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2-Aminopurine inhibits RNA and protein synthesis and reduces catecholamine desensitization in C6-2B rat glioma cells

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We previously proposed that intracellular cyclic AMP accumulation induces a putative, rapidly turning over protein inhibitory to further hormone activation of adenylate cyclase. In the present study, 2-aminopurine, which has been reported to selectively block c-*fos* gene expression, was used to test the hypothesis that c-fos protein might be involved in the desensitization process. Indeed, a reduction in heterologous desensitization to catecholamines was observed in 2-aminopurine-treated C6-2B rat glioma cells. However, we found 2-aminopurine to inhibit, in a concentration-dependent manner, total cellular RNA and protein synthesis in C6-2B, HeLa, Swiss 3T3 and BALB/c cells. mRNA synthesis was also markedly reduced in 2-aminopurine-treated cells. These unexpected findings, while supporting our hypothesis of a protein synthesis-sensitive step in the development of refractoriness, raise concern about the specificity of action of 2-aminopurine to inhibit c-*fos* induction and thus any cellular process, including desensitization, which might be regulated by c-*fos* gene expression.

c-*fos*; Cell biosynthesis; Refractoriness

1. INTRODUCTION

Our laboratory has previously shown in several cell systems that inhibitors of RNA and protein synthesis, such as actinomycin D, cycloheximide and diphtheria toxin, reduce or reverse cyclic AMP (cAMP)-mediated heterologous catecholamine desensitization [1–3]. Therefore, our working hypothesis is that stimulation of intracellular cAMP accumulation induces a rapidly turning over protein which inhibits subsequent hormonal responses.

As we previously showed, in rat C6-2B glioma cells, the beta-adrenergic agonist (−)isoproterenol, causes a transient intracellular cAMP accumulation [4], accompanied by an increase in c-*fos* mRNA content, which is also transient and peaks at 30 min [5]. This finding prompted us to investigate the possible role played by the early inducible protein c-fos in the desensitization process. To address this question, we used 2-aminopurine (2-AP), a purine analog which has been reported to selectively block c-*fos* gene expression [6]. 2-AP has also been shown to selectively inhibit the induction of the interferon gene [7], interferon-inducible genes [8], human tumor necrosis factor gene [9], and to enhance the expression of exogenous genes [10]. Previous studies from other laboratories have stated that prolonged treatment with 2-AP at the routinely used concentration of 10 mM did not significantly affect cellular metabolism [7,10]. We have previously shown that 2-AP, at 10 mM concentration, inhibited the increase in c-fos nuclear protein immunoreactivity induced by isoproterenol in C6-2B cells [5]. We now report that 2-AP also partially prevents cAMP-mediated catecholamine desensitization in C6-2B cells. However, in contrast to what was stated in other reports [7,10], 2-AP causes a concentration-dependent inhibition of total RNA, mRNA and protein synthesis, as measured by [^{3}H]uridine and [^{35}S]methionine incorporation into cellular RNA and proteins.

Abbreviations: 2-AP, 2-aminopurine; Bt$_2$cAMP, N^6,$O^{2'}$-dibutyryladenosine 3',5'-cyclic monophosphate; IBMX, 3-isobutyl-1-methylxanthine.

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2. MATERIALS AND METHODS

2.1. *Materials*

2-Aminopurine (A-3509, lots 109F-7703 and 129F-0180), cycloheximide, actinomycin-D, (−)isoproterenol HCl, Bt$_2$ cAMP and IBMX were purchased from Sigma Chemical Co. (St. Louis, MO). Forskolin was from Calbiochem (Behring Diagnostic, La Jolla, CA). [^{35}S]Methionine (>800 Ci/mmol) and [^{3}H]uridine (40 Ci/mmol) were obtained from Amersham Corp. (Arlington Heights, IL). Tissue culture products were from Grand Island Biological Company (Grand Island, NY).

2.2. *Cell culture and cyclic AMP measurement*

Cells were grown as monolayer cultures in Ham's F-10 nutrient medium (C6-2B cells) or Dulbecco's Modified Eagle Medium (HeLa, Swiss 3T3 and BALB/c cells) with 10% calf serum. 2-AP stock solutions (150 mM) were prepared in phosphate-buffered saline (NaCl 137 mM; KCl 2.7 mM; Na_2HPO_4 8 mM; KH_2PO_4 1.4 mM) containing glacial acetic acid (200:1, v/v), as described [7]. All experiments included control cells treated with the vehicle used to prepare 2-AP

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solutions; the final pH of control and 2-AP solutions was adjusted to 7.0–7.2. Upon drug treatment at 37°C in serum free-medium containing the phosphodiesterase inhibitor IBMX (0.1 mM), the intracellular cAMP content was determined as previously described [3], using the Atto-Flo automated radioimmunoassay system [11], (Atto Instruments, Inc., Rockville, MD). Proteins were measured by the Bradford assay [12] using bovine serum albumin as standard.

2.3. *RNA and protein synthesis determination*

C6-2B and HeLa cells were seeded on 24-well clusters at the density of $1-2 \times 10^4$ cells/well and used while 80% confluent, unless otherwise indicated. Swiss 3T3 and BALB/c cells were plated either at 2.5×10^4 cells/well, refed at day 2, and used at day 7, when confluent and quiescent, or at 3.4×10^3 cells/well, refed at day 2, and used 2 days later while exponentially growing [13]. Cells were labeled at 37°C for 60 min with [^{3}H]uridine (RNA synthesis) or 30 min with [^{35}S]methionine (protein synthesis) at 1–2 μCi/ml/well. Precipitation with 10% ice-cold trichloroacetic acid was followed by solubilization of the cells with 1 ml/well of 0.2 M NaOH. 50 μl/well aliquots were tested for protein content by the Bradford assay [12]. RNA and protein synthesis were measured by the incorporation of [^{3}H]uridine and [^{35}S]methionine, respectively (dpm/mg protein per well) and expressed as a percent of control, vehicle-treated cells. In many experiments, inhibition of RNA and protein synthesis by actinomycin-D (10 μM) and cycloheximide (30 μM), respectively, was tested as an internal control and found to be about 95%. Finally, for mRNA isolation, subconfluent C6-2B cells, grown in 150-mm tissue culture plates, were exposed for 3 h to 10 mM 2-AP at 37°C, and given a 60 min [^{3}H]uridine pulse (2 μCi/ml). Control and 2-AP treated cells were then harvested and poly(A)$^+$ RNA extracted by using the Fast Track mRNA isolation kit (Invitrogen Corporation, San Diego, CA) according to the manufacturer's instructions.

3. RESULTS

As shown in Table 1, stimulated cAMP accumulation in C6-2B cells was not appreciably affected by the presence of 10 mM 2-AP when measured after a 30 min exposure to (−)isoproterenol (Iso), yet was 2-fold higher in 2-AP-treated cells after a 3-h Iso exposure. Also, a pretreatment with 2-AP (10 mM) for 3 h did not significantly affect cAMP accumulation induced by a 30-min challenge with 10 μM Iso (Fig. 1). However, after a 3-h Iso treatment and a 30-min washing in the absence of 2-AP, C6-2B cells showed desensitization such that only 11% of the original response to a subsequent re-challenge with 10 μM Iso was observed, as shown in Fig. 1. When the cells were co-incubated for 3 h with 10 mM 2-AP and Iso, a 2–4-fold greater cAMP response to a second challenge was observed (Fig. 1). However, if, following a 3-h co-incubation with 2-AP and Iso, 2-AP was omitted from the washing and rechallenge solutions, cell responsiveness was decreased by 10%, compared to cells which had been washed and re-challenged in the continuous presence of 2-AP, suggesting that the 2-AP effect is reversible (data not shown). Furthermore, 2-AP reduced the desensitization to Iso induced by a 3-h exposure to either the cell-permeable cAMP analogue Bt_2cAMP (1 mM) or forskolin (0.1 mM), a diterpene believed to directly activate the catalytic subunit of adenylate cyclase (Fig. 1).

Table 1

Effect of 2-aminopurine on isoproterenol-stimulated C6-2B cell cAMP content

Time	cAMP, pmol/mg protein	
	Control	2-AP treated
30 min	6817 ± 766	5937 ± 288
3 h	650 ± 40	1351 ± 65

C6-2B cells were incubated with isoproterenol (10 μM) in the presence or absence of 2-AP (10 mM). Basal cell cAMP levels were 20–25 pmol/mg protein. The data are presented as the mean ± SEM of 3 separate experiments done in triplicate.

