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Effect of Pharmacological Agents on Human Keratinocyte Mitosis *in Vitro*

I. Inhibition by Adenine Nucleotides (38241)

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(Introduced by Frederick Urbach)

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In the last few years many studies have suggested that cyclic adenosine 3′:5′ monophosphate (cyclic AMP) may be specifically involved in regulation of proliferation and differentiation of many types of mammalian cells *in vitro* (1–4). In organ cultures of mouse skin, the reduction of epidermal mitosis by epinephrine is associated with an increase in adenyl cyclase activity (5, 6). This finding supports results in other *in vitro* systems showing that increased cyclic AMP levels are inhibitory to cellular proliferation (1, 4). Recently, Voorhees *et al.* (7), showed that dibutyryl cyclic AMP, but not 5′-AMP, inhibited epidermal cell division in mouse skin in organ culture. Using HeLa and L cells, Ryan and Heidrick (1) reported inhibition of cell growth by cyclic AMP but not by 5′-AMP or adenosine. Others who have investigated the role of cyclic AMP in cellular function either found no effect of 5′-AMP, ADP and ATP, or found that these nucleotides are toxic to cells (1, 2, 8). Thus, specificity of cyclic AMP has been implied. However, we report here a study, using primary cell cultures of normal human epidermal keratinocytes, which indicates that nucleotides other than cyclic AMP may affect a specific physiological function, namely mitosis, in the absence of observable toxic effects.

Materials and Methods. Normal human abdominal skin was obtained during surgery and cell cultures of epidermal keratinocytes were made from skin explants on glass coverslips according to a method described elsewhere (9). Each explant gives rise to an outgrowing sheet of epithelial cells whose origin from epidermal keratinocytes has previously been determined (9, 10). Four to five explants were placed on each coverslip. Cultures were immersed in Eagle's minimum essential medium supplemented with 10% fetal calf serum and 100 U/ml each of penicillin, streptomycin and mycostatin. Cultures were incubated at 37° in a high humidity incubator in a mixture of 5% CO_2 in air.

Cultures 7–9 days old were used for all experiments. In each experiment the test compound was dissolved in culture fluid and then an appropriate aliquot was added to each test dish in order to give the desired final concentration. Each nucleotide was of highest purity and purchased from Sigma Chemical Co. To examine the effect of each compound on mitosis, the metaphase arrest agent, colcemid (2 μg/ml), was added along with the test compound. After 4 hr the cultures were fixed in 10% buffered formalin and the explants removed, leaving the outgrowths on the coverslip. Outgrowths were stained in acid hematoxylin. Controls containing only colcemid were run with each experiment. The entire number of arrested metaphases and the total number of cells in each outgrowth were determined by a method described by Chopra (11) and expressed as the mitotic index (MI). For each dish, the MI was obtained for each outgrowth and then the mean MI and standard deviation were calculated. The data in

TABLE I. Inhibition of Human Keratinocyte Mitosis *in Vitro* by Adenine Nucleotides.

Nucleotide	Conc (*M*)	MI ± SD*		Percent inhibition
		Control	Test	
Cyclic AMP	1×10^{-2}	17.8 ± 2.2	6.9 ± 1.4 ($P < 0.001$)	61%
	1×10^{-3}	14.5 ± 2.2	8.0 ± 1.7 ($P < 0.001$)	45%
	1×10^{-4}	14.5 ± 2.2	9.3 ± 3.4 ($P < 0.025$)	36%
	1×10^{-5}	23.5 ± 1.8	25.7 ± 2.9	0% (+11%)
Dibutyryl cyclic AMP	1×10^{-3}	24.7 ± 3.0	11.0 ± 2.8 ($P < 0.001$)	55%
	1×10^{-4}	14.5 ± 2.2	7.3 ± 2.1 ($P < 0.001$)	50%
	1×10^{-5}	14.5 ± 2.2	9.1 ± 3.3 ($P < 0.025$)	37%
5′-AMP	1×10^{-3}	24.7 ± 3.0	10.2 ± 2.9 ($P < 0.001$)	59%
	1×10^{-4}	17.8 ± 2.2	12.1 ± 3.1 ($P < 0.025$)	31%
	1×10^{-5}	17.8 ± 2.2	19.1 ± 3.2	0% (+7%)
ADP	1×10^{-3}	19.9 ± 4.7	4.1 ± 1.1 ($P < 0.001$)	79%
	1×10^{-4}	20.2 ± 5.8	10.1 ± 2.1 ($P < 0.001$)	50%
	1×10^{-5}	17.8 ± 2.2	15.1 ± 3.3 ($P > 0.100$)	15%
ATP	1×10^{-3}	24.7 ± 3.0	10.3 ± 1.1 ($P < 0.001$)	58%
	1×10^{-4}	24.7 ± 3.0	10.8 ± 2.5 ($P < 0.001$)	56%
	1×10^{-5}	24.7 ± 3.0	20.8 ± 2.5 ($P > 0.250$)	16%

* Values with $P < 0.050$ are statistically significant.

