Biochimica et Biophysica Acta, 613 (1980) 229—237

BBA 68959

DETERMINANTS OF THE STIMULATION OF FAT CELL ADENYLATE CYCLASE BY HIGH CONCENTRATIONS OF SODIUM AND MAGNESIUM SALTS

IMPLICATIONS FOR THE ROLE OF MAGNESIUM IN REGULATION OF ENZYME ACTIVITY

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(Received October 10th, 1979)

Key words: Adenylate cyclase stimulation; Guanine nucleotide; Na^+ regulation; Mg^{2+} regulation; (Fat cell)

Summary

Stimulation of adenylate cyclase by high concentrations of Mg^{2+} has been attributed to either occupation of a divalent cation site on the enzyme or Mg^{2+} binding of inhibitory species of uncomplexed ATP. However, the stimulation of adenylate cyclase by other salts led us to reexamine the alleged specificity of Mg^{2+}. The stimulatory effects of high concentrations of magnesium and sodium salts on adenylate cyclase were compared in rat fat cell membranes ('ghosts'). Under standard assay conditions of 5 mM $MgCl_2$ and 1 mM ATP, added $MgCl_2$ (45 mM), NaCl (90 mM), $MgSO_4$ (45 mM), and Na_2SO_4 (45 mM) stimulated basal activity in a temperature-dependent manner. At 30°C all four salts produced 2—3-fold stimulation with linear time courses to 10 min. At 37°C time courses were nonlinear, and although $MgCl_2$ and NaCl were stimulatory over 10 min, sulfate salts of both cations had little or no effect. When 10^{-4} M GTP was added, however, all four salts became stimulatory at 37°C with linear time courses. In the presence of GTP, $MgCl_2$ and NaCl were equally effective at equimolar concentrations (100 mM) of Cl^-. $MgCl_2$ and NaCl were stimulatory (in the absence or presence of GTP) over the same range of Cl^- concentrations up to 200 mM. In contrast to the results with GTP, magnesium salts

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Abbreviation: GMP-P(NH)P, 5′-guanylyl-imidophosphate.

enhanced stimulation by the GTP analog 5'-guanylyl-imidodiphosphate (GMP-P(NH)P) much more than did sodium salts. $MgCl_2$ and $MgSO_4$ each approximately tripled GMP-P(NH)P stimulation, while NaCl and Na_2SO_4 had only minimal stimulatory effect. None of the salts increased 10^{-4} M epinephrine-stimulated adenylate cyclase activity in the absence or presence of GTP.

Our results show that the effects of 'high Mg' are, under certain conditions, approximated by high concentrations of another cation (Na) and are in part determined by the accompanying anion. Stimulation by high $MgCl_2$, therefore, appears to be a generalized salt effect rather than a specific effect of Mg^{2+}. In addition, the degree of stimulation of adenylate cyclase of fat cell ghosts by salts, including those of Mg^{2+}, is dependent on temperature and the presence or absence of guanine nucleotide and hormone.

Introduction

Adenylate cyclases of a number of mammalian tissues are stimulated by magnesium salts at high concentrations greatly in excess of those required to form the active Mg-ATP^{2-} substrate complex [1—8]. It has been suggested that Mg^{2+} at these high concentrations activates adenylate cyclase either by occupying a divalent cation binding site on the enzyme [1—4,9] or by binding inhibitory species of uncomplexed ATP [5,6,10]. Recently stimulation by both Mg^{2+} and Mn^{2+} * salts has been found to be independent of the ratio of free to complexed ATP. This finding is thought to be evidence in favor of divalent cation stimulation at a metal ion binding site rather than activation by metal binding of uncomplexed ATP [12].

Both theories of Mg^{2+} activation of adenylate cyclase assume that stimulation by high concentrations of magnesium salt is a divalent cation effect. Recently, however, high concentrations of anions have also been found to stimulate adenylate cyclase activity [13—15]. The question then arises of whether stimulation by 'high Mg' concentration is due only to Mg^{2+} or whether the accompanying anion is also involved. We have found that in rat fat cell membrane ('ghost') preparations stimulation of adenylate cyclase by sodium salts is often equivalent to that by magnesium salts and that stimulation by Mg and Na salts depends on the accompanying anion species. In this paper we compare the effects of Mg and Na salts (chloride and sulfate) on enzyme activities in the unstimulated (basal) state and in the presence of guanine nucleotides and hormone. Approximation of the effects of 'high Mg' by multiple salts suggests that the concept of 'Mg' stimulation of adenylate cyclase must be revised to take into acount the stimulatory effects of both anions and cations.

Materials and Methods

Isolated fat cells were prepared from rat epididymal fat pads by collagenase

* Manganese salts are known to stimulate adenylate cyclase at much lower concentrations than are required with magnesium salts. Mn^{2+} has been thought to act at the same divalent cation binding site as Mg^{2+} [11,12].

digestion as described by Rodbell [16]. Fat cell ghosts were prepared by the method of Birnbaumer et al. [1] with the exception that 1 mM dithiothreitol was included in the lysing and suspending media. Fat pads from 2—3 outbred Wistar rats (2 months old) were used for each experiment. Adenylate cyclase activity was assayed as previously described [17], and cyclic AMP product was isolated by the method of Salomon et al. [18]. Standard assay conditions in 10 min incubations at 30 or 37°C were 25 mM Tris-HCl (pH 8.2—8.5), 1 mM ATP, 2 mM cyclic AMP, 10 mM theophylline, and an ATP regenerating solution of 11 mM creatine phosphate and 1 mg/ml creatine phosphokinase. At least 5 mM $MgCl_2$ was always present. The effects of Mg and Na salts on adenylate cyclase were compared at salt concentrations added to give equivalent anion concentrations in the final assay system. Specifically, 90 mM chloride (45 mM $MgCl_2$ or 90 mM NaCl) or 45 mM sulfate (45 mM $MgSO_4$ or Na_2SO_4) were added. Additions of epinephrine, GTP, or the GTP analog GMP-P(NH)P, individually or in combination, were included in the assay as specified in Results.

Salts of the highest available purity were obtained from Baker ($MgSO_4$ and Na_2SO_4), Sigma ($MgCl_2$), and Research Organic/Inorganic Chemical Corp., Belleville, NJ U.S.A. (NaCl). The sources of other materials are listed elsewhere [17]. Mean values from multiple experiments are presented ±S.E.

Results

Fig. 1 shows that high concentrations of both Na and Mg salts stimulated adenylate cyclase of rat fat cell ghosts and that the effects of the salts were in part temperature dependent. At 30°C all four salts tested stimulated basal activity by about 2—3-fold with linear time courses to 10 min (Fig. 1A). In five experiments stimulation at 10 min by $MgCl_2$ (2.4-fold ± 0.2 relative to basal activity) was significantly greater ($P < 0.01$, paired Student's t test) than that by NaCl (1.8-fold ± 0.2). In the two experiments of Fig. 1A, 10-min stimulations by $MgSO_4$ and Na_2SO_4 were 2.6- and 2.2-fold, respectively.

Stimulation by salts was not increased by increasing the incubation temperature to 37°C. In fact, although chloride salts of Mg and Na stimulated substantially at the higher temperature, sulfate salts of both cations had little or no effect (Fig. 1B). In seven experiments at 37°C stimulations by $MgCl_2$ and NaCl at 10 minutes (2.4 ± 0.2 and 1.8-fold ± 0.1 relative to basal, respectively) were the same as at 30°C (see above), with $MgCl_2$ effect being significantly greater ($P < 0.001$) than that of NaCl. At 37°C 10-min stimulations by $MgSO_4$ and Na_2SO_4 were 1.5-fold ± 0.2 (n = 5) and 1.2-fold ± 0.2 (n = 4), respectively; these effects of sulfate salts were significantly less ($P < 0.02$, unpaired Student's t test) than the above mentioned stimulations by Cl salts of the same cations. Temperature differences of salt stimulation at 10 min were unaffected by basal activity, which was unchanged at 30°C vs 37°C (data not shown). Fig. 1 shows that unlike the results at 30°C, time courses at 37°C were nonlinear in the absence and presence of salts. Nonlinear time courses clearly limit assessment of the magnitude of salt effects at 37°C based on activities at a single 10 min time point. Nevertheless, 10-min salt stimulations were generally representative of stimulations relative to basal at earlier time points (see Fig. 1B).