Treatment with 2-AP severely impaired total RNA and protein synthesis in C6-2B and HeLa cells in a time- and concentration-dependent manner. Time-course experiments of the effect of 10 mM 2-AP on the incorporation of [^{3}H]uridine and [^{35}S]methionine were carried out and the results from a representative experiment are shown in Fig. 2. In both cell lines, the maximal inhibitory effect was reached after a 3-h exposure to 2-AP, although at earlier time points 2-AP inhibited [^{35}S]methionine incorporation more efficaciously in HeLa than in C6-2B cells. A similar time-dependency was observed for the inhibition of [^{3}H]uridine incorporation by 10 mM 2-AP. A treatment with 2-AP, at concentrations ranging from 0.01–50 mM for 3 h, increasingly inhibited both [^{3}H]uridine and [^{35}S]methionine incorporation in both C6-2B and HeLa cells, as shown in Fig. 3. Exposure of the cells to 50 mM 2-AP for 3 h, the time-frame of our experiments, resulted in drastic changes in cell morphology and occasional cell detachment, whereas no apparent cytotoxicity was detected after a 3 h

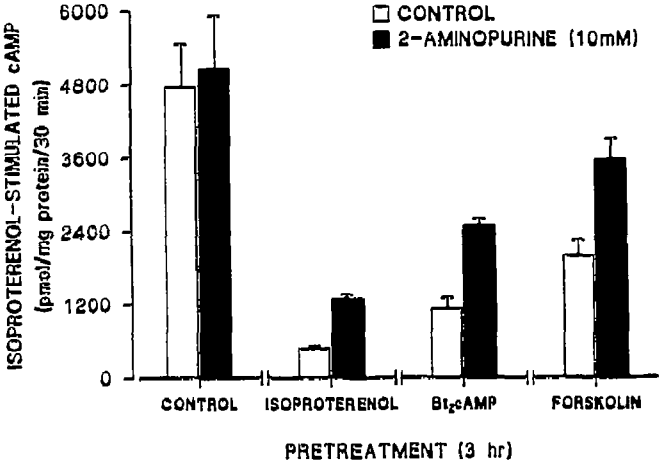

Fig. 1. 2-AP reduces isoproterenol-, Bt_2cAMP- and forskolin-induced refractoriness. C6-2B cells were incubated for 3 h with (solid bar) or without (open bars) 10 mM 2-AP, in control serum-free medium, in medium containing 10 μM (−)isoproterenol, 1 mM Bt_2cAMP or 0.1 mM forskolin. Cells were then washed 5-times with serum-free medium (or 2-AP-containing serum-free medium for cells pretreated with 2-AP) and cAMP was measured after a 30 min challenge with 10 μM (−)isoproterenol in the presence or in the absence of 2-AP. The data represent the mean ± SEM of at least 3 separate experiments.

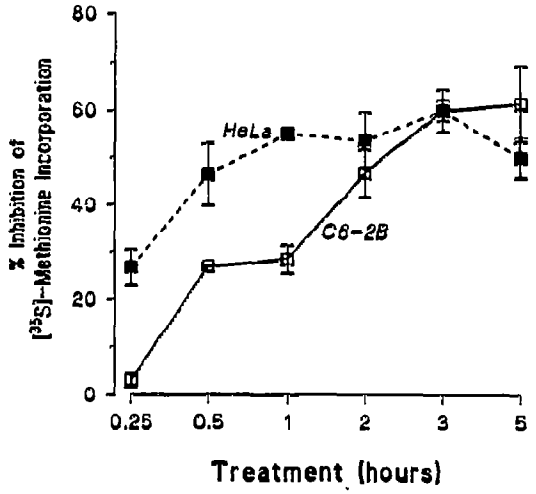

Fig. 2. Time-course of the inhibition of [^{35}S]methionine incorporation by 2-AP in C6-2B and HeLa cells. Cells were incubated in the presence or absence of 10 mM 2-AP for the indicated time points and a 30 min pulse with [^{35}S]methionine was given (15 min for the 0.25 h time point).

exposure to 10 mM 2-AP. The extent of inhibition of both [^{3}H]uridine and [^{35}S]methionine incorporation was slightly greater in HeLa than in C6-2B cells, whereas no significant differences in 2-AP efficacy in inhibiting both [^{3}H]uridine and [^{35}S]methionine were observed between subconfluent and confluent C6-2B or HeLa cultures (Fig. 3B). The inhibitory effect of 2-AP was reversible since washing out 2-AP readily restored the ability of the cells to incorporate both [^{3}H]uridine and [^{35}S]methionine (data not shown). Furthermore, in non-tumor cells, such as Swiss 3T3 and BALB/c mouse fibroblasts, 2-AP also inhibited RNA and protein synthesis in a concentration-dependent manner. However, both Swiss 3T3 and BALB/c cells exposed to 10 mM 2-AP, showed mild changes in cell morphology, although to a much lesser degree than that observed in C6-2B and HeLa cells which had been exposed to 50 mM 2-AP. A 10-fold greater incorporation of [^{3}H]uridine and [^{35}S]methionine (dpm/μg protein) was detected in exponentially growing cultures compared to confluent, quiescent cultures. In both Swiss 3T3 and BALB/c lines, the extent of inhibition of [^{3}H]uridine incorporation by 2-AP was generally greater in exponentially growing cells compared to confluent cells. As shown in Fig. 4A, a rightward shift in the dose–response curve of the inhibition of [^{3}H]uridine incorporation by 2-AP was observed in confluent compared to exponentially growing Swiss

