MEASUREMENTS OF ADENOSINE 3':5'-CYCLIC MONOPHOSPHATE AND MEMBRANE POTENTIAL IN NEUROBLASTOMA × GLIOMA HYBRID CELLS: OPIATES AND ADRENERGIC AGONISTS CAUSE EFFECTS OPPOSITE TO THOSE OF PROSTAGLANDIN E_1

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1. Introduction

Hybrid cells obtained by fusion of rat glioma and mouse neuroblastoma cells display many properties characteristic of differentiated neurons [1,2]. On treatment with N^6-2'-*O*-dibutyryl adenosine-3':5'-cyclic monophosphate (dibutyryl cyclic AMP) they extend long processes [1–4]. In containing choline acetyltransferase of high specific activity [1,2,5,6] and dopamine-β-hydroxylase [3] they have marker enzymes of both cholinergic and adrenergic nerves. They fire action potentials on electrical stimulation or when acetylcholine is applied to their plasma membrane. The response to acetylcholine can be blocked by the cholinergic antagonists d-tubocurarine, atropin or α-bungarotoxin [1,2]. When exposed to prostaglandin E_1, neuroblastoma [7,8] and hybrid cells [9] increase their intracellular concentration of cyclic AMP. In both cell types this effect can be antagonized by morphine [10]. The basal level of cyclic AMP in unstimulated cells or the elevated level in cells exposed to a phosphodiesterase inhibitor is lowered in the presence of noradrenaline (NA) [9].

In extension of this work we now report that in the hybrid cells NA at low concentrations also prevents the increase of intracellular levels of cyclic AMP evoked by PGE_1. In doing so it is a more potent agent than dopamine, phenylephrine and isoproterenol (order of decreasing potency). The adrenergic α-blocker phentolamine antagonizes the action of NA more effectively than the β-blocker propranolol. The data suggest the adrenergic receptor to be classified as α-receptor [11]. Also opiates, in a highly stereospecific way, and cholinergic agonists [12] antagonize the action of PGE_1 on the hybrid cells. In view of the membrane depolarizing action of acetylcholine and of the comparable behavior of NA, morphine and acetylcholine in antagonizing the action of PGE_1 on the levels of cyclic AMP, the effect of morphine, NA and PGE_1 on the membrane potential of the hybrid cells was tested. As expected, morphine and NA (like acetylcholine) depolarize, whereas PGE_1 hyperpolarizes the plasma membrane.

2. Methods

2.1. *Chemicals*

L-noradrenaline hydrochloride and D, L-propranolol hydrochloride were purchased from Sigma, dopamine·HCl and D, L-isoproterenol·HCl from Serva, L-phenylephrine·HCl from C. Roth and morphine·HCl from Boehringer, Ingelheim. Phentolamine·HCl was a gift from Ciba-Geigy, haloperidol from Janssen Pharmaceutica, levorphanol and dextrorphan from Hoffmann-La Roche and Professor A. Herz, isobutylmethylxanthine from Searle and Co. and prostaglandins from Dr J. Pike, The Upjohn Co.

2.2. *Cell culture and experimental incubation*

Line N4TG3 is a 6-thioguanine resistent mutant [6] of line N4 [13] derived from mouse neuroblastoma C-1300. 108CC5 and 108CC15 are clonal mouse neuroblastoma × rat glioma hybrid lines [1,2]. Clone 108CC25 was obtained after fusion of 108CC5 cells with each other [14]. The cells were cultured in

