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Beta Adrenergic Regulation of Glycogen Phosphorylase Activity and Adenosine Cyclic 3',5'-Monophosphate Accumulation in Control and Desensitized C-6 Astrocytoma Cells

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SUMMARY

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(−)-Isoproterenol (0.01-10 nM) caused the rapid, complete conversion of glycogen phosphorylase from the *b* to the *a* form in cultured C-6 astrocytoma cells. This was associated with a 60-fold elevation in cellular cyclic 3',5'-AMP content. Both effects were blocked stereoselectively by (−)-propranolol. The *beta* adrenoceptor partial agonists (±)-salbutamol (1 nM-10 μM) and (±)-hydroxybenzylpindolol (0.1 nM-10 μM) also caused full conversion of phosphorylase *b* to *a*, although their maximal effects on cellular cyclic AMP content were only 80% and 17.5%, respectively, of that obtainable with (−)-isoproterenol. Prostaglandin E_1 (PGE_1), which caused only a 1.6-fold elevation in cyclic AMP content, gave rise to 54% conversion of phosphorylase *b* to the *a* form. Prior incubation of cells with 3-isobutyl-1-methylxanthine caused a 1.4-fold elevation of cyclic AMP and converted 36% of phosphorylase *b* to the *a* form. Under those conditions dibutyryl cyclic AMP fully activated phosphorylase *b* to *a*, and the effects of *beta* adrenergic agonists on both cyclic AMP levels and phosphorylase *b* to *a* conversion were enhanced. These results suggest that *beta* adrenoceptor agonists and PGE_1 convert phosphorylase *b* to *a* by a mechanism involving cyclic AMP. However, conversion of phosphorylase *b* to *a* was essentially complete when the cellular cyclic AMP content had risen by only 6% of the maximum amount attainable by *beta* adrenoceptor stimulation. Because of this relationship between cell cyclic AMP content and phosphorylase conversion, cells that had been desensitized to *beta* adrenoceptor stimulation by prior incubation with low concentrations of (−)-isoproterenol still responded to a subsequent addition of (−)-isoproterenol with full phosphorylase *b* to *a* conversion. However, in these cells complete conversion required 5-15-fold higher concentrations of (−)-isoproterenol than control cells. The divalent cation ionophore A23187, which did not produce a measurable rise in cell cyclic AMP levels, activated phosphorylase by 50%. This effect, unlike the action of *beta* adrenergic agonists, was inhibited by lowering the extracellular divalent cation concentration and may therefore occur by a different, cyclic AMP-independent mechanism.

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INTRODUCTION

The majority of cells in the mammalian brain are of glial origin, and the function of these cells is still unclear (1). One type

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of glial cell, the astrocyte, contains the majority of brain glycogen (2) and is thought to play a major role in brain carbohydrate metabolism. The isolation of astrocytoma cell lines in culture has facilitated the study of astrocyte function, and the finding of a *beta* adrenergic receptor controlling cyclic 3′,5′-AMP levels in rat C-6 astrocytoma cells (3–6) has raised the possibility that astrocytes respond to the release of neurotransmitters at adjacent synapses, and thus have a close functional relationship with neuronal cells.

Subsequent work has demