EFFECTS OF CHOLINERGIC AGENTS AND SODIUM IONS ON THE LEVELS OF GUANOSINE AND ADENOSINE 3′:5′-CYCLIC MONOPHOSPHATES IN NEUROBLASTOMA AND NEUROBLASTOMA × GLIOMA HYBRID CELLS

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1. Introduction

Although both cyclic nucleotides, adenosine and guanosine 3′:5′-cyclic monophosphates (cyclic AMP and cyclic GMP, respectively) occur in high concentrations in nervous tissue [1], little is known about their functions. The complexity of the structure of nervous tissue makes the interpretation difficult of data obtained from biochemical and pharmacological experiments with this material. Clonal cell lines derived from nervous tissue can serve as simple model systems, since they retain differentiated functions of the nervous system. Thus, neuroblastoma × glioma hybrid cells can be induced to extend neurite-like processes [2,3], they have high activities of choline acetyltransferase [2] and contain clear and dense core vesicles [4]. Their membranes are excitable by depolarizing current [2] or by the neurohormones acetylcholine [2] and noradrenaline [5]. In the hybrid and neuroblastoma cells the intracellular level of cyclic AMP strongly increases in response to prostaglandin E_1 [6]. This action of prostaglandin E_1 is potently obviated by noradrenaline [5], acetylcholine [7] and opiates [8–10]. The effect of opiates is probably mediated by cyclic GMP [11].

In the present communication we report that in the hybrid and neuroblastoma cells low concentrations of the cholinergic agonists carbamoylcholine (Carb), tetramethylammonium (TMA) and pilocarpine (Pil) increase the intracellular concentration of cyclic GMP, while high concentrations of acetylcholine may slightly elevate the level of cyclic AMP. The responses to Carb and TMA are more pronounced in the presence of high (154 mM) than of low (5 mM) concentrations of Na^+. The effects of Pil at high Na^+ are even inverse to those at low Na^+ concentration. Thus, while 1 μM Pil lowers the level of cyclic GMP and elevates that of cyclic AMP in the former case, it does the reverse in the latter case.

2. Materials and methods

Acetylcholine chloride was purchased from E. Merck, carbamoylcholine chloride from Fluka, tetramethylammoniumhydroxide·5 H_2O and pilocarpine·HCl from Sigma.

Neuroblastoma × glioma hybrid line 108CC15 was obtained by fusion of line N18TG2 and rat glioma line C6-BU-1 [2]. The clonal lines N4TG3, N18TG2, NS2OY and N1E-115 were derived from mouse neuroblastoma C-1300 [12]. N4TG3 [13] and N18TG2 [14] are 6-thioguanine resistant mutants of N4 and N18, respectively. Since NS2OY contains choline acetyltransferase, N1E-115 tyrosine hydroxylase and N4 and N18 neither enzyme, these lines were classified as cholinergic, adrenergic and 'inactive' cell lines, respectively [12].

The cells were cultured in plastic dishes 85 mm in diameter [15] and subcultured by using isotonic solutions [16] of 0.005 % trypsin (crystallized twice, Boehringer/Mannheim).

One hour before the experimental incubation, the growth medium was replaced by 5 ml of incubation

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medium [17]. Two kinds of incubation media were employed containing either 154 or 5 mM Na^+. The osmolality of the latter was adjusted to 320 mosM by the addition of choline chloride. The cholinergic agents were added as described [8]. After the extraction, separation and purification, cyclic GMP [11] and cyclic AMP [8] were determined by protein binding assays.

3. Results

Exposure of the hybrid cells to Carb causes a strong and rapid increase in the intracellular level of cyclic GMP (fig.1, curves I and II). The response is highest after only 1 min of incubation (fig.1A). With prolonged exposure to Carb (fig.1B and C) the level of cyclic GMP falls progressively. The maximal effect of the cholinergic agent is seen at already 0.1 nM (fig.1A, B and C). A further increase in concentration reduces the level of cyclic GMP found. Thus, after 10 min exposure to 1 μM Carb the intracellular concentration of cyclic GMP is no longer different from

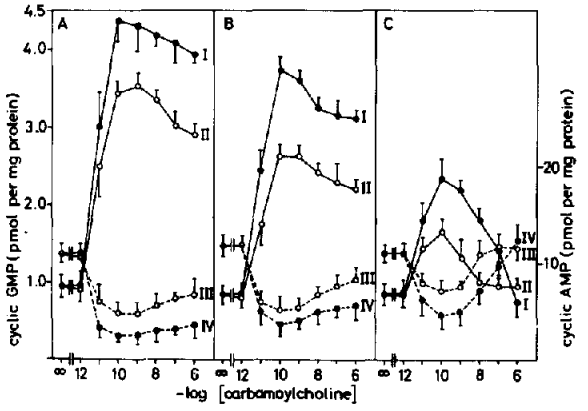

Fig.1. Influence of the concentration of Carb on the levels of cyclic GMP (solid line) and cyclic AMP (broken line) in clonal hybrid line 108CC15 during various times of incubation at high (filled symbols) and at low (open symbols) concentrations of Na^+. Mean values of 3 replica plates ±S.D. 1.2 × 10^6 viable cells and 1.3 mg protein per plate, viability 94%, passage number 10. The cells were incubated at 37° with Carb for 1 min (A), 5 min (B) and 10 min (C). Incubations for 1 min were performed by immersing the plates into a water bath, those at 5 and 10 min were carried out in a cell incubator.