Tables I–III represent one experiment for each concentration listed. However, for each compound tested at the various concentrations, two or more experiments were carried out using skin obtained from different patients. While control values differed from experiment to experiment, comparable results (% inhibition) were obtained in every case. Statistical evaluation was carried out using Student's paired *t* test. Statistical significance was considered to be present at the 5% level or less.

Theophylline was not used in any of the experiments for two reasons. First, we were able to obtain significant inhibition without the compound. Second, it has been shown by a number of investigators that theophylline and other methylxanthines are not specific in their action (12–14).

Results. The data in Table I clearly shows that mitosis in human keratinocytes *in vitro* can be inhibited not only by cyclic AMP and its dibutyryl derivative, but also by ATP, ADP, and 5′-AMP. The rapid effect (within 4 hr) indicates the mechanism to be via a block in the G_2 phase of the cell cycle (15), which has been shown to have a value of about 7 hr in this system (16). Strong inhibition was exhibited by all the adenine nucleotides at 1×10^{-3} *M* with ADP being the best mitotic inhibitor. At 1×10^{-4} *M*, the nucleotides still showed inhibition with ATP exhibiting the strongest effect. Dibutyryl cyclic AMP was the only

TABLE II. Effect of Nonadenine Nucleotides on Human Keratinocyte Mitosis *in Vitro*.

Nucleotide	Conc (M)	MI ± SD*		Percent inhibition
		Control	Test	
5′-GMP	1×10^{-3}	23.2 ± 1.8	20.1 ± 1.7 ($P < 0.025$)	13%
Cyclic GMP	1×10^{-3}	23.2 ± 1.8	24.2 ± 4.0	0% (+4%)
Dibutyryl cyclic GMP	1×10^{-3}	21.8 ± 4.0	19.2 ± 2.7 ($P > 0.100$)	12%
GDP	1×10^{-3}	23.2 ± 1.8	26.8 ± 2.2	0% (+16%)
GTP	1×10^{-3}	17.8 ± 2.2	10.2 ± 1.4 ($P < 0.001$)	43%
	1×10^{-4}	17.8 ± 2.2	13.4 ± 3.5 ($P > 0.050$)	25%
5′-CMP	1×10^{-3}	18.7 ± 1.3	16.5 ± 2.7 ($P > 0.100$)	12%
CDP	1×10^{-3}	23.2 ± 1.8	20.7 ± 0.9 ($P > 0.350$)	11%
CTP	1×10^{-3}	20.2 ± 5.8	10.3 ± 3.3 ($P < 0.001$)	49%
	1×10^{-4}	20.2 ± 5.8	18.5 ± 2.0 ($P > 0.350$)	9%
5′-TMP	1×10^{-3}	23.7 ± 4.1	24.0 ± 4.9	0%
TTP	1×10^{-3}	20.2 ± 5.8	20.0 ± 6.9	0%
5′-UMP	1×10^{-3}	23.7 ± 4.1	23.4 ± 2.4	0%
UTP	1×10^{-3}	17.8 ± 2.2	12.7 ± 2.2 ($P < 0.025$)	29%
	1×10^{-4}	17.8 ± 2.2	17.4 ± 2.2	0%

* Values with $P < 0.050$ are statistically significant.

adenine nucleotide to show inhibition at 1×10^{-5} M. Sodium phosphate and sodium butyrate were tested at 1×10^{-3} M with no mitotic inhibition seen.

Nucleotides other than adenine nucleotides were tested for possible antimitotic activity (Table II). Only GTP, CTP, and possibly UTP at 1×10^{-3} M exhibited mitotic inhibition. At 1×10^{-4} M, these nucleotides had little effect.

In Table III, effect of nucleotide metabolites on human keratinocyte mitosis is shown. Only adenosine at 1×10^{-3} M was found to inhibit mitosis to any degree. This effect dropped to around zero at 1×10^{-4} M.

It was possible that the block of mitosis in this *in vitro* cell system might be due to permanent cell damage. To answer this question, keratinocytes were grown in the presence of 1×10^{-4} M dibutyryl cyclic AMP for 3 days. Colcemid was then added to control and test cultures for 4 hr. While strong mitotic inhibition (>50%) was found in the test system, no cell damage was observed based on light microscopic examination. At the same time, duplicate cell cultures were washed and fresh culture fluid was added. The mitotic block was found to be released which resulted in a rise in the MI over that in the controls. At the end of 3 days, the treated cultures were approximately equal to controls in terms of outgrowth size and cell density. 5′-AMP at 1×10^{-3} M was also tested with no toxic effect seen.

Discussion. It is clear from the data presented that the inhibition of normal human keratinocyte mitosis *in vitro* is not a specific effect of the exogenous addition of cyclic AMP. If one compares the cell response to the various nucleotides at 1×10^{-3} M, all the adenine nucleotides as well as GTP, CTP and possibly UTP show strong mitotic inhibition. However, if one now uses a presumably more physiological concentration,

TABLE III. Effect of Nucleotide Metabolites on Human Keratinocyte Mitosis *in Vitro*.

