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Voltage-sensitive calcium channels regulate guanosine 3′,5′-cyclic monophosphate levels in neuroblastoma cells

(membrane depolarization/muscarinic receptor/veratridine/D600)

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ABSTRACT **Veratridine or high potassium concentration increased guanosine 3′,5′-cyclic monophosphate (cGMP) levels in neuroblastoma cells of clone N1E-115 without affecting levels of adenosine 3′,5′-cyclic monophosphate (cAMP). The increases in cGMP appear to be a direct result of the depolarizing action of these agents and not due to the action of substances released from the cells upon depolarization. The increase in cGMP produced by depolarization was dependent upon extracellular calcium and could be prevented by the calcium channel blockers D600 and cobalt. Carbachol, acting on muscarinic acetylcholine receptors, also caused a calcium-dependent increase in cGMP in these cells. The carbachol and potassium effects were additive from 5 to 100 mM potassium and from 1 to 3 mM calcium. The carbachol response was nearly as sensitive as the potassium response to inhibition by D600 but was much less sensitive to inhibition by cobalt. The results suggest that depolarization increases cGMP levels in these cells by opening voltage-sensitive calcium channels and that activation of muscarinic receptors opens separate, voltage-insensitive calcium channels.**

Cellular levels of guanosine 3′,5′-cyclic monophosphate (cGMP) can be increased by the activation of certain receptors on the cell surface (1), but the mechanism of this response is unclear. The mechanism by which receptor activation stimulates the enzyme that synthesizes cGMP, guanylate cyclase [GTP pyrophosphate-lyase (cyclizing), EC 4.6.1.2], is probably indirect: the enzyme exists primarily in soluble form in most tissues, including neuroblastoma cells (2, 3), and no coupling between the receptor and guanylate cyclase has been demonstrated in broken cells. Increases in cellular cGMP levels produced by receptor activation have been repeatedly observed to depend on extracellular calcium (2, 4–6). The divalent cationophore A23187 can mimic these calcium-dependent increases in cGMP in various tissues (2, 7–9). These observations support the hypothesis (4, 10) that calcium translocation, caused by activation of hormone or neurotransmitter receptors, serves as an intermediate step in stimulating cGMP synthesis.

Because translocation of calcium by A23187 is sufficient to increase cGMP levels, it is possible that physiologic stimuli that induce calcium influx may also regulate cGMP levels. Many neural cells exhibit inward calcium currents in response to depolarization of the surface membrane (11, 12). Calcium-dependent increases in cGMP levels have been reported in brain slices in response to depolarizing agents such as veratridine and high-potassium medium (6, 7). However, the fact that brain slices retain some functional neural connections makes it difficult to determine whether these cGMP increases are due to released neurotransmitters.

Murine neuroblastoma cells of clone N1E-115 exhibit many neural properties, including the ability to generate action potentials (13) and to be depolarized by veratridine and high-potassium medium (14). These cells also possess a muscarinic acetylcholine receptor, as measured by increases in cGMP levels (2, 15–17) and by binding of [^{3}H]quinuclydinyl benzylate (15, 18). This paper presents evidence that depolarizing agents increase cGMP levels in these cultured cells by a direct, nonsynaptic mechanism. The results indicate that depolarization and muscarinic receptor activation increase cGMP levels by distinct calcium-dependent processes.

MATERIALS AND METHODS

Noradrenergic neuroblastoma cells of clone N1E-115 were used between 20 and 34 subcultures after isolation as a single cell (19). Differentiated monolayer cultures were maintained in 3% fetal calf serum as described (2). Experiments were performed at 37°C. The standard preincubation buffer (pH 7.4; 336 mosM) contained: 129 mM NaCl; 5 mM KCl; 5 mM Hepes; 0.5 mM 3-isobutyl-1-methylxanthine (IBMX); 1 mM $MgSO_4$; 3 mM $CaCl_2$; 5.2 mM glucose; and 46 mM sucrose. The cells were preincubated for 10 min in this buffer with various receptor antagonists or channel blockers as indicated. For incubations, the buffer was then replaced by an identical buffer with or without 1 mM carbachol, 100 μM veratridine (added as freshly made 10 mM solution in 50 mM HCl), or high-potassium medium. When potassium concentrations were greater than control levels (5 mM), the concentration of NaCl was decreased to maintain constant ionic strength. Incubations were carried out for 1 min (unless otherwise indicated) and terminated by using 7% (wt/vol) trichloroacetic acid as described (2). The trichloroacetic acid extract contained >90% of the cellular cyclic nucleotides. cGMP was assayed by the method of Steiner *et al.* (20) and cAMP, by the method of Brown *et al.* (21). All cGMP assays were performed with antisera that were not sensitive to interfering substances present in these cells (15). Cellular protein was dissolved in 0.4 M NaOH and measured by the method of Lowry *et al.* (22) with bovine serum albumin as standard. Statistical significance was calculated by using Student's *t* test (two-tailed).

The phosphodiesterase inhibitor IBMX produced approximately a 6-fold increase in basal cGMP levels in two typical experiments. Veratridine (100 μM) and potassium (100 mM) also increased cGMP in the absence of IBMX.

The control levels of cGMP, as well as the increase produced by carbachol and by depolarizing agents, varied between experiments but were consistent within each experiment. In contrast to the response to carbachol, which decreased with

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Abbreviations: cGMP, guanosine 3′,5′-cyclic monophosphate; cAMP, adenosine 3′,5′-cyclic monophosphate; TTX, tetrodotoxin; EGTA, ethylene glycol bis(β-aminoethyl ether)*N,N,N′,N′*-tetraacetic acid; IBMX, 3-isobutyl-1-methylxanthine.

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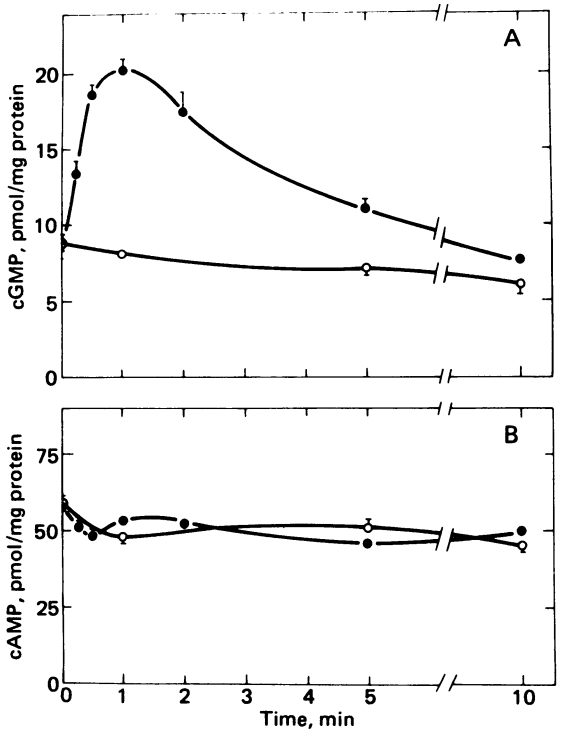

FIG. 1. Effect of high-potassium medium on levels of cGMP (*A*) and cAMP (*B*) in N1E-115 neuroblastoma cells as a function of time. Monolayer cultures were preincubated in buffer containing 5 mM K^+ and then were incubated in buffer containing 5 mM K^+ (○) or 100 mM K^+ (●) for the indicated times. Both cGMP and cAMP were determined for each culture. Data represent the mean ± SEM for three cultures, each assayed in triplicate.

successive subcultures (2), variations in the response to depolarization did not correlate with subculture.

Drugs were obtained from the following sources: carbachol chloride, atropine sulfate, and phentolamine hydrochloride, Sigma; veratridine and IBMX, Aldrich; pyrilamine maleate (mepyramine), Smith, Kline and French; tetrodotoxin (TTX), Calbiochem; D600 hydrochloride, Knoll AG (Ludwigschafen am Rhein, West Germany). All other chemicals were reagent grade.

RESULTS

Exposure to high-potassium medium (100 mM) caused cGMP levels to increase in N1E-115 cells by the earliest time (15 sec) when accurate measurement was possible (Fig. 1*A*). Levels of cGMP were maximal at about 1 min and returned to control levels after 10 min. Slight changes in cAMP levels were seen in this experiment (Fig. 1*B*), but no changes were seen in others.

Veratridine, a depolarizing agent that opens voltage-sensitive sodium channels, also increased cGMP levels in N1E-115 cells

Table 1. Effect of veratridine, high-potassium medium, and tetrodotoxin on cGMP levels in N1E-115 neuroblastoma cells

Incubation conditions	cGMP, pmol/mg protein	
	No TTX	With TTX
Control	16.0 ± 1.8	19.0 ± 3.2
Veratridine (100 μM)	48.3 ± 10.0	11.8 ± 2.3
High potassium (100 mM)	37.6 ± 6.4	40.2 ± 4.7

Tetrodotoxin (TTX), 1 μM, was present throughout the preincubation and incubation periods. Data represent the mean ± SEM for three cultures, each assayed in triplicate.

