labeling intracellular stores of GTP, the cyclic nucleotide precursor. With this new assay technique, which is described here, we have shown that certain psychotropic drugs block muscarinic receptor-mediated cyclic GMP formation, and from this blockade equilibrium dissociation constants for these drugs and the muscarinic receptor were determined[13, 14].

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To understand better this receptor-mediated cyclic GMP response in these nerve cells, we have characterized further this function of the muscarinic receptor by studying its ionic requirements and the effects on this process of local anesthetics which may affect permeability of certain ions into cells[15, 16]. We show that carbamylcholine-stimulated cyclic GMP formation by these cells is markedly dependent on external calcium ions and that local anesthetics antagonize the effects of carbamylcholine apparently by competing at the muscarinic receptor site.

METHODS AND MATERIALS

Cells and culture conditions

Mouse neuroblastoma clone N1E-115 (subculture 10–23) was cultured in Dulbecco–Vogt modification of Eagle's medium (Grand Island Biological Co., Grand Island, NY, Cat. No. H-21) supplemented with 10% (v/v) fetal calf serum (Colorado Serum Co., Denver, CO) without antibiotics (medium I) at 37° in an atmosphere of 10% CO_2 and 90% humidified air. Cells were grown in flasks (75 cm/250 ml, Costar, Cambridge, MA) with 20 ml medium and maintained in stationary phase (requiring daily medium change) for at least 1 week prior to assay. Subculture of neuroblastoma cells was achieved by incubation in a modified Puck's D_1 solution[17] (medium II). Cells were negative for mycoplasma (PPLO) in three tests: microbiological assay, fluorescent microscopy using Hoechst 33258 stain[18], and scanning electron microscopy.

Assays

Receptor-mediated [^{3}H]cyclic GMP formation. Cyclic GMP was assayed using intact cells which were washed free of medium I and dissociated from the surface of flasks by incubation with medium II; collected by low-speed centrifugation (500 rev/min in a Damon/IEC CRU-5,000 centrifuge); and resuspended at a density of approximately 8 to 10×10^6 cells/ml in phosphate-buffered sodium chloride solution (medium III) containing the following: 110 mM-NaCl, 5.3 mM-KCl, 1.8 mM-$CaCl_2$, 1.0 mM-$MgCl_2$, 2.0 mM-Na_2HPO_4, 25 mM-glucose and 70 mM-sucrose. This solution was titrated to pH 7.4 and adjusted to an osmolality of 335–340 mOsM with sucrose. The cell suspension was placed in a 25-ml Erlenmeyer flask, [8-^{3}H]guanine (1μM final concn, 10μCi/ml) added and then the suspension was rotated at 37° for 45 min at 80 rev/min (Gyratory shaker, model G2, New Brunswick Scientific Co., Inc., New Brunswick, NJ). Cells were then collected by low-speed centrifugation, washed three times with 10 ml of medium III (or a modification of medium III) to remove extracellular radioactivity, and resuspended in medium III (or a modification of medium III). For the final steps in the assay (addition of antagonists and/or agonist) the radioactive cell suspension was distributed in 250-μl aliquots into wells of a multi-well tray (Disposo trays FB16-24TC, Bellco Glass Inc., Vineland, NJ) and incubated at 37° at 80 oscillations/min in a shaker bath (model 25, GCA/Precision Scientific Co., Chicago, IL). In experiments to determine the equilibrium dissociation constant (K_B) or inhibitor constant (K_i) for a local anesthetic, 30μl of the compound in medium III was added to the cells for 10 min prior to the addition of carbamylcholine chloride in 20μl medium III. (Lidocaine was first dissolved in ethanol before dilution in medium III. Since ethanol was found to reduce the cyclic GMP response, control cells were assayed at concentrations of ethanol [0.1%, v/v] similar to that for lidocaine-treated cells.) After 30 sec of incubation with the agonist, the reaction was stopped by the addition of 30μl of ice-cold 50% (w/v) trichloroacetic (TCA). The suspension was briefly sonicated (3 sec at setting No. 3, Ultrasonic Cell Disrupter, Kontes Glass Co., Vineland, NJ), and [8-^{14}C]cyclic GMP (0.05 nCi) was added as internal standard. Each sample was then passed through a (0.8×8 cm) column of Dowex 50-H^+ (AG50W-X2, 200–400 mesh, Rio-Rad Labs., Richmond, VA) which had been washed with water. Additionally, each well was rinsed once with 500μl of 5% TCA and this wash was passed through the ion-exchange column. Radioactive cyclic GMP was isolated from each column as described by others[19]. Thus, each column was washed with 4.4 ml of 0.1 N HCl and 1.0 ml water successively before eluting cyclic GMP with the next 1.5 ml water, which was usually collected directly into a scintillation vial. Efficiencies for counting tritium and carbon-14 using 10 ml Quantafluor (Mallinckrodt, Inc., St. Louis, MO) and a double-label program on a Searle Isocap/300 scintillation counter were 35 and 45 per cent in their respective channels. The calculated dis./min for each sample incorporated corrections for quench (using external standard ratio), for c.p.m. of [8-^{14}C]cyclic GMP in the tritium channel (efficiency 20 per cent) and for recovery of [8-^{14}C]cyclic GMP (85–95 per cent).