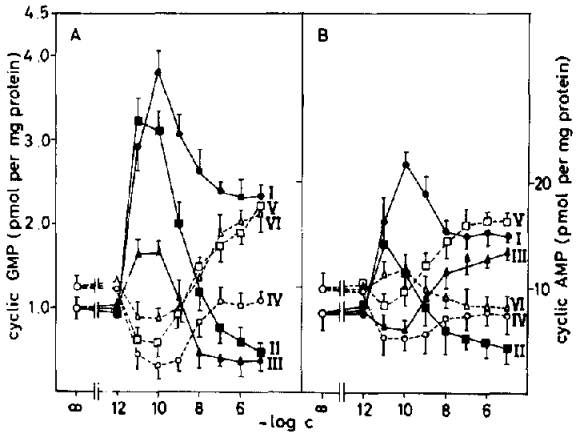

Fig.2. The effect of the cholinergic agonists Carb (circles), TMA (squares) and Pil (triangles) on the levels of cyclic GMP (closed symbols and solid line) and cyclic AMP (open symbols and broken line) at high (A) and low (B) concentration of Na^+. Incubation time was 5 min. 1.4 × 10^6 viable cells and 1.5 mg protein per replica plate, viability 93%, passage number 12.

that of the controls (fig.1C, curve I). At high concentrations of Na^+ the concentration of cyclic GMP is raised to higher values than at low concentrations of Na^+ (fig.1, curves I and II). In all cases the changes of the levels of cyclic AMP (fig.1, curves III and IV) are inversely related to those of cyclic GMP. Results, not presented here, with acetylcholine as stimulant, were qualitatively similar to those obtained with Carb.

In order to obtain an indication whether nicotinic or muscarinic cholinergic receptors were involved in these effects, the hybrid cells were exposed for 5 min to 3 types of cholinergic agonists [18]. At concentrations up to 0.1 nM all 3 agents cause an increase of the level of cyclic GMP and a concomitant decrease of the level of cyclic AMP (fig.2A, curves I to III), if high concentrations of Na^+ are present. The maximal effect of Carb (fig.2A, curve I) is somewhat higher than that of TMA (fig.2A, curve II), while that of Pil (fig.2A, curve III) is much less pronounced. As the concentrations are raised from 0.1 nM to 10 μM, the level of cyclic GMP approaches a plateau at a reduced value. For Carb (fig.2A, curve II) this value is well above that of the control (absence of drug), but for the other 2 agents (fig.2A, curves II and III) it is only a fraction of that of the control. Again, the levels of cyclic AMP (fig.2A, curves III to VI) change inversely

to those of cyclic GMP. It is specially striking that at high concentrations TMA (fig.2A, curve V) and Pil (fig.2A, curve VI) elevate the levels of cyclic AMP above base line. At low concentration of Na^+ (fig.2B), the maximal level of cyclic GMP reached in the presence of Carb (fig.2B, curve I) or TMA (fig.2B, curve II) is markedly reduced, if compared to the results obtained at high Na^+ concentration (fig.2A). Otherwise the curves (fig.2B, curves I, II, IV and V) are qualitatively similar to the corresponding ones in fig.2A. Lowering the Na^+ concentration exerts a remarkable effect on the response evoked by Pil. At concentrations up to 0.1 nM Pil depresses the level of cyclic GMP (fig.2B, curve III) while it slightly elevates that of cyclic AMP (fig.2B, curve VI), and at concentrations further up to 10 μM it does just the reverse. Thus, if the ligand of the cholinergic receptor is Pil, it appears that the change in the Na^+ concentration caused adenylate and guanylate cyclases to exchange their roles.