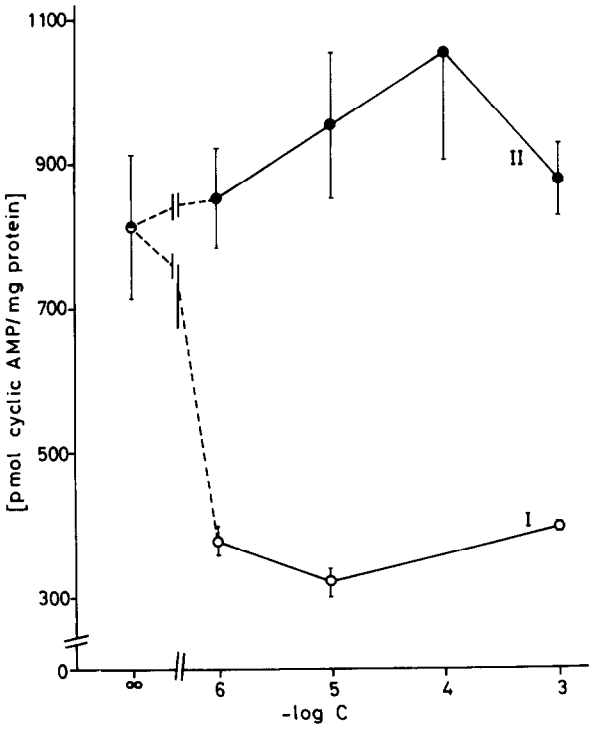

Fig.1. Formation of cyclic AMP in the presence of 3 μM PGE_1 and varying concentrations of levorphanol (I) or dextrorphan (II). The control values in the absence of PGE_1 are between 9.6 and 13.1 pmol cyclic AMP/mg protein. Replica plates; 1.6 × 10^6 108CC15 cells per plate; viability 90%; passage number 18.

plastic petri dishes 8.5 cm in diameter (Greiner, Nürtingen) [9].

Experimental incubations in the presence of various drugs were for 10 min at 37°C as described [15]. Isolation and quantitation of cyclic AMP were done as reported [15]. Cells of clones 108CC5, 108CC15 and 108CC25 were stimulated to extend processes (differentiated hybrid cells) by culturing in medium containing 1 mM dibutyryl cyclic AMP [1–3].

2.3. *Electrophysiology*

Intracellular recording from and stimulation of single differentiated cells was via glass electrodes filled with 3 M KCl. Drugs were applied to the cells in one of two ways. They were either iontophoretically released from micropipettes to the surface of the cells, or they were added as concentrated solutions to the surface of the medium, from were they had to diffuse down to the impaled cell. Details will be given elsewhere [17].

3. Results and discussion

When hybrid cells 108CC15 are exposed to PGE_1, the intracellular level of cyclic AMP increases strongly. The effect is antagonized by levorphanol (fig.1, curve I), a congener of morphine, but not by dextrorphan, the biologically inactive enantiomer of levorphanol (fig.1, curve II). This result demonstrates the stereospecificity

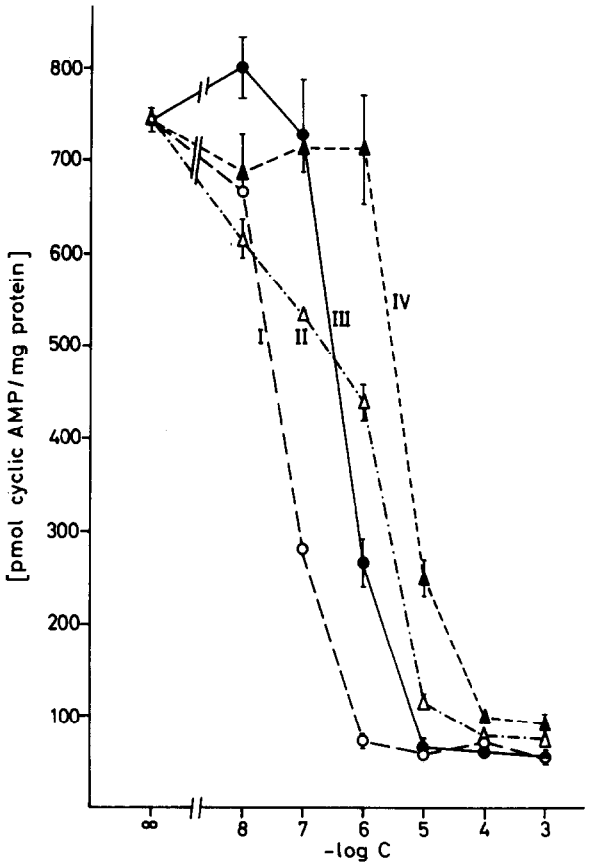

Fig.2. Formation of cyclic AMP in the presence of 3 μM PGE_1 and varying concentrations of NA (I), phenylephrine (II), dopamine (III) and isoproterenol (IV). The control values in the absence of PGE_1 are between 5.5 and 8.6 pmol cyclic AMP/mg protein. Replica plates; 2 × 10^6 108CC15 cells per plate; viability 91%; passage number 6.

Table 1
Effect of adrenergic agonists and antagonists on the intracelullar levels of cyclic AMP (pmol/mg protein) in hybrid cell line 108CC15 stimulated by PGE_1.

Antagonist	Concentration (μM)	Agonist: None	L-NA (10 μM)	Dopamine (10 μM)	D,L-isoproterenol (20 μM)
None	–	1210 ± 70	151 ± 35	228 ± 24	488 ± 40
Phentolamine	10	–	543 ± 46	1040 ± 70	1040 ± 80
	100	1350 ± 30	957 ± 61	1040 ± 90	1040 ± 70
Haloperidol	10	1440	165 ± 12	395 ± 27	665 ± 46
	100	–	340 ± 40	765 ± 35	1290 ± 20
Propranolol	10	–	176 ± 18	298 ± 32	817 ± 73
	100	1630 ± 24	237 ± 33	643 ± 133	1230 ± 100

Data (± standard deviations) are mean values obtained with 3 plates. All incubations were in the presence of 3 μM PGE_1 and combinations of agonists and antagonists. 1.5×10^6 viable cells per plate; viability 98%; passage number 10. The control values (pmol cyclic AMP/mg protein) found in the absence of PGE_1 are: no additions 12; 10 μM NA 13; 10 μM dopamine 22; 200 μM D,L-isoproterenol 19; 100 μM phentolamine 17; 100 μM haloperidol 12; 200 μM D,L-propranolol 11.

Table 2
Effects of PGE_1, morphine, NA and IBMX and some combinations of these compounds on the intracellular levels of cyclic AMP (pmol/mg protein) in neuroblastoma and hybrid cells. The values given without standard deviations were obtained from incubation of single plates. In the cases in which 2 concentrations are given, they refer to the first and second compound, respectively, that had been added to the incubation medium.

Addition	Concentration (μM)	Cell line: N4TG3	108CC15	108CC25
None	–	6	10	16
PGE_1	3	122	811 ± 109	116 ± 16
NA	100	5	14 ± 0	9 ± 3
IBMX	500	–	41	42
Morphine	10	–	–	15
Morphine	100	–	–	8
PGE_1 + NA	3100	45	120 ± 7	45 ± 1
PGE_1 + morphine	3100	–	–	90
PGE_1 + morphine	3100	–	–	106
PGE_1 + IBMX	3500	–	–	1233
NA + IBMX	100 500	–	–	24
Viable cells per plate $\times 10^{-6}$		8	1.6	0.8
Viability %		–	90	94
Protein per plate (mg)		7	3.1	1.8

of the opiate action on the hybrid cells and is in agreement with similar findings on PGE_1-stimulated adenyl cyclase [18], with stereospecific opiate binding in brain homogenates [19–21], synaptic plasma membranes from brain [22], intact neuroblastoma cells [23] and homogenates of neuroblastoma × glioma hybrid cells [24].

If in an experiment analogous to the one discussed the opiate is substituted by NA, qualitatively the same phenomenon is observed. The increase in the intracellular concentration of cyclic AMP due to the activity of PGE_1 is strongly inhibited (fig.2). The susceptibility of the cells to aromatic amines decreases in the sequence NA, (curve I, concentration at which 50% inhibition occurs, IC50:0.05 μM) dopamine (curve III, IC50:0.5 μM), isoproterenol (curve IV, IC50:5 μM). The response to phenylephrine (curve II, IC50:1.2 μM) appears to be biphasic. The fact that the sensitivity of the cells to NA is much higher than that to isoproterenol, suggests the presence of adrenergic α-receptors rather than β-receptors. This view is supported by the results shown in table 1. The responses to NA, dopamine or isoproterenol are much more strongly inhibited by the adrenergic α-blocker phentolamine than by the dopamine antagonist haloperidol or the β-blocker propranolol. Interestingly, propranolol and haloperidol are more powerful in blocking the actions of dopamine or isoproterenol than of NA.

NA antagonizes the effect of PGE_1 also in the neuroblastoma line N4TG3 and the hybrid line 108CC25 (table 2). The latter cells are only slightly sensitive to morphine. The phosphodiesterase inhibitor IBMX elevates the level of cyclic AMP in line 108CC25. The effect is partially inhibited by NA. A similar phenomenon had been observed with lines 108CC5 and 108CC15 [9]. This result indicates that the depression of accumulation of cyclic AMP in the presence of NA is not due to an increase in phosphodiesterase activity, but rather to reduced adenylate cyclase activity. Since morphine, acetylcholine and NA stimulate the levels of intracellular guanosine 3′:5′-cyclic monophosphate (cyclic GMP) in neuroblastoma and hybrid cells [25], our working hypothesis is that the depression of formation of cyclic AMP by these compounds is mediated by cyclic GMP.

The hybrid cells stimulated by PGE_1 to high levels of cyclic AMP can be screened for hormone receptors controlling guanylate cyclase activity. Usually the ratio of the level of cyclic nucleotide in stimulated to that in unstimulated cells is higher for cyclic AMP than for cyclic GMP. Therefore, the depression of cellular cyclic AMP concentrations, in the presence of hormonally elevated levels of cyclic GMP, provides a more sensitive assay for the action of these hormones than measuring cyclic GMP levels directly. For assessing extracellular influences mediated by intracellular cyclic GMP this approach could be useful with other cells, too, provided there are hormones that cause a strong increase in the levels of cyclic AMP. Our previous findings [9] and the present communication are the first reports of a cyclic AMP depressing action of NA or related compounds on neuroblastoma and their hybrid cells. Previous workers had found no influence of 0.1 mM isoproterenol or dopamine on levels of cyclic AMP in mouse neuroblastoma cells [26], or dopamine was observed to stimulate (rather than inhibit) adenylate cyclase in homogenates of mouse neuroblastoma cells more effectively than NA [27]. These differences in the results on neuroblastoma cells might be due to the use of different clones, albeit of the same tumor. A relationship between PGE_1 (causing increase of cellular cyclic AMP) and NA (causing increase of cyclic GMP and decrease of cyclic AMP) that is completely analogous to the one reported here, has been observed in human platelets [28]. In the ductus deferens of the rat, NA slightly increases the level of cyclic GMP. However, in contrast to our findings, NA does not lower the level of cyclic AMP [29]. In rat pituitary glands the accumulation of cyclic AMP evoked by PGE_2 is depressed in the presence of somatostatin [30]. This suggests that the action of somatostatin is mediated by cyclic GMP. There are a number of examples in which prostaglandins behave as antagonists of NA or other hormones, although with exchanged roles. The elevation of intracellular cyclic AMP by NA or other hormones is inhibited by PGE_1 [31–35]. An antagonism between PGE and NA at synapses has been found in vas deferens [36,37] and at cerebellar Purkinje cells [38]. At the present it is not clear whether the systems are comparable at all [39] and to what extent our findings are related to the ones obtained with these two tissues.

In order to assess the effects on membrane potentials of the compounds influencing levels of

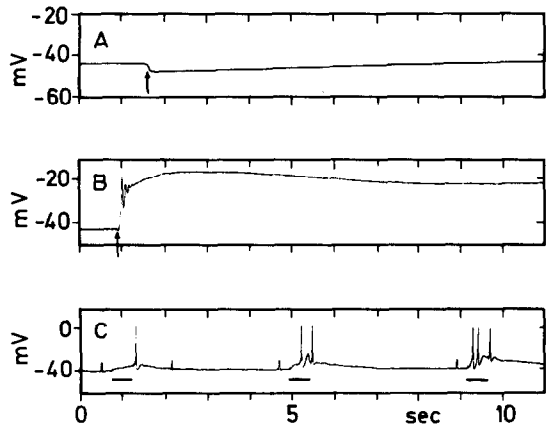

Fig.3. Influence of PGE_1, morphine and NA on the membrane potential of hybrid cells 108CC15 treated with dibutyryl cyclic AMP. A) Influence of PGE_1. At the time indicated (arrow) 2 μl of 1 mM PGE_1 were added to the surface of the medium above the cell. 5 cells were tested, yielding the same result. B) Influence of morphine. Same cells as in A. 2 μl 10 mM morphine was added (arrow). 31 cells were tested with identical results. C) Influence of NA applied iontophoretically from micropipettes filled with 0.1 M NA·HCl in 0.1 M HCl. Iontophoretic application of 0.1 M HCl or NaCl was without effect. A total of 11 cells was tested, showing the same result.

cyclic AMP, hybrid cells treated with dibutyryl cyclic AMP were impaled with a microelectrode. Addition of a solution of PGE_1 to the medium above the cell evokes a slow and transient hyperpolarization of maximally 10 mV (fig.3A), which lasts about 10 sec. Sometimes it is followed by a less pronounced slow depolarization of 1–3 mV. Also $PGF_{1\alpha}$, but not PGA_1, causes a slight hyperpolarization. On the other hand, addition of morphine is followed by pronounced depolarization (fig.3B), which lasts up to several minutes. The high rate of depolarization allows the development of an action potential followed by oscillation of the membrane potential. After the membrane potential has returned to its original value, the cells do no longer fire action potentials on electrical stimulation, in contrast to their behavior before the addition of morphine. However, if the plasma membrane is hyperpolarized electrically prior to electrical stimulation, the cells fire action potentials again. Membrane depolarizations by catecholamines of hybrid cells 108CC15 were also observed by others [40].

A similar depolarizing effect was caused when NA was pipetted to the medium above the cell tested. Iontophoretic application of NA resulted in a comparatively fast depolarization, which was rapid enough to evoke action potentials (fig.3C). Similar effects were obtained with dopamine.

In different preparations of nerve, muscle or brown fat cells PGE_1 is known to depolarize [38,41,42], hyperpolarize [43], first hyper- then depolarize [44], or not influence resting membrane potentials [45]. In two of the systems, NA and PGE_1 antagonize each other [38–43], one [43] showing responses to PGE_1 and NA like our hybrid cells do. Morphine excited or inhibited various nerve cells in the brain stem [46], but showed only an excitatory effect on Renshaw cells [47]. An antagonism between PGE_1 and morphine in the action on small intestine is well documented. The contraction stimulated by PGE_1 is inhibited by morphine [48,49].

The present investigation suggests a relationship between the effects of neurohormones on cellular levels of cyclic nucleotides and on membrane potential, i.e. ion permeability of the plasma membrane. PGE_1 causes hyperpolarization and an increase of the level of cyclic AMP; acetylcholine [12], NA and morphine evoke depolarizations and depression of elevated levels of cyclic AMP, possibly mediated by cyclic GMP [25]. The common action of the latter three agents suggests as a primary event, the increase of the permeability of the plasma membrane for the same ion. Work is in progress to elucidate this aspect.

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