When medium III was modified (e.g. by decreasing or increasing the concentration of certain ions), isotonicity was maintained by adjustments in the concentration of sucrose unless otherwise stated. Medium III with 20 mM HEPES (*N*-2-hydroxyethyl-piperazine-*N'*-ethane sulfonic acid) buffer thus contained 50 mM sucrose.

Radioimmunoassay of cyclic GMP. Aliquots of the Dowex column eluate were assayed for cyclic GMP using a kit from New England Nuclear (Boston, MA). Counting of iodine-125 was done using a Packard Auto-Gamma 5210 with an efficiency of 73 per cent.

Cell counts and protein assays. Protein was assayed by a modification of the method of Lowry *et al.*[20] with bovine serum albumin as a standard. Cell counts were determined using an electronic cell counter (model Z_F, Coulter Electronics, Hialeah, FL).

Identification of radioactivity from cells

Preparation of extracts. To obtain extracts from whole cells, the supernatant fractions from triplicate samples of TCA-treated cells (as described above) were pooled, extracted seven times with equal volumes of ether to remove TCA, lyophilized, and dissolved in 40μl of 0.01 M-KH_2PO_4 (pH 3.1). To obtain extracts of cellular material subjected to Dowex column fractionation, TCA-treated cells were passed through columns (as described above) and the fractions containing cyclic GMP from triplicate samples were pooled, freeze dried and dissolved in 40μl of a solution of guanine, guanosine and their nucleotides (0.4 mM each in water).

Analysis of extracts. [8-^{3}H]guanine and its radioactively labeled derivatives in 10μl extract were separated by high-pressure liquid chromatography (h.p.l.c.) on a Glenco HPLC system I (Glenco Scientific, Inc., Houston, TX) equipped with a digital programmer (DP 810), an automatic stream selector (SSV-6), an optical unit (5480) for u.v. absorption at 254 nm, and an anion-exchange resin (Partisil −10 SAX, Reeve Angel, Clifton, NJ). Adsorbed compounds were eluted using a solvent program which was modified from a system described by others[21]. This program comprised a series of isocratic runs with intermediate step changes. Eluent compositions and program were as follows:

Duration (min)	Buffer (KH_2PO_4)
9	0.01 M, pH 3.1
13	0.10 M, pH 4.2
4	0.25 M, pH 4.2
7	0.40 M, pH 4.2
7	0.50 M, pH 4.2
5	0.01 M, pH 3.1

The column flow rate was 3 ml/min and the column was at room temperature. The quantities of GTP in the whole cell extracts were determined by comparison of peak heights in samples to that of 1 nmole of GTP standard.

Chemicals and drugs

Radioactive compounds were from Amersham/Searle (Arlington Heights, IL). The following compounds were obtained from Sigma Chemical Co. (St. Louis, MO): butacaine hemi-sulfate, carbamylcholine chloride, choline chloride, ethyl aminobenzoate, guanine hydrochloride, guanosine, guanosine 5'-mono-, di- and triphosphoric acids, cyclic GMP, HEPES, procaine hydrochloride and tetracaine hydrochloride. Dibucaine hydrochloride was obtained from ICN Pharmaceuticals, Inc. (Irvine, CA); lidocaine, from Astra Pharmaceutical Products, Inc. (Worcester, MA); and cesium chloride, from Alfa Products (Beverly, MA).

RESULTS

The assay

The entry of [^{3}H]guanine into mouse neuroblastoma cells rotating in suspension at 37° was linear for 40–50 min (data not shown). Cells incubated for 45 min with [^{3}H]guanine, washed free of extracellular radioactivity and then incubated for 30 sec at 37° without (basal) or with 1 mM carbamylcholine (stimulated) contained mostly [^{3}H]GTP (Fig. 1 and Table 1). The data from Fig. 1 were normalized to 100 per cent recovery of [^{14}C]cyclic GMP to obtain the results in Tables 1 and 2. Additionally, since there were some peaks of radioactivity from the h.p.l.c. (Fig. 1) which were unidentified, the total radioactivity recovered was greater than the sum found in guanine and derivatives (Table 1) by 3–5 per cent. The quantities of [^{3}H]GTP and non-radioactively labeled GTP in the aliquots of cell extracts (Fig. 1B and C) yielded specific activities for [^{3}H]GTP of 1.1×10^5 dis./min/1.1 nmole (or 45 mCi/m-mole) and 1.0×10^5 dis./min/0.9 nmole (or 49 mCi/m-mole) for basal and stimulated cells respectively. This was nearly a 200-fold lower specific activity than that of the original material. Based upon previous calculations of cell water for these cells ($5.6\mu l/1 \times 10^6$ cells)[22], the concentration of GTP within these cells was around 1 mM. For stimulated cells, 5 per cent of the total radioactivity was [^{3}H] cyclic GMP; for basal cells, 0.1 per cent (Table 1). The per cent of the total radioactivity in [^{3}H]GTP + [^{3}H]cyclic GMP for stimulated cells was almost equal to that for basal cells (Table 1), implying a stoichiometric relationship between the quantities of these nucleotides within the cells.

Identification of the radioactivity from the cyclic GMP fraction of the Dowex 50 column showed that this fraction contained not only [^{3}H]cyclic GMP but also [^{3}H]guanine and its other derivatives (Fig. 1D and E, Table 1). For basal cells, only 40 per cent of the total radioactivity was [^{3}H]cyclic GMP; however, for stimulated cells it was 94 per cent. Similar results were obtained using thin-layer chromatography and high voltage electrophoresis[13, 14]. The absolute amounts of radioactivity from contaminants in the Dowex column fraction were nearly equal for basal and stimulated cells (Table 1), so that the difference

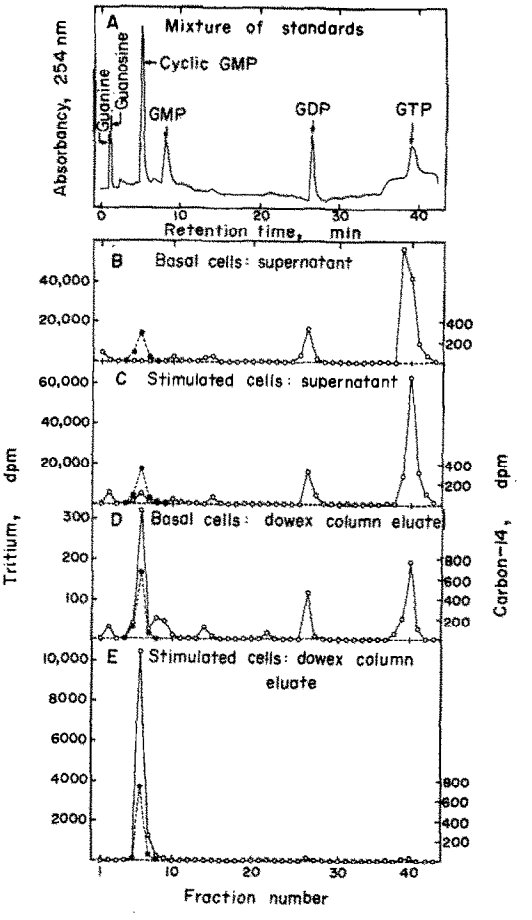


Fig. 1. Identification by high-pressure liquid chromatography (h.p.l.c.) of radioactively labeled material from mouse neuroblastoma cells incubated with [8-^{3}H]guanine. Clone N1E-115 cells (subculture 10) were assayed for carbamylcholine-stimulated [^{3}H]cyclic GMP formation and prepared for h.p.l.c. as described in Methods and Materials. Cell supernatant fractions were either directly analyzed by h.p.l.c. (B and C) or first passed through a Dowex 50-H$^+$ column and the fractions containing cyclic GMP then analyzed by h.p.l.c. (D and E) as described in Methods and Materials. The column flow rate was 3 ml/min and the fraction collector was set at 1-min intervals. The entire fraction was counted for radioactivity using 15 ml INSTA-GEL (Packard Instrument Co., Inc., Downers Grove, IL) with the efficiencies for counting tritium and carbon-14 averaging 37 and 45 per cent in their respective channels using a double label setting on the scintillation counter. Corrections for cpm of the [^{14}C]cyclic GMP internal standard in the tritium channel were made. Each aliquot (10μl) of material analyzed by h.p.l.c. was from approximately 3.7×10^5 cells (~1 mg protein). Panel A: mixture of standards: strip-chart recording of u.v. absorbance at 254 nm for 4 nmoles each of guanine, guanosine, cyclic GMP, GMP, GDP and GTP. Panels B and C: radioactivity from basal (no incubation with carbamylcholine (B) and stimulated (30-sec incubation with 1 mM carbamylcholine (C) cell supernant fractions. Panels D and E: radioactivity from Dowex column fractions after passing through basal (D) and stimulated (E) cell supernatant fractions.

Key: —○—, tritium; and —●—, carbon-14.

Table 1. Radioactively labeled compounds synthesized by mouse neuroblastoma clone N1E-115 cells from [8-^{3}H]guanine*

Additions to cells	Method of isolation of radioactivity	Total radioactivity recovered dis/min (per cent total)	Guanine and guanosine	GMP	GDP	GTP	Cyclic GMP
			dis/min (per cent recovery)				
None (basal)	High-pressure liquid chromatography	428,000 (114)	16,500 (4)	9500 (2)	61,300 (14)	322,000 (75)	630 (0.1)
	Cation exchange Resin†/h.p.l.c.‡	1450 (78)	50 (3)	140 (10)	180 (12)	420 (30)	560 (40)
Agonist§ (stimulated)	High-pressure liquid chromatography	342,000 (96)	14,700 (4)	7,800 (2)	51,600 (15)	235,000 (69)	17,800 (5)
	Cation exchange Resin/h.p.l.c.	18,300 (98)	40 (0.2)	40 (0.2)	220 (1)	410 (2)	17,300 (94)

*These data were derived from those presented in Fig. 1 after corrections were made for recovery of [^{14}C]cyclic GMP.
†Dowex 50-H$^+$.
‡High-pressure liquid chromatography as described in Methods and Materials.
§Carbamylcholine (1 mM) for 30 sec.

between stimulated and basal values (analogous to subtraction of a 'no enzyme' or 'no substrate' blank in a radiometric enzyme assay) was essentially identical to those differences derived from cell extracts directly analyzed by h.p.l.c. or from Dowex column fractions further analyzed by h.p.l.c. (Table 2). Thus, with the large stimulation of [^{3}H]cyclic GMP formation by muscarinic-receptor stimulation of these cells, a one-step fractionation of this cyclic nucleotide with a cation exchange resin appeared feasible.

With the addition of a precipitation step [23, 24] after Dowex column fractionation, recovery of radioactivity from basal and stimulated cells was reduced (Table 3) as a result of the removal of >90 per cent of [^{3}H]GDP and around 60 per cent of [^{3}H]GTP (data now shown) without affecting recovery of cyclic GMP (Table 3, legend). Thus, with the precipitation step, which added approximately 30 min to the assay overall, the apparent stimulation over basal radioactivity was increased from around 5- to around 30-fold (Table 3). Results from dose–response curve experiments for agonists using both isolation techniques were superimposable when data were plotted minus basal radioactivity (data not shown).

Our previous results using this assay technique [13, 14] were very similar to those obtained by others using a radioimmunoassay for cyclic GMP [6]. However, to further validate our assay technique, a dose–response curve for carbamylcholine-stimulated cyclic GMP formation was determined by assaying for radioactively labeled and for immunologically active cyclic GMP. For this experiment the Dowex column fraction was divided in half for scintillation count-

Fig. 2. Carbamylcholine dose–response curves for radioactively labeled and immunologically active cyclic GMP formation by mouse neuroblastoma cells. Clone N1E-115 cells (subculture 11) were assayed in triplicate for [^{3}H]cyclic GMP formation at the indicated concentrations of carbamylcholine as described in Methods and Materials except that one half of the Dowex column fraction containing cyclic GMP (750 μl) was analyzed for radioactivity (—○—) and an aliquot of the remainder was analyzed in quadruplicate for cyclic GMP by radioimmunoassay (—□—). Standard errors of the mean are designated by vertical lines. Arrows indicate the concentrations of carbamylcholine causing 50 per cent of maximum stimulation. There were approximately 3×10^5 cells and 1 mg protein/assay.

Table 2. [^{3}H]cyclic GMP formation by cultured mouse neuroblastoma cells using different methods for isolation of cyclic nucleotide

Additions to cells	Method of isolation of [^{3}H]cyclic GMP		
	Cation exchange resin* (dis/min)	Cation exchange resin/h.p.l.c.† (dis/min)	High-pressure liquid chromatography (dis/min)
None (basal)	2200	560	630
Agonist‡ (stimulated)	19,700	17,300	17,800
Δ	17,500	16,740	17,170

*Data from the cation exchange resin (Dowex 50-H$^+$) were obtained from replicate samples of cells in the same experiment as that used for cation exchange resin/h.p.l.c. and h.p.l.c. (Fig. 1 and Table 1).

†High-pressure liquid chromatography as described in Methods and Materials.

‡Carbamylcholine (1 mM) for 30 sec.

Table 3. [^{3}H]cyclic GMP formation by cultured mouse neuroblastoma cells using cation exchange resin alone or with a precipitation step to isolate the cyclic nucleotide*

Additions to cells	Method of isolation of [^{3}H]cyclic GMP	
	Cation exchange resin† (dis/min ± S.E.M.)	Cation exchange resin followed by precipitation‡ (dis/min ± S.E.M.)
None (basal)	3700 ± 950	600 ± 25
Agonist§ (stimulated)	21,000 ± 700	17,000 ± 1500
Δ	17,300 ± 1200	16,400 ± 1500

*Mouse neuroblastoma clone N1E-115 cells (subculture 16) were assayed for [^{3}H]cyclic GMP formation as described in Methods and Materials except for the addition of the precipitation step as described below. Recovery of [^{14}C]cyclic GMP (internal standard) was (± S.E.M.) 85 ± 6 per cent for Dowex alone and 83 ± 7 per cent for Dowex plus precipitation. There were approximately 2×10^5 cells/assay.

†Dowex 50-H$^+$.

‡Twenty-five microliters each of 3.2 M $ZnSO_4$ and 3.2 M Na_2CO_3 were added to the 1.5-ml fraction from the cation exchange resin column. After sedimentaiton of the precipitate (by centrifugation for 3 min in a Beckman Microfuge B), the supernatant fraction was quantitatively removed and assayed for radioactivity.

§Carbamylcholine (1 mM) for 30 sec.

ing and for radioimmunosassay. The results (Fig. 2) showed that, with each assay technique, similar slopes of the curves and concentrations of carbamylcholine causing 50 per cent maximum stimulation were obtained. That is, results with this new assay technique were comparable to those obtained by radioimmunoassay.

Ionic requirements for receptor-mediated cyclic GMP formation

The results of experiments to determine the ionic requirements for the muscarinic receptor-mediated cyclic GMP response in clone N1E-115 showed that choline chloride (70 mM) and HEPES (20 mM) inhibited the receptor-mediated response (Table 4). Both of these quaternary amine compounds, which probably caused their effects by competition for the receptor site, therefore should be used with caution as osmotic filler or buffer, respectively, when studying the muscarinic receptor. Another amine buffer, Tris (2-amino-2-hydroxymethyl-1,3-propanediol), has been shown to antagonize the effects of acetylcholine in neurons of *Aplysia californica* [25].

In the absence of external sodium ions, the cyclic GMP response was most reduced when choline chloride was used as osmotic filler (Table 4 and Fig. 3A); this apparent sodium ion requirement was not as prominent when sucrose or cesium chloride was used as osmotic filler (Table 4). The results with cesium chloride (Table 4 and Fig. 3B) most likely reflect the true sodium ion requirement for this process since this osmotic filler maintains the ionic strength of the medium, and cesium ions are not readily permeable through sodium channels [26]. Thus, increasing the external sodium ion concentration ($[Na^+]_e$) while decreasing the choline chloride concentration to maintain isotonicity resulted in a relation (Fig. 3A) which probably reflects both the sodium ion dependence of the cyclic GMP response and the inhibitory effects of choline at the muscarinic receptor. A similar experiment utilizing cesium chloride as osmotic filler (Fig. 3B) showed that there was little

Table 4. Muscarinic receptor-mediated cyclic GMP formation—effects of ions, HEPES, and osmotic fillers*

Component omitted	Incubation medium: Buffer	Incubation medium: Osmotic filler	Carbamylcholine-Stimulated cyclic GMP formation as per cent control (± S. E. M.)†
None‡	Na_2HPO_4	Sucrose	100
None	Na_2HPO_4	Choline	36 (±1)§
None	Na_2HPO_4, HEPES	Sucrose	50 (±3)§
NaCl, Na_2HPO_4	KH_2PO_4	Sucrose	37 (±2)§
NaCl, Na_2HPO_4	KH_2PO_4	Choline	3 (±1)§
NaCl, Na_2HPO_4	KH_2PO_4	CsCl, sucrose	61 (±2)§
KCl	Na_2HPO_4	Sucrose	130 (±6)‖
$CaCl_2$	Na_2HPO_4	Sucrose	9 (±6)§
$MgCl_2$	Na_2HPO_4	Sucrose	130 (±13)‖

*Mouse neuroblastoma clone N1E-115 cells (subculture 21) were prepared for assay as described in Methods and Materials. At the end of the incubation period with [^{3}H]guanine, cells were divided into nine aliquots and washed with the appropriate incubation medium, as listed in the table. After a 10-min incubation at 37°, cells were stimulated by the addition of 20 μl of 10 mM carbamylcholine in water for 30 sec; unstimulated cells received 20 μl water. Each condition was determined in triplicate. There was an average of 3.1×10^5 cells and 660 μg of protein assay.

†For the control the average radioactivity was 2250 and 17,300 dis/min for basal and stimulated cells respectively. The difference between the stimulated and basal levels represents carbamylcholine-stimulated cyclic GMP formation. Basal values were not affected by the alterations in the composition of the incubation medium.

‡The composition of the incubation medium without omissions (medium III) is described in Methods and Materials.

§$P < 0.0005$ vs control.

‖Non-significant vs control.

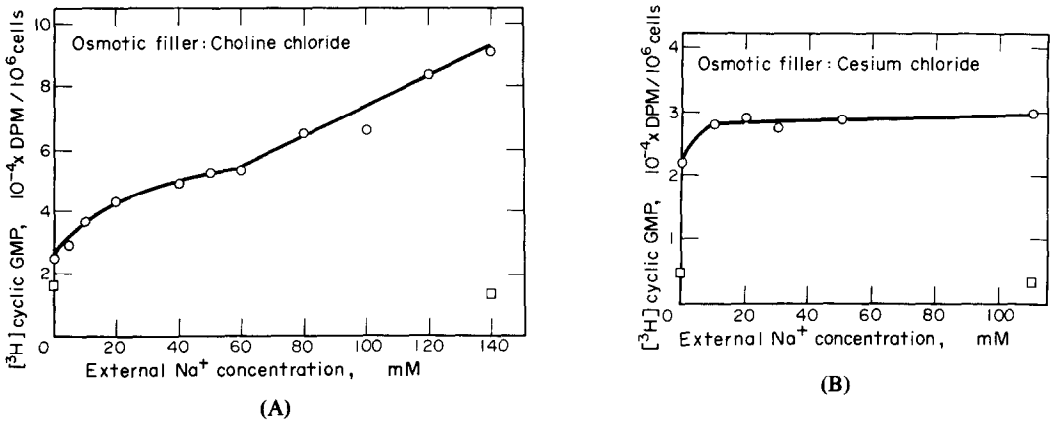


Fig. 3. Effect of external sodium ion concentration on muscarinic receptor-mediated cyclic GMP formation by mouse neuroblastoma cells using choline chloride (A) or cesium chloride (B) as osmotic filler. Mouse neuroblastoma clone N1E-115 cells (A, subculture 18; B, subculture 23) were assayed in duplicate at the indicated concentrations of sodium ions for [^{3}H]cyclic GMP formation as described in Methods and Materials. (A) 1.6×10^5 Cells and 670 μg protein/assay; (B) 2.4×10^5 cells and 680 μg protein/assay. Key: ○, carbamylcholine chloride (1 mM final concn) for 30 sec; and □, no agonist.

difference in the cyclic GMP response with 10 or 110 mM sodium chloride present. The removal of potassium or magnesium ions (Table 4) also had no significant effect on receptor-mediated cyclic GMP formation. In addition [^{3}H]cyclic GMP formation stimulated by carbamylcholine (1 mM) in the presence of tetrodotoxin (5 μM) was not different from control (47,000 dis/min/10^6 cells vs 49,000 dis/min/10^6 cells respectively), suggesting that activation of fast sodium channels is not required for the response.

In contrast to the effects of Na^+, absence of calcium ions in the incubation medium resulted in about 90 per cent reduction in the muscarinic response (Table 4). The optimal calcium ion concentration for the effect of carbamylcholine on cyclic GMP formation was around 5 mM (Fig. 4). In addition, with increasing amounts of external calcium ion there was an apparent increase in basal cyclic GMP formation (Fig. 5). While the results presented in Fig. 4 were obtained in the presence of HEPES buffer with no phosphate (to assure solubility of calcium ions), similar results were found with phosphate buffer alone (data not

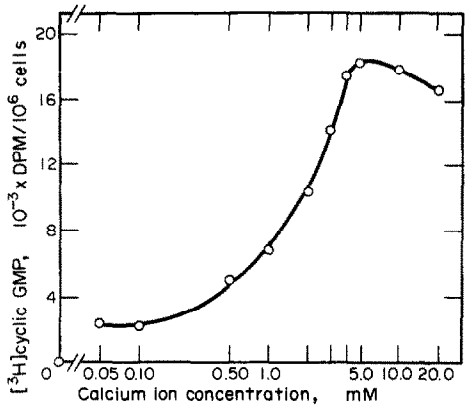


Fig. 4. Effect of external calcium ion concentration on muscarinic receptor-mediated cyclic GMP formation by mouse neuroblastoma cells. Clone N1E-115 cells (subculture 14) were radioactively labeled in medium III as described in Methods and Materials; washed in medium III without calcium ions; and resuspended in medium III containing 20 mM Hepes buffer, no potassium phosphate and the indicated concentrations of calcium ions. Basal and carbamylcholine-stimulated levels of [^{3}H]cyclic GMP were then determined in duplicate. The data are [^{3}H]cyclic GMP formation after 30 sec with 1 mM carbamylcholine chloride minus the basal values at each calcium ion concentration. At $[Ca^{2+}]_e = 5$ mM, carbamylcholine caused a 180 per cent increase in [^{3}H]cyclic GMP formation over basal levels. There were approximately 2×10^5 cells and 670μg protein/assay.

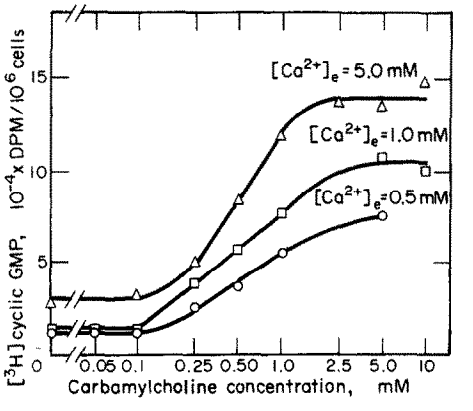


Fig. 5. Effect of external calcium ion concentration ($[Ca^{2+}]_e$) on the dose–response curve for carbamylcholine and cyclic GMP formation by mouse neuroblastoma cells. Clone N1E-115 cells (subculture 13) were assayed in duplicate for [^{3}H]cyclic GMP formation at the indicated concentrations of carbamylcholine chloride as described in the legend to Fig. 4 and in Methods and Materials. There were approximately 3×10^5 cells/assay.

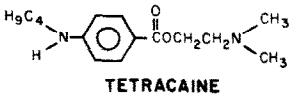
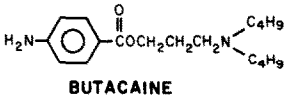
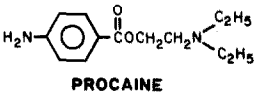
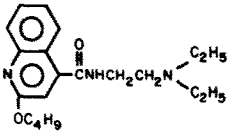
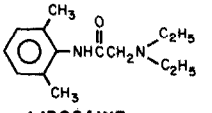
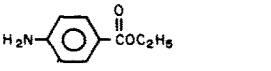
shown). Finally, calcium ions had no discernible effect on the affinity of carbamylcholine for the muscarinic receptor since there was no shift in the dose–response curves for carbamylcholine when $[Ca^{2+}]_e$ was varied (Fig. 5).

Effects of local anesthetics on muscarinic receptor-mediated cyclic GMP formation

The precise mechanism of action of local anesthetics on excitable membranes is not understood, but interactions with calcium ions have been demonstrated [15, 16]. We, therefore, tested the effects of a group of local anesthetics (Table 5) on the muscarinic receptor-mediated cyclic GMP formation.

Procaine hydrochloride (0.1 mM) caused a rapid inhibition of the muscarinic response to carbamylcholine with maximum inhibition occurring after about 5 min (Fig. 6). Procaine (Fig. 7) and all other local anesthetics (Table 5) were apparent competitive inhibitors of carbamylcholine at the muscarinic receptor. Equilibrium dissociation constants (K_B) which were determined from the displacements of the dose–response curves for carbamylcholine (e.g. Fig. 7) gave the following rank order of the affinity for the muscarinic acetylcholine receptor: tetracaine = butacaine = procaine > dibucaine = lidocaine $\gg$ ethyl aminobenzoate. In addition, procaine and all the other local anesthetics were apparent competitive inhibitors of the effects of external calcium ions when the carbamylcholine concentration was 1 mM (e.g. Fig. 8). For these compounds the apparent K_i values vs. $[Ca^{2+}]_e$ (range 20–400μM) were greater than their K_B values for the muscarinic receptor. Thus, as pointed out by a reviewer, at 1 mM carbamylcholine, blockade of the muscarinic receptor by the local anesthetics would

Table 5. Local anesthetics and the muscarinic acetylcholine receptor

COMPOUND	K_B* (μM)
 TETRACAINE	6
 BUTACAINE	6
 PROCAINE	6
 DIBUCAINE	20
 LIDOCAINE	20
 ETHYL AMINOBENZOATE	250

*EQUILIBRIUM DISSOCIATION CONSTANT

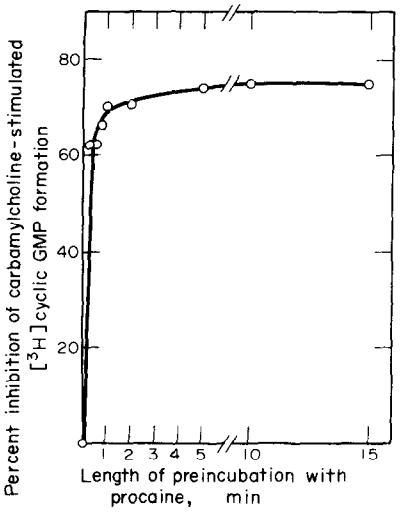


Fig. 6. Inhibition by procaine of muscarinic receptor-mediated cyclic GMP formation by mouse neuroblastoma cells: time course. Clone N1E-115 cells (subculture 10) were assayed in triplicate for carbamylcholine-stimulated [^{3}H]cyclic GMP formation as described in Methods and Materials. Cells were incubated with procaine hydrochloride (0.1 mM final concn.) for the indicated times prior to the addition of carbamylcholine chloride (1 mM final concn) for 30 sec. In the absence of procaine (control), carbamylcholine increased [^{3}H]cyclic GMP 6-fold above basal values. There were approximately 2.2×10^5 cells and 480μg protein/assay.

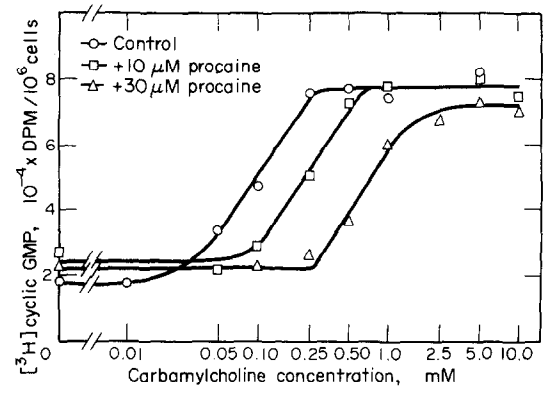


Fig. 7. Effect of procaine on the carbamylcholine dose-response curve for cyclic GMP formation by mouse neuroblastoma cells. Clone N1E-115 cells (subculture 11) were assayed in duplicate for [^{3}H]cyclic GMP formation as described in Methods and Materials at the indicated concentrations of carbamylcholine chloride and procaine hydrochloride. There were approximately 1.6×10^5 cells and 450μg protein/assay.

also be expected. To overcome this muscarinic receptor blockade by the local anesthetics, these experiments were repeated at 20 mM carbamylcholine; under these conditions no effect of local anesthetics on external calcium could be demonstrated. In other experiments, procaine also inhibited the carbamylcholine response in the absence of external sodium ions but did not affect cyclic GMP formation stimulated by 1 mM sodium azide (data not shown).

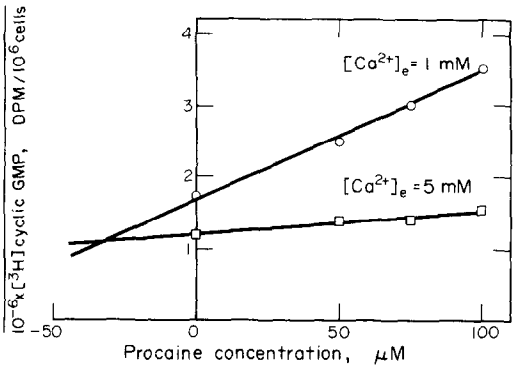


Fig. 8. Inhibition by procaine of muscarinic receptor-mediated cyclic GMP formation by mouse neuroblastoma cells: effects of external calcium ion concentration. Clone N1E-115 cells (subculture 10) were assayed in duplicate for carbamylcholine-stimulated cyclic GMP formation as described in the legend to Fig. 4 and in Methods and Materials at the indicated concentrations of Ca^{2+} and procaine hydrochloride, which was added 10 min prior to the addition of agonist (1 mM final concn). The increase above basal levels of [^{3}H]cyclic GMP formation caused by carbamylcholine was plotted by the method of Dixon[27]. The inhibitor constant (K_i) for procaine vs Ca^{2+} was 40μM. There were approximately 2×10^5 cells and 750μg protein/assay.

DISCUSSION

Our assay technique measuring [^{3}H]cyclic GMP synthesis from [^{3}H]GTP by intact mouse neuroblastoma cells was developed to facilitate studies of muscarinic-receptor effects on these cells and was modeled after an analagous technique developed to study cyclic AMP formation by brain slices[28] and cultured cells[29]. In this latter assay, [^{14}C]adenine was used to radioactively label intracellular stores of ATP. Radioimmunoassay for cyclic GMP in these mouse neuroblastoma cells required prior purification of cellular material to remove interfering substances and an incubation period with antibody and radioactively labeled antigen for 18–20 hr. Thus with radioimmunoassay techniques, results for an experiment would be obtained 1 day later than similar results obtained by the techniques described here (e.g. Fig. 2). However, our assay measures relative increases in the amount of cyclic GMP formed under various conditions. It was not designed to measure absolute levels of cyclic GMP which require techniques such as radioimmunoassay.

The results of studies using this new assay define some of the ionic requirements for muscarinic receptor-mediated cyclic GMP formation by mouse neuroblastoma clone N1E-115. The most prominent effect was seen with removal of calcium ions from the incubation medium, which caused nearly complete abolition of the receptor-mediated response (Table 4). Thus, like almost every other cyclic GMP system which has been studied[5, 25–31], muscarinic receptor-mediated cyclic GMP formation by intact clone N1E-115 cells requires the presence of external calcium ions. This muscarinic receptor effect may therefore be added to the list of differentiated properties of this cell line, which includes such characteristics as electrically excitable membranes and

certain enzymes involved in the synthesis and catabolism of catecholamines[12].

Many processes which are apparently mediated by the muscarinic receptor are dependent on calcium ions[32]. For the cyclic GMP response, this ionic requirement was found for smooth muscle[30, 31], thyroid[33], neutrophils[34], fat cells[35], liver cells[36], renal cortex[37] and autonomic ganglia[5], while the only exception was with guinea pig pancreatic acinar cells[38]. It was shown with smooth muscle that calcium ions can alter the affinity of carbamylcholine for the muscarinic receptor[39]; thus, experiments were done to test this possibility (Fig. 5). The data which were in agreement with those of receptor-binding studies[32] showed no effect of calcium ion concentration on the affinity of carbamylcholine for the muscarinic receptor. The reason for the difference in our results from those of Burgen and Spero[39] is not known.

The results with local anesthetics (Table 5) demonstrate that these compounds interacted with mouse neuroblastoma cells to perturb muscarinic acetylcholine receptor function. The least potent compound in our series was the neutral local anesthetic ethyl aminobenzoate, and the equilibrium dissociation constants for all compounds (Table 5) were in a concentration range that would be expected to cause anesthetic block.

Previously, competition between local anesthetics and calcium[15, 16, 40–43], as well as between local anesthetics and cholinergic agonists[44–46] for sites on membranes, was demonstrated. More specifically, for example, local anesthetics were shown to antagonize competitively calcium-induced contractions of smooth muscle and to inhibit calcium binding to isolated rat muscle sarcolemma[15]; additionally, these compounds bind to the nicotinic acetylcholine receptor site on membrane fragments from *Electrophorus* and *Torpedo* electric organs[44, 45]. Superficially, our data appear to suggest that the local anesthetics also could act by competing with the agonists at the muscarinic receptor-binding sites but we could not demonstrate an interaction of these compounds with calcium ions.

Weber and Changeux[44] found that local anesthetics bind to the nicotinic receptor site at concentrations much higher than that required to block the electrical response to cholinergic agonists. Thus, they concluded that the pharmacological effect of these compounds was not a result of nicotinic receptor blockade. Our data taken with those of Weber and Changeux[44] are sufficient to suggest that the local anesthetics may be acting in a non-specific manner. While there are no data which define the action of local anesthetics on sodium transport, the current consensus is that they alter the surface charge[47, 48] and possibly the fluidity of cell membranes[49, 50]; both these effects could markedly affect the binding of agonists to receptors.

A further possibility exists: biological membranes are widely assumed to be asymmetric with respect to phospholipid compositions such that the inner leaflet of the bilayer tends to be more negatively charged when compared to the outer leaflet. Such asymmetry of charge led Sheetz and Singer[51] to propose that drugs which were charged could asymmetrically affect the lipid bilayer (so-called bilayer-couple hypothesis). This concept, taken with the mobile-receptor proposal[52, 53], suggests that the combined action of local anesthetics on fluidity and on the bilayer couple[51] may result in dislocation of the receptor and effector components in the muscarinic receptor–guanylate cyclase system.

Finally, in recent experiments [54, 55] we have demonstrated in clone N1E-115 the presence of histamine H_1 receptors which mediate the formation of cyclic GMP. With the information at hand about ionic requirements for muscarinic receptor-mediated cyclic GMP formation and the effects of local anesthetics in the process, we have begun similar studies on the histamine H_1 receptor.

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