The response to the cholinergic agents of 4 neuroblastoma lines is very similar to that of the neuroblastoma X glioma hybrid clone 108CC15. This implies that the 4 lines show no striking differences between them. As shown in fig.3 (A,C,E,G), at high Na^+ concentration a maximal increase of the level of cyclic GMP is reached in the range of 0.1 to 1 nM agonist. As the concentrations of the cholinergic agents rise further, the elevations of cyclic GMP levels fade away and eventually are turned into depressions. Again, considerable elevations are seen only in the presence of Carb or TMA (fig.3A,C,E,G, curves I and II), whereas Pil causes only a slight increase at low concentrations, but the strongest depression at 10 μM concentration (fig.3A,C,E,G, curves III). At low Na^+ concentration the reduced response to Carb or TMA is seen again (fig.3B,D,F,H, curves I and II). As described for the hybrid cells, at low Na^+ concentration Pil shows a concentration dependency of its effects which is inverse to that of the other 2 agents. Low concentrations lower and high concentrations elevate the concentration of cyclic GMP (fig.3B,D,F, H, curves III).

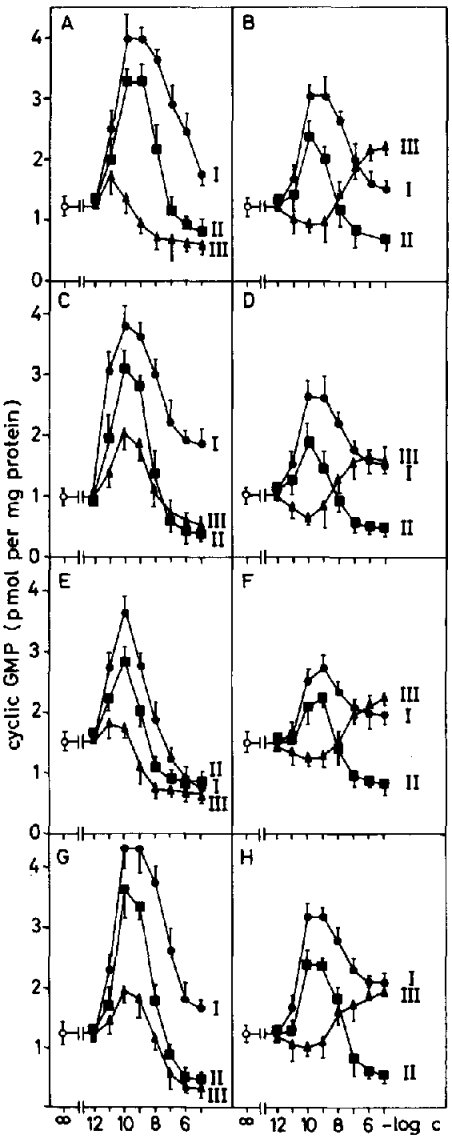

Fig.3. The effect of cholinergic agonists Carb (circles), TMA (squares) and Pil (triangles) on the production of cyclic GMP in mouse neuroblastoma lines N4TG3 (A, B), N18TG2 (C, D), NS2OY (E, F) and N1E-115 (G, H) in the presence of high (A, C, E, G) and low Na^+ concentration (B, D, F, H). The numbers of viable cells (in millions per plate), viabilities and passage numbers, respectively, were: N4TG3, 1.7, 93%, 20; N18TG2: 1.3, 90%, 15; NS2OY: 1.2, 96%, 10; N1E-115: 1.4, 94%, 9. Time of incubation was 5 min.

4. Discussion

Eight interrelated aspects of the data shall be discussed. The first, the elevation of the level of cyclic

GMP in the hybrid cells, had been expected, since cholinergic agonists attenuate the increase of the level of cyclic AMP caused by prostaglandin E_1 [7] or adenosine [21]. Such action of prostaglandin E_1 is also obviated by noradrenaline [5] and morphine [8,19], 2 agents known to increase the intracellular levels of cyclic GMP [11,20] in the hybrids. Cholinergic agonists increase the level of cyclic GMP in heart [22,23], brain [23–25], liver and thyroid [26], ileum [25], vas deferens [27], fat cells [28] and lung [29] and partially prevent the increase of the concentration of cyclic AMP by isoproterenol or glucagon in heart [30] or by adrenaline in uterus [31].

In the hybrid cells, the action of the cholinergic agents is already half maximal at the very low concentration of 10 pM. On the other hand, the concentration of acetylcholine causing 50% inhibition of the effect of prostaglandin E_1 is 0.1 μM. This discrepancy is the second aspect of these data. It may be due to the elevation of the level of cyclic AMP in the presence of prostaglandin E_1. Work is under way to elucidate this phenomenon.

The third aspect is that at concentrations above 0.1 to 1 nM the elevation of the level of cyclic GMP by the cholinergic agonists becomes progressively smaller. One explanation could be that in the cell surface there exist 2 types of receptors with markedly different binding constants for cholinergic agonists. If a ligand bound to the high affinity receptor, an activation of guanylate cyclase would ensue; if it occupied the low affinity receptor, activation of adenylate cyclase, an increase in the level of cyclic AMP and a decrease in the rate of formation of cyclic GMP would be the consequence. Another, perhaps simpler explanation is derived from the observation that high doses of acetylcholine desensitize the membranes of the hybrid cells [2]. Earlier studies of the desensitization phenomenon in other systems led to the conclusion that the nicotinic acetylcholine receptor can exist in 2 different states [32]. Binding of an agonist by the 'active' receptor causes a depolarization of the membrane, while binding to the 'desensitized' does not. Thus, in the hybrid cells the stimulation of the formation of cyclic GMP by low concentrations of the cholinergic agonists may be mediated by the 'active' form of the receptor. Increasing concentrations of the agonists will allow more and more receptors to switch to the 'desensitized' form [32], and thus, with the decrease in the population of 'active' receptors also the rate of formation of cyclic GMP drops. Ligand binding studies using membrane fragments from the electric organ of Torpedo [33] or from intestinal smooth muscle of guinea pig [34] support the view of the existence of the receptor in 2 different states. Also in other systems biological responses (including the elevation of the level of cyclic GMP) that occur at low concentrations of cholinergic agonists are not observed at high concentrations [28,35].

Another, the fourth aspect of this work is the transient response inspite of the continuous presence of the cholinergic agonist. This unexplained effect, which has also been observed in other systems [20, 22,23,28,36] is the object of further studies in our laboratory.

The fifth aspect is related to the previous one and deals with the increase of the level of cyclic AMP, at high concentrations of the agonists (cf. fig.2B). In the hypothesis just mentioned this would mean that occupation of the 'desensitized' form of the receptor is followed by enhanced adenylate cyclase activity. Thus, the striking inverse relationship in the levels of cyclic AMP and cyclic GMP would find a partial explanation. In those cases, in which the level of cyclic GMP is depressed below baseline while that of cyclic AMP rises well above the controls, an additional mechanism must be at work. Probably the elevated level of cyclic AMP either causes an enhanced rate of degradation or a decreased rate of synthesis of cyclic GMP. Qualitatively analogous to the responses evoked by cholinergic agonists were those caused by opiates: elevation of cyclic GMP at low, of cyclic AMP levels at high concentrations and inverse changes of the levels of the 2 cyclic nucleotides [11].

The elevation of the level of cyclic AMP at high agonist concentrations is specially pronounced if the Na^+ concentration is low. This takes us to the sixth aspect, the influence of Na^+ ions. At low concentrations of both Na^+ and agonist, the elevation of cyclic GMP is less than in the presence of high Na^+ concentration. It appears as if at the low concentration of Na^+ the 'desensitized' state of the cholinergic receptor was more populated than at high Na^+ concentration. Na^+ enhances the binding of angiotensin to its receptor [36]. It also plays an important role in the

action of opiates, although in comparison to the cholinergic agents the situation appears to be reversed. At low Na^+ concentrations the binding of opiate agonists to their receptors is stronger than that of antagonists. At high Na^+ concentrations the reverse holds [37]. In agreement with this is the observation that in the hybrid cells the concentration of opiates causing a half maximal increase in the level of cyclic GMP is much lower at low than at high Na^+ concentrations [20]. Other explanations for the effect of Na^+ on the hybrids must also be considered. E.g., high Na^+ concentration could increase the constant for the binding of cholinergic agonists to their receptor or could enhance the activity of the guanylate cyclase. The report that cholinergic agonists, in homogenates of neuroblastoma cells, activate adenylate cyclase [38] seems to contradict our findings [7]. However, the discrepancy may be resolved in the light of the effects of Na^+ reported here, for the adenylate cyclase activities had been measured in the absence of or at very low concentrations of Na^+ [38].

The seventh aspect of this communication is the difference in the action of the 3 cholinergic agents used. The highest elevation of the level of cyclic GMP was brought about by Carb, which stimulates both muscarinic and nicotinic receptors [18] and by TMA that is considered a nicotinic agonist [18]; a comparatively small effect was seen in the presence of the muscarinic agonist Pil [18]. At low Na^+ the concentration dependency of the action of Pil was running inversely to that of the 2 other agonists. At present we do not have an explanation for this phenomenon. Thus, on the basis of these results a classification of the cholinergic receptor(s) of the hybrid cells as nicotinic or muscarinic is not feasible. It is interesting to note that in depressing the effect of prostaglandin E_1 the action of cholinergic agents was clearly mediated by a receptor sensitive only to the muscarinic antagonist atropine, but not to nicotinic antagonists [7]. A detailed study of the effects of cholinergic antagonists is in progress.

The eighth aspect is the extremely similar behaviour of all cell lines tested. Thus, besides the difference in their make-up of neurotransmitter enzymes, the cell lines, including the hybrids, have many properties in common. One among these properties is the increase in the level of cyclic AMP in the presence of prostaglandin E_1 [15].

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