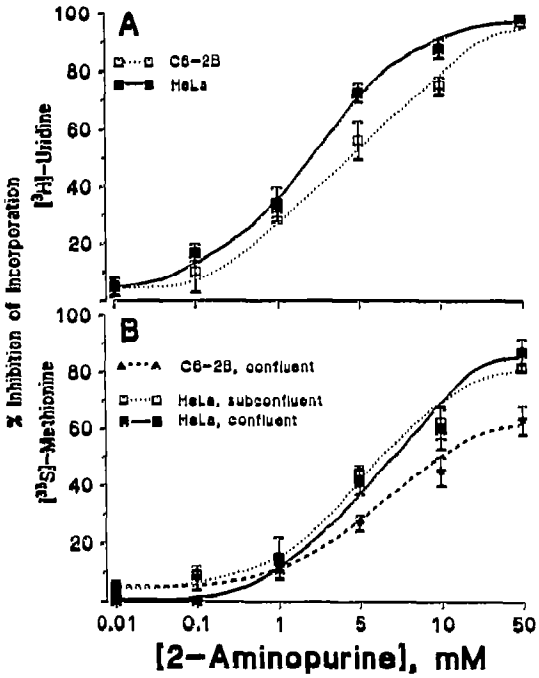

Fig. 3. 2-AP concentration-dependent inhibition of [^{3}H]uridine and [^{35}S]methionine incorporation into C6-2B and HeLa cells. Cultures were exposed to increasing concentrations of 2-AP for 3 h at 37°C and pulse labelling was performed as described in Materials and Methods. Values represent the mean ± SEM of 3 determinations.

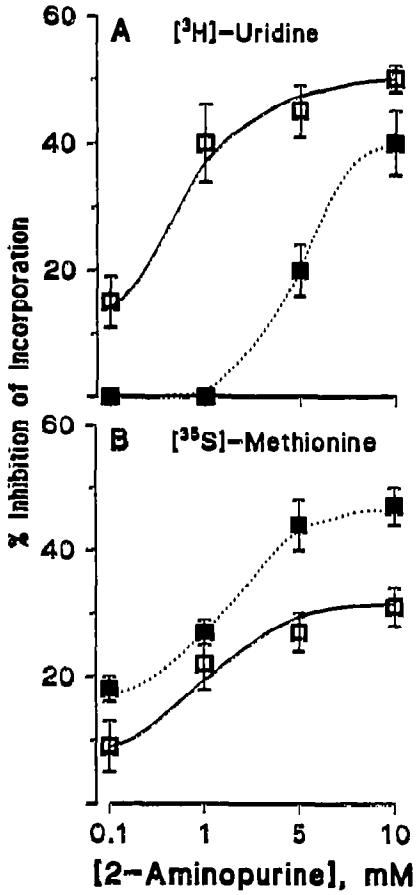

Fig. 4. Concentration-dependent inhibition of [^{3}H]uridine and [^{35}S]methionine incorporation by 2-AP in Swiss 3T3 mouse fibroblasts. Exponentially growing (□) and confluent/quiescent cells (■) were treated with 2-AP at the indicated concentrations, and a 60 min pulse with [^{3}H]uridine (A) or a 30 min pulse with [^{35}S]methionine (B) at 2 μCi/ml/well was given. Total 2-AP treatment time was 3 h; protein content was 10–20 μg (exponentially growing cultures) and 100–120 μg (confluent cultures) per well.

Table II

Effect of 2-aminopurine on [^{3}H]uridine incorporation into C6-2B cell poly(A)$^+$RNA

	Control	2-AP-treated
dpm/μg poly(A)$^+$ RNA	40042 ± 528	10334 ± 1640

Subconfluent C6-2B cells were incubated for 3 h with serum-free medium in the presence or absence (control) of 2-AP (10 mM). [^{3}H]Uridine pulse and RNA extraction were performed as described in Materials and Methods.

3T3 cells. [^{35}S]methionine incorporation was usually inhibited to a similar extent in actively growing and quiescent cultures, although high concentrations (5–10 mM) of 2-AP caused a greater inhibition in confluent than in exponentially growing Swiss 3T3 cells (Fig. 4B). Similar results were obtained with BALB/c fibroblasts.

Finally, 2-AP was found to inhibit mRNA synthesis. As shown in Table II, the specific activity (dpm/μg mRNA) of [^{3}H]uridine-labeled poly(A)$^+$ RNA isolated from 2-AP-treated C6-2B cells was reduced 74% compared to control. poly(A)$^+$ RNA yield was similar in control and 2-AP-treated cells; three 150-mm plates with equal cell density yielded 10.5 (control) and 11.2 (2-AP) μg of poly(A)$^+$ RNA, as determined from the absorption at 260 and 280 nm.

4. DISCUSSION

In this study, 2-AP, a blocker of c-*fos* gene expression [6], was used to investigate the possible role played by the early inducible protein c-fos in the cAMP-mediated desensitization in C6-2B cells. 2-AP did not significantly affect acute cAMP accumulation elicited by a 30-min challenge with isoproterenol, but retarded the decline in cellular cAMP at longer time points. Furthermore, 2-AP partially prevented homologous as well as heterologous desensitization to catecholamines in C6-2B cells. However, a concentration-dependent inhibition of total RNA and protein synthesis was observed in C6-2B cells treated with 2-AP for up to 3 h, the time-frame of our experiments. This unexpected result suggested to us that the reduction in desensitization caused by 2-AP, rather than being mediated by the selective inhibition of c-*fos* gene expression, could be accounted for by the general inhibition of the RNA and protein synthesis, as we proposed earlier [1–3]. The lack of data, at least in the literature we were aware of, describing inhibitory effects of 2-AP on cell metabolism, led us to extend our studies to other cell lines. We chose human carcinoma HeLa cells because this cell line had been used in studies where other investigators had stated that 2-AP was not associated with inhibition of RNA and protein synthesis [7,10]. Indeed, 2-AP inhibited RNA and protein synthesis in HeLa cells with a time–course and a concentration-dependency similar to those observed in C6-2B cells. As 2-AP is a purine analog, one could expect it to be a more potent inhibitor in rapidly dividing than in stationary, resting cultures. No significant differences in 2-AP efficacy were observed between subconfluent and confluent C6-2B and HeLa cultures. However, in C6-2B and HeLa cells, cell confluency does not temporally coincide with arrested growth and quiescence because, due to their tumoral nature, these cell lines are not contact-inhibited. Therefore, to appropriately address the question of whether 2-AP inhibits RNA and protein synthesis more strongly in actively growing rather than quiescent cultures, we extended our studies to Swiss 3T3 and BALB/c mouse fibroblasts. In these cell lines, 2-AP inhibited the incorporation of labelled uridine more efficaciously in exponentially growing than in confluent cultures, whereas the incorporation of labelled methionine was similar in both growth states. Interestingly, 2-AP appears to be more cytotoxic in non-tumoral than in tumoral cells, although the maximal inhibition of RNA and protein synthesis was generally greater in C6-2B and HeLa than in Swiss 3T3 and BALB/c cell lines. To investigate whether these differential effects on tumor vs. non-tumor systems are due to the purine analog properties of 2-AP or to unique mode of action of the drug itself, although interesting, is beyond the scope of this paper.

Finally, we showed that messenger RNA synthesis is also markedly inhibited in 2-AP-treated C6-2B cells. Thus, since 2-AP inhibits mRNA synthesis, its action might be expected to be more demonstrable on short half-life proteins or on genes undergoing active transcription, such as the early inducible gene c-*fos* or the putatively induced gene(s) which we suggested are involved in some aspect of the cAMP-mediated desensitization of hormonal responses. However, since 2-AP blocks general RNA and protein synthesis, a conclusion about the role of c-*fos* in the refractoriness process cannot be drawn from our present results and different studies are needed to properly address this question. Nevertheless, it is clear that the inhibitory activity on RNA and protein synthesis must be taken into account when 2-AP is used to pinpoint a role for c-*fos* in any cellular process. Finally, 2-AP is also a protein kinase inhibitor whose only two in vitro substrates known to date are the heme-regulated kinase and the double-stranded RNA-dependent eukaryotic initiation factor (eIF)-2α kinase [14–16]. Although there is no evidence that 2-AP might be a general inhibitor of cellular kinases, the possibility that 2-AP acts through an as yet unknown phosphorylating protein, whose activity might be required for continuous responsiveness to hormonal stimulation or RNA and protein synthesis, cannot be ruled out.

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