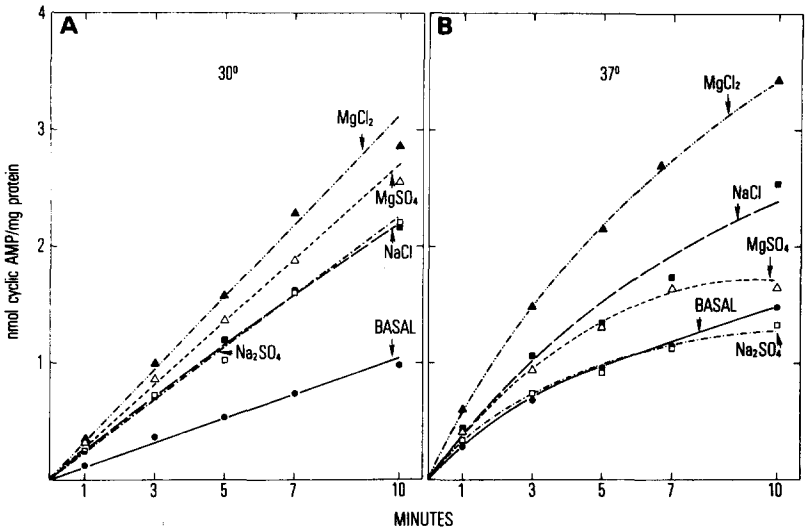

Fig. 1. Stimulation of adenylate cyclase in rat fat cell ghosts by Mg and Na salts. Effects of temperature. Panel A shows the mean values from two experiments; panel B shows the mean values of duplicate determinations from a single, representative experiment. Absolute activities shown at 30 vs. 37°C should not be compared because the experiments at 30 and 37°C were performed with different fat cell ghost preparations at different times.

GTP is known to regulate the activity of rat fat cell adenylate cyclase [19]. Fig. 2 compares the effects of Mg and Na salts in our system at 37°C in the presence of 10^{-4} M GTP. GTP alone stimulated basal activity after an initial inhibitory lag phase *. Salt effects in the presence of GTP differed from their effects on basal activity in several respects. First, time courses of stimulation by each of the four salts were linear at 37°C to 10 min in the presence but not in the absence of GTP. Second, GTP activity was increased by Mg and Na salts to a greater extent than was basal activity (cf. Figs. 1B and 2). Finally, unlike the results in the absence of GTP (see above) $MgCl_2$ and NaCl had equivalent effects in the presence of GTP at equimolar concentrations of Cl^- (90 mM). In six experiments at 37°C GTP stimulated basal activity by 1.7-fold ± 0.1 at 10 min, while $MgCl_2$ and NaCl increased GTP stimulation to 3.4-fold ± 0.2 and 3.7-fold ± 0.3, respectively. Similarly, $MgSO_4$ increased 10-min GTP stimulation to 3.7-fold ± 0.1 relative to basal ($n = 4$). Na_2SO_4 effect was somewhat lower, increasing GTP stimulation to 2.8-fold ± 0.2 ($n = 3$). Since basal activity was nonlinear at 37°C, the numerical values of these stimulated activities relative to basal represent only the 10 min point and do not represent the degree of stimulation throughout the entire 10 min incubation.

Stimulation of enzyme activities by added Mg and Na salts was dose-dependent over a wide range of salt concentrations. Fig. 3 shows that with or

* Although the initial lag phase of guanine nucleotide action is well recognized [6], GTP has usually been found to inhibit basal activity of the rat fat cell enzyme [19]. However, at conditions of higher temperature, i.e., 37°C, we have found that in our system GTP is stimulatory. This finding is in agreement with a previous study of fat cell adenylate cyclase conducted at 37°C [20]. The influence of incubation temperature on GTP effect is considered in a separately submitted manuscript.

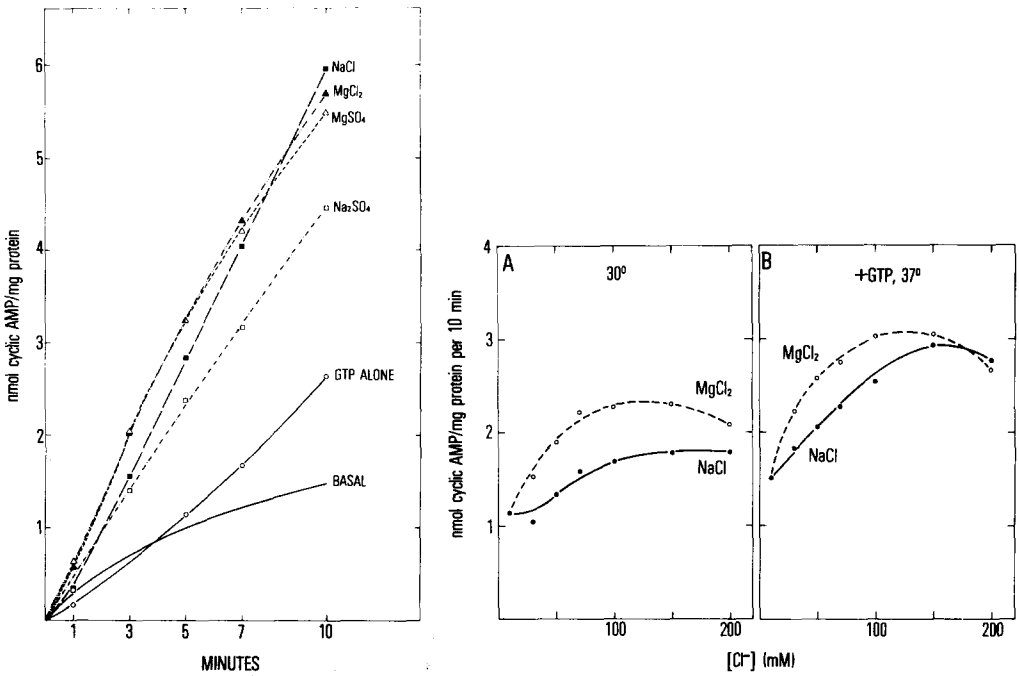

Fig. 2. Stimulation of adenylate cyclase in rat fat cell ghosts by Mg and Na salts in the presence of GTP. Concentration of GTP was 10^{-4} M. Each point is the mean of duplicate determinations from a single, representative experiment at 37° C. This experiment was performed simultaneously with the experiment of Fig. 1B, using the same fat cell ghost preparation and assay conditions (except for the addition of GTP). Basal curves presented in the two figures are therefore identical, but for reasons of clarity salt stimulations in the absence of GTP are presented only in Fig. 1B.

Fig. 3. Dose-responses of $MgCl_2$ and NaCl stimulation of adenylate cyclase in rat fat cell ghosts. Concentration of GTP in panel B, 10^{-4} M. Cl^- concentrations shown on the abscissa include the 10 mM Cl^- added to the standard assay as 5 mM $MgCl_2$. Panel A shows the mean values from two experiments; panel B shows the mean values of duplicate determinations from a single, representative experiment. Experiments A and B were performed at different times. In experiment B in the presence of GTP $MgCl_2$ had a greater effect than did NaCl at Cl^- concentrations up to 100 mM. However, in six experiments, including experiment B, increase of GTP activity by the two salts (at 100 mM Cl^-) showed no statistically significant difference (see Results).

without added GTP both $MgCl_2$ and NaCl were stimulatory over the same range of Cl^- concentrations, with threshold stimulation at about 25 mM Cl^- and maximum effect at 100—150 mM Cl^-.

In contrast to the equivalent effects of $MgCl_2$ and NaCl in the presence of GTP (see above and Fig. 2), interaction of salts with the GTP analog GMP-P(NH)P showed specificity for the Mg^{2+} cation. Fig. 4 shows the effects of Mg and Na salts on adenylate cyclase activity in the presence of GMP-P(NH)P. Like GTP the analog alone stimulated basal after an initial lag phase. $MgCl_2$ and $MgSO_4$ each markedly increased GMP-P(NH)P effect throughout the entire 10 min time course, while the Na salts had minimal effect. In three experiments (37°C, 10 min) GMP-P(NH)P stimulated enzyme activity by 3.8-fold ± 0.2 relative to basal. $MgCl_2$ and $MgSO_4$ approximately tripled this stimulation, to 11.5 ± 1.9 and 13.4-fold ± 2.1, respectively. On the other hand, NaCl and