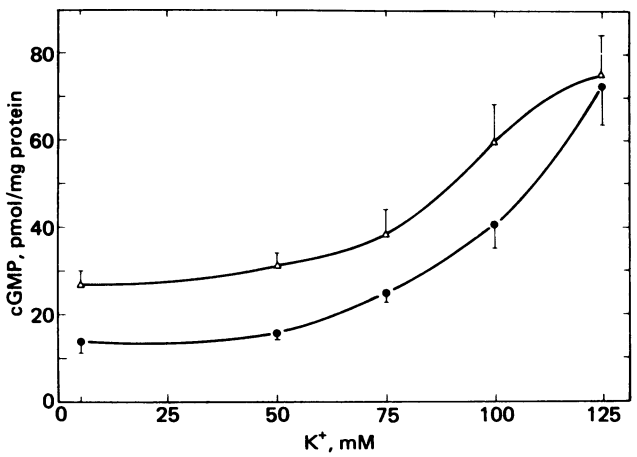

FIG. 2. Effect of various concentrations of potassium on levels of cGMP in N1E-115 neuroblastoma cells, in the presence (△) or absence (●) of 1 mM carbachol. Data represent the mean ± SEM for three cultures, each assayed in triplicate.

(Table 1). This response was also maximal at 1 min (data not shown). The increase in cGMP was inhibited by tetrodotoxin (TTX) which specifically blocks these sodium channels. The cGMP response induced by 100 mM potassium was not affected by TTX, consistent with the expectation that under these conditions the cells are depolarized without involvement of sodium channels (Table 1). Increases in cGMP levels produced by carbachol were also not affected by TTX (23).

Intracellular cGMP levels increased with increasing potassium concentration over the range 75–125 mM (Fig. 2). Concomitant treatment of cells with 1 mM carbachol, a maximally stimulating concentration (15, 17), caused a constant increment in cGMP level above that produced by 5–100 mM potassium. At 125 mM potassium, cGMP levels were not significantly different in the absence or presence of carbachol.

Responses to high-potassium medium, veratridine, and carbachol were dependent on the extracellular calcium concentration. No cGMP responses were seen when extracellular calcium was absent during the preincubation and incubation periods (Table 2). Under control conditions, cGMP levels increased about 10-fold as extracellular calcium was increased from 0 to 30 mM (Fig. 3). Increases in cGMP levels produced by high-potassium medium were maximal at 3 mM calcium, and declined to control levels at 30 mM calcium. Carbachol increased levels of cGMP at all calcium concentrations tested. The effects of carbachol and 100 mM potassium were additive up to 3 mM calcium. This additivity was seen in each of seven

Table 2. Effect of removal of extracellular calcium on cGMP levels in N1E-115 neuroblastoma cells

Incubation condition	cGMP, pmol/mg protein		
	With calcium	No calcium	No calcium; with EGTA
Control	4.11 ± 0.23	1.25 ± 0.24	0.94 ± 0.10
High potassium (100 mM)	21.12 ± 3.88	1.00 ± 0.18	0.73 ± 0.17
Veratridine (100 μM)	18.43 ± 2.08	1.23 ± 0.05	0.86 ± 0.15
Carbachol (1 mM)	7.81 ± 0.44	1.20 ± 0.14	0.86 ± 0.15

Where indicated, calcium (3 mM) or ethylene glycol bis(β-aminoethyl ether)*N,N,N',N'*-tetraacetic acid (EGTA) (0.1 mM) was present throughout the preincubation and incubation periods. Data represent the mean ± range of two cultures, each assayed in triplicate.

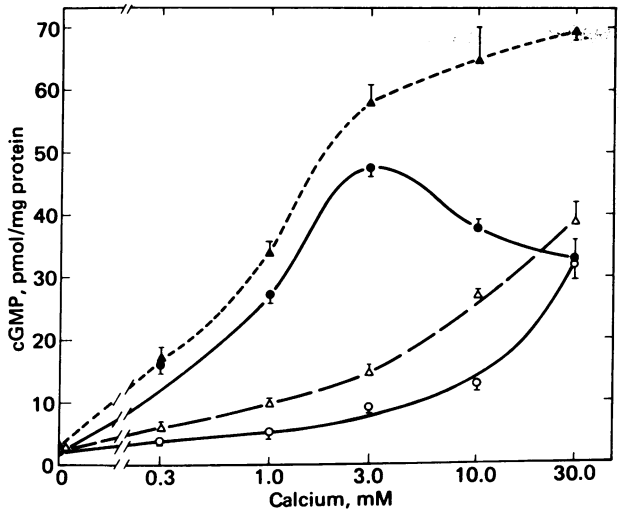

FIG. 3. Effect of various concentrations of calcium on levels of cGMP in N1E-115 neuroblastoma cells. The indicated calcium concentrations were present throughout the preincubation and incubation periods. The incubation conditions were: control (5 mM potassium) (○); high-potassium (100 mM) medium (●); 5 mM potassium plus 1 mM carbachol (△); or 100 mM potassium plus 1 mM carbachol (▲). At calcium concentrations greater than 3 mM, the sodium concentration was decreased to maintain osmolarity and ionic strength. Results similar to those shown here were obtained when the sodium concentration was not decreased. Data represent the mean ± SEM for three cultures, each assayed in triplicate.

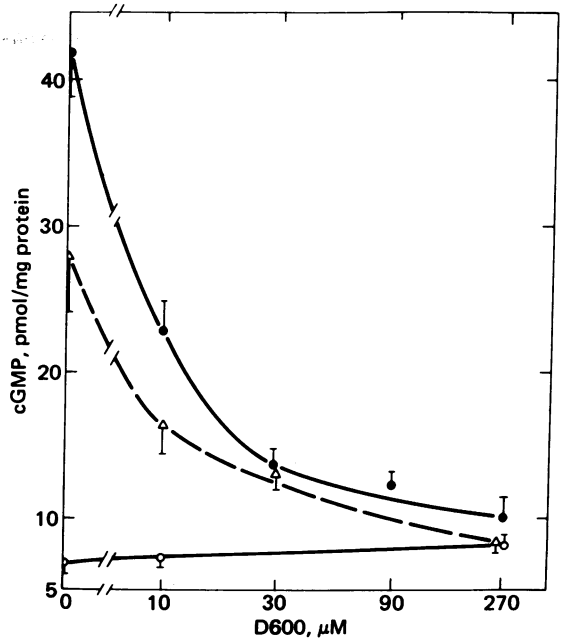

FIG. 4. Effect of various concentrations of D600 on levels of cGMP in N1E-115 neuroblastoma cells. D600 was present throughout the preincubation and incubation periods. D600 (as the HCl salt) was dissolved in ethanol and added to the buffer so that the final ethanol concentration was 0.1%. This concentration of ethanol had no effect on basal or stimulated levels of cGMP. The incubation conditions were: control (5 mM potassium) (○); high-potassium (100 mM) medium (●); or 1 mM carbachol (△). Data represent the mean ± SEM for three cultures, each assayed in triplicate.

experiments using 1 and 3 mM calcium (data not shown). At calcium concentrations from 3 to 30 mM, the combined response to carbachol and 100 mM potassium continued to increase slightly, in contrast to the decrease in cGMP levels seen with 100 mM potassium alone.

Depolarization of N1E-115 cells activates a "slow" calcium current in addition to sodium and potassium currents (24). This calcium current is thought to result from the opening of voltage-sensitive calcium channels. The ability of veratridine or high-potassium medium to increase cGMP levels could be due to opening of these channels. This hypothesis was tested by using specific calcium-channel blockers. D600, an organic blocker of calcium channels (25), antagonized the responses both to high-potassium medium and to carbachol without a statistically significant effect on control levels of cGMP (Fig. 4). The cGMP response to high-potassium medium was slightly more sensitive than that to carbachol, half-maximal inhibitions occurring at about 10 and 20 μM D600, respectively. This apparent difference in sensitivity was seen in each of three experiments.

Cobalt ions also block calcium channels in neuroblastoma and other cell types (25–27). The cGMP response to high-potassium medium was completely blocked by 10 mM cobalt with a half-maximal effect at less than 1 mM (Fig. 5; note that the ordinate is on a logarithmic scale). At 30 mM cobalt, the cGMP level in the presence of high-potassium medium was less than that of control; this may be due to a toxic effect of a small amount of cobalt entering the cells through voltage-sensitive channels. In contrast, the response to carbachol was relatively insensitive to blockade by cobalt. Cobalt at 3–30 mM decreased both the control levels of cGMP and the increases due to carbachol; a 2- to 3-fold response to carbachol remained in the presence of up to 30 mM cobalt.

These results are consistent with the idea that depolarizing agents increase cGMP levels by allowing calcium to enter through voltage-sensitive calcium channels, thus leading to increased guanylate cyclase activity. However, cGMP accumulation could be a result of some other effect of these agents. One such mechanism could involve a reduction in outward pumping of calcium. In many central and peripheral neurons, internal calcium is maintained at a low concentration by an outward pumping of calcium driven by the electrochemical gradient for sodium (28). If this is true for N1E-115 cells, a reduction of the sodium gradient across the membrane, produced either by lowering external sodium (in high-potassium buffer) or by increasing internal sodium (by influx in response to veratridine or high-potassium medium), could decrease the out-

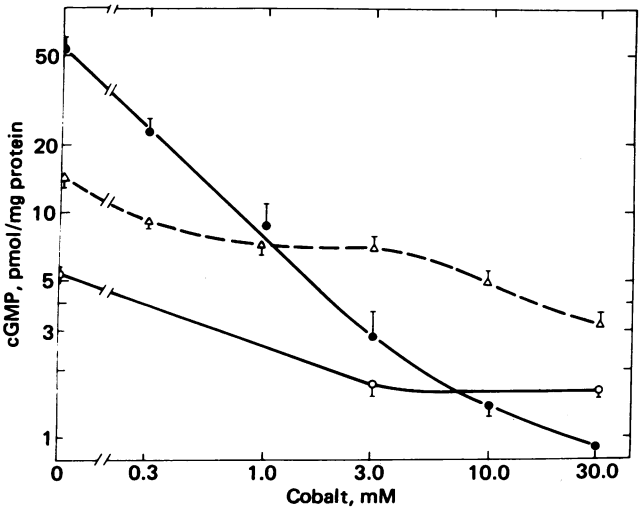

FIG. 5. Effect of various concentrations of cobalt on levels of cGMP in N1E-115 neuroblastoma cells. Cobalt chloride was present throughout the preincubation and incubation periods. The incubation conditions were: control (5 mM potassium) (○); high-potassium (100 mM) medium (●); or 1 mM carbachol (△). Data represent the mean ± SEM for three cultures, each assayed in triplicate.

Table 3. Effect of receptor antagonists on cGMP increases produced by high-potassium medium in N1E-115 neuroblastoma cells

Incubation condition	cGMP, pmol/mg protein
Control	16.0 ± 1.8
High potassium (100 mM)	37.6 + 6.4
High potassium + atropine	36.1 ± 2.5
High potassium + phentolamine	36.0 ± 3.8
High potassium + mepyramine	45.2 ± 3.8

Antagonists (10 μM) were present throughout the preincubation and incubation periods. Data represent the mean ± SEM for three cultures, each assayed in triplicate.

ward pumping of calcium. The resulting increases in internal calcium could stimulate cGMP synthesis, independently of depolarization. This possibility was tested for the case of high-potassium medium by an experiment in which the extracellular sodium was decreased from 129 to 34 mM and replaced by either potassium or choline. In this experiment, high-potassium (100 mM) medium increased cGMP from a mean (± SEM) of 5.7 ± 0.5 pmol/mg of protein in control cells to 25.7 ± 1.5 pmol/mg of protein ($P < 0.001$). When sodium was replaced instead by 95 mM choline, cGMP was increased only to 9.1 ± 1.2 pmol/mg of protein ($P < 0.025$ vs. control). Thus, a small portion of the cGMP response to high-potassium medium may be due to a decrease in the sodium gradient, but the response is primarily due to the potassium itself, probably from its depolarizing effect.

Another mechanism by which depolarizing agents might act could be by causing the release of a cellular substance which would then interact with an appropriate receptor and lead to an increase in cGMP synthesis. To test this possibility, cells were preincubated with blockers of neurotransmitter receptors known to be associated with increases in cGMP [N1E-115 cells have histamine H_1-receptors (ref. 29; unpublished data), as well as muscarinic receptors; an α-adrenergic receptor antagonist was included because these cells synthesize catecholamines (19, 30)]. Increases in cGMP produced by 100 mM potassium were not affected by 10 μM atropine (which blocks muscarinic receptors), mepyramine (which blocks histamine H_1-receptors), or phentolamine (which blocks α-adrenergic receptors) (Table 3). However, the possibility that some other, as yet unidentified, substance is responsible for the increase in cGMP is not ruled out by this experiment. If such a substance were released into the medium upon depolarization, then the medium from depolarized cells might be able to increase cGMP levels in non-depolarized cells. This possibility was tested by using veratridine. Conditioned medium from veratridine-treated cultures was added to other cultures, in the presence of TTX (1 μM) added to block further depolarization by veratridine. This conditioned medium did not increase cGMP levels in the second group of cultures. These results suggest that, if substances are released into the surrounding medium, they do not cause increases in cGMP levels. However, if such substances are degraded very rapidly or require high local concentrations to exert an effect, their action would not have been detected.

DISCUSSION

Our results suggest that high-potassium medium or veratridine can increase cGMP levels in N1E-115 neuroblastoma cells via calcium influx through voltage-sensitive calcium channels. The cGMP response to depolarization was abolished by removal of extracellular calcium or by the calcium-channel blockers D600 and cobalt. The potencies of these blocking compounds are similar to those seen for calcium fluxes in N1E-115 cells measured with electrophysiological methods (24, 26) and in other cell types by electrophysiological or radiocalcium flux methods (12, 25, 27). Therefore, the calcium channels that are involved in the cGMP increase may be the same as those observed by other methods.

An inward flux of calcium appears to be sufficient to increase cGMP levels, because the ionophore A23187 also produces a large calcium-dependent increase in cGMP in neuroblastoma cells (2). Studies on barnacle muscle have also shown an accumulation of cGMP due to calcium influx in the absence of receptor activation (31). These observations support the idea that calcium entry triggers the cGMP accumulation seen in response to depolarizing agents.

Calcium influx through voltage-sensitive calcium channels might be involved in the increase of cGMP levels in two ways: by acting as an intermediate in the stimulation of guanylate cyclase activity, or by causing the release of a substance that acts at a receptor on the cell surface to increase cGMP. This second possibility appears unlikely. Although N1E-115 cells form axon-like processes and synthesize and store catacholamines, no evidence of functional synapses or depolarization-induced release of endogenous neurotransmitters has been reported. Furthermore, our experiments with conditioned medium and with receptor antagonists argue against the involvement of a released substance.

Increases in cGMP levels resulting from depolarization were maximal after about 1 min and then gradually decayed. The "slow" calcium current observed by Moolenaar and Spector (24) in voltage-clamped N1E-115 cells appears to become inactivated after about 150 msec of depolarization. Such a short-lived calcium influx could explain the time course of the cGMP increases reported here in the presence of veratridine or high-potassium medium.

Because in many types of neurons the ionic currents that give rise to action potentials have a component of calcium influx through voltage-sensitive calcium channels, it is possible that intracellular levels of cGMP reflect to some extent the firing activity of these cells. Consistent with this possibility, activation of excitatory pathways to the Purkinje cells in the cerebellar cortex increases cGMP levels in this region, whereas activation of inhibitory pathways to these cells decreases cGMP levels (32, 33). Depolarizing agents increase cGMP in slices of cerebellum from various mammalian species (34). Recently, Llinas and coworkers (35) have shown that Purkinje cells of mammalian cerebellum exhibit calcium spikes. Thus, excitatory and inhibitory transmitters in the cerebellar cortex may regulate cGMP by controlling the firing of Purkinje cells (and perhaps other neurons). Although it is not yet known whether firing alone can increase cGMP levels in neurons, the results presented here indicate that, in N1E-115 cells, sustained depolarization is sufficient.

The cGMP responses to muscarinic agonists and to depolarizing agents have some properties in common. Both depend on external calcium and are sensitive to the calcium-channel blocker D600. It is possible that D600 could inhibit the muscarinic response by interaction with carbachol-binding sites rather than with calcium channels. This is unlikely because the potency of D600 in blocking increases in cGMP levels is similar to its known affinity for calcium influx sites while its binding affinity for muscarinic receptors is much lower [as determined for heart membranes (36)].

Other evidence suggests that muscarinic agonists and depolarizing agents increase cGMP by distinct calcium-dependent mechanisms. The response to a maximally effective concentration of carbachol (1 mM) is additive with that of high-po-

tassium medium over a range of potassium and calcium concentrations. This additivity is probably not due to further depolarization by carbachol because muscarinic agonists and acetylcholine do not depolarize N1E-115 cells (ref. 15; C. Palfrey, personal communication). Moreover, the increase in cGMP produced by carbachol and that produced by high-potassium medium were affected differently by changes in concentrations of external calcium and cobalt. Our findings can be explained by the existence of two types of calcium channels, one linked to the muscarinic receptor and voltage-insensitive and the other not receptor-linked but voltage-sensitive.

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