Metabolite	Conc (M)	MI ± SD*		Percent inhibition
		Control	Test	
Adenine	1×10^{-3}	22.2 ± 3.0	23.5 ± 4.4	0% (+6%)
Adenosine	1×10^{-3}	22.2 ± 3.0	11.0 ± 2.3 ($P < 0.001$)	50%
	1×10^{-4}	22.2 ± 3.0	20.6 ± 3.2 ($P > 0.200$)	7%
Guanine	1×10^{-4}	23.2 ± 1.8	24.7 ± 3.7	0% (+6%)
Guanosine	1×10^{-3}	15.0 ± 5.5	11.9 ± 3.1 ($P > 0.050$)	22%
	1×10^{-4}	15.9 ± 5.5	18.0 ± 5.0	0% (+13%)
Hypoxanthine	1×10^{-3}	22.2 ± 3.0	22.5 ± 6.6	0%
Inosine	1×10^{-3}	23.2 ± 1.8	25.8 ± 5.0	0% (+16%)
5′-IMP	1×10^{-3}	17.8 ± 2.2	18.5 ± 4.3	0%
Cytidine	1×10^{-3}	16.5 ± 3.6	12.8 ± 2.4 ($P > 0.050$)	22%
	1×10^{-4}	16.5 ± 3.6	14.6 ± 2.7 ($P > 0.200$)	12%

* Values with $P < 0.050$ are statistically significant.

i.e., 1×10^{-4} M, only the adenine nucleotides retain antimitotic activity with ATP being the strongest inhibitor. Thus, mitotic inhibition in our cell system seems specific for the adenine nucleotides.

The explanation for the mitotic inhibition by these nucleotides is unknown at this time. Inherent in this explanation lies the problem of cell permeability to nucleotides. While dibutyryl cyclic AMP may enter the cell unchanged (20) it is generally believed that naturally occurring nucleotides do not cross the plasma membrane intact, but are first dephosphorylated (17–19). It has been shown by Kaukel and Hilz (20) using HeLa cells, that cyclic AMP is rapidly degraded extracellularly to various metabolites which were taken up by the cells and converted to purine nucleotides. This does not seem to be the case in our cell system since no nucleotide metabolite was capable of producing mitotic inhibition at the same level exhibited by the adenine nucleotides (Table III). The mitotic block in our system by nucleotides other than cyclic AMP may be related to indirect effects that raise intracellular cyclic AMP. Recently, it has been shown by a number of investigators that various nucleotides can activate membrane adenyl cyclase as well as cause an effect on membrane structure (21–24). Moreover, ATP has been found to be formed at the membrane surface of cells in culture (25). Thus it is possible that extracellular ATP could act as the immediate substrate for adenyl cyclase, leading to increased intracellular cyclic AMP and inhibition of mitosis. This explanation would be compatible with the findings in the present study. One cannot overlook, however, the possible effect that the adenine nucelotides may have on cyclic GMP which is an antagonist of cyclic AMP in bidirectionally controlled systems (26). An imbalance in the cyclic GMP—cyclic AMP ratio in favor of the latter (whether due to a rise in cyclic AMP or a fall in cyclic GMP) might induce the mitotic effects seen in our system. This will be determined in future studies designed to measure intracellular cyclic AMP and cyclic GMP following addition of the adenine nucleotides.

The explanation for the mitotic inhibition exhibited by adenosine at 1×10^{-3} M in our cell system may also be related to the activation of membrane adenyl cyclase. Recently, it has been shown by Blume *et al.* (27) that the exogenous addition of adenosine elevates cyclic AMP in cultured mouse neuroblastoma cells.

Regardless of the final explanation, it is clear that with respect to causing a mitotic block in the G_2 part of the cell cycle, nucleotides other than cyclic AMP are highly effective. The apparent specificity of cyclic AMP reported by others measuring different aspects of cell activity must be considered in terms of the particular cell system and response studied. More specifically, the effect of nucleotides has been studied on many different parameters including cell morphology (2), cell-substratum adhesion (28), doubling time (3), cell movement (29), and mitotic blockade (7). Thus, analysis of differences between experimental systems is difficult. Except for the work of Voorhees (7), most investigators have not specifically examined the effect of adenine nucleotides on events within the cell cycle. Moreover, different *in vitro* systems have been used by different investigators. In particular, most work has been performed on so-called "normal" as well as transformed cells that have been established in culture for long periods of time. According to the parameters being measured, the failure to note a response to nucleotides other than cyclic AMP may be related to changing cell properties, especially those concerning the plasma membrane. Our system employed primary cell cultures whose existence *in vitro* was of relatively short duration (<2 weeks). Observations made in such a system may confer certain advantages over long term cultures for studying the adenyl cyclase system.

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