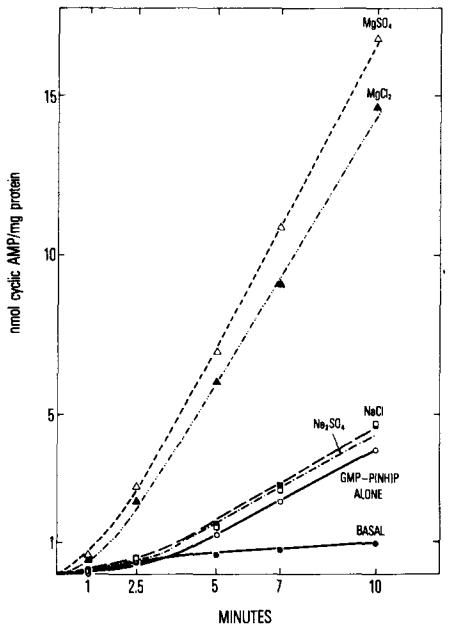

Fig. 4. Effects of Mg and Na salts on adenylate cyclase activity in rat fat cell ghosts in the presence of GMP-P(NH)P. Concentration of GMP-P(NH)P, 10^{-4} M. Each point is the mean of duplicate determinations from a representative experiment at 37°C. Salt stimulation curves in the absence of GMP-P(NH)P were similar to those in Fig. 1B; 10-min salt-stimulated activities were 3.2 nmol/mg protein for $MgCl_2$, 1.9 for $MgSO_4$, 2.4 for NaCl, and 1.4 for Na_2SO_4.

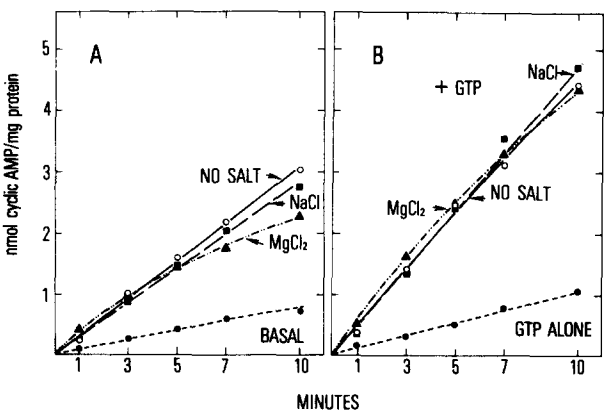

Fig. 5. Epinephrine-stimulated adenylate cyclase activity in rat fat cell ghosts in the absence and presence of $MgCl_2$ and NaCl. Epinephrine (EPI), 10^{-4} M; GTP in panel B, 10^{-4} M. Both panels show the mean values of duplicate determinations from a representiative experiment at 37°C. Unlike previous findings in fat cell ghosts of GTP inhibition of EPI-stimulated adenylate cyclase [19], GTP enhanced EPI stimulation under our assay conditions (cf. A and B). This stimulatory effect of GTP has also been shown by others working with rat fat cell membranes at 37°C [20].

Na_2SO_4 increased GMP-P(NH)P stimulation only to 4.6 ± 0.2 and 4.5-fold ± 0.5. In all experiments Mg salt effect on GMP-P(NH)P stimulation was partly dependent on the accompanying anion, with SO_4 salt showing somewhat greater effect than Cl salt. The reason for different salt effects in the presence of GTP vs GMP-P(NH)P (cf. Figs. 2 and 4) is unclear, but in this case the analog is apparently inadequate to study the role of the naturally occurring nucleotide in enzyme regulation.

Hormone-stimulated activity in the absence and presence of high concentrations of salts was also examined. Fig. 5 shows that unlike salt stimulation of basal and GTP activities (Figs. 1 and 2), $MgCl_2$ and NaCl had no effect on stimulation of adenylate cyclase by 10^{-4} M epinephrine in the absence or presence of 10^{-4} M GTP.

Discussion

Our results show that under certain assay conditions stimulation of adenylate cyclase by high concentrations of Mg salt can be approximated by high concentrations of Na salt. Accordingly, the stimulation in a number of tissues previously attributed to 'high Mg' [1—12] appears to be at least partly a salt effect not specific for Mg^{2+}. The degree of stimulation by salts is dependent on both cation (Figs. 1 and 4) and anion (see Results and Figs. 1B, 2, and 4). Furthermore, degree of stimulation by salt is temperature-dependent (Fig. 1) and varies with the absence or presence of other enzyme activators, i.e., guanine nucleotides and/or hormone (Figs. 1, 2, 4, and 5).

Descriptions of 'Mg' stimulation of adenylate cyclase must take these findings into account. The similarities between stimulatory effects of Mg and Na salts cast doubt on the validity of the specific mechanisms of enzyme activation formerly attributed to high Mg^{2+} concentration. Activation by high concentrations of salts probably did not occur by cation binding of inhibitory species of uncomplexed ATP as has been previously postulated [5,6,10]. For Na salts at least, the stability constants of Na-ATP complexes appear to be so low relative to those of Mg-ATP complexes [21] that in the presence of the 5 mM $MgCl_2$ in the standard assay only a very small fraction of ATP would be bound to Na, even at 90 mM added NaCl. Previous studies have already questioned whether uncomplexed ATP is a potent inhibitor of enzyme activity [9,12]. Although Mg^{2+} has alternatively been thought to stimulate adenylate cyclase by binding to a divalent metal ion binding site on the enzyme [1—4, 9,12], this model of activation by Mg^{2+} does not account for our observations of equivalent effects of $MgCl_2$ and NaCl, at least in the presence of GTP (see Fig. 2), and the dependence of Mg salt effect on the accompanying anion (see Results and Figs. 1B, 2, and 4).

The mechanism(s) by which high concentrations of salts, including Mg salts, stimulate adenylate cyclase remains to be determined. Salt effects are conceivably mediated at single or multiple cation and/or anion binding sites within the enzyme, or, more generally, within the cell membrane. In this regard stimulation and inhibition of beef thyroid adenylate cyclase by polycations over a wide range of concentrations are thought to be due to a direct polycation effect on the membrane based on charge interactions [22]. Binding of

these polycations to intact cells is known to change the properties of the cell membrane [22]. Salts might therefore induce specific changes in the conformational structure of adenylate cyclase or affect enzyme activity indirectly by altering the membrane to which the enzyme is bound. Stimulation of activity by salts might reflect alteration of a regulatory component of the enzyme as indicated by increased GTP effect in the presence of salts (Fig. 2). In any event, in our system the only circumstance in which stimulation of adenylate cyclase by salts might be mediated by a mechanism specific for Mg^{2+} cation is in the presence of GMP-P(NH)P, where activity is selectively augmented by Mg and not Na salts (Fig. 4).

Epinephrine stimulated activity was unaffected by high concentrations of salts (Fig. 5). This finding may be accounted for in previous studies of adenylate cyclases from a number of tissues [1,7,8,12], where the apparent affinities of the enzyme for stimulatory Mg salts were increased in the presence of hormone. The results shown in Fig. 5 are compatible with an epinephrine-induced shift of maximum salt effect to a low salt concentration (i.e., that already present in the standard assay medium) although our studies did not specifically address this possibility. In several tissues the affinity of adenylate cyclase for Mg salt was also increased in the presence of guanine nucleotides [7,8,12]. However, our observation of apparently greater salt effect on GTP-stimulated activity than on basal activity (cf. Figs. 1B vs. 2) was not due to a shift of salt dose-response, which was essentially the same in the absence vs. presence of GTP (Fig. 3). Alteration of salt effects by hormones and guanine nucleotides are currently being studied further.

While this work was in progress, Roy et al. [23] reported different effects of sulfate and chloride ions on vasopressin-sensitive adenylate cyclase of pig kidney plasma membranes. These authors found that $MgCl_2$ at high concentrations had greater stimulatory effect on basal activity than did equivalent concentrations of $MgSO_4$. This finding in kidney is similar to our own observation in fat that chloride salts (both Mg and Na) stimulated basal activity at 37°C more than did sulfate salts (see Results and Fig. 1B). On the other hand, the two tissue systems appear to differ in the interaction of salts with hormone-stimulated activity. In kidney membranes high concentrations of salt increased vasopressin-stimulated activity, whereas in our fat cell ghosts added salt had no apparent effect on epinephrine-stimulated activity (Fig. 5). Roy et al. [23] concluded that it was difficult to define a precise role of Mg^{2+} in the regulation of adenylate cyclase because the effect of Mg salt on basal activity depended on the accompanying anion. We agree with this conclusion. However, by comparing the stimulatory effects of different cations (Mg^{2+} vs. Na^+) and anions (Cl^- vs. SO_4^{2-}), we have been able to relate, at least in part, the effects of 'high Mg' to the more generalized phenomenon of stimulation of adenylate cyclase by high concentrations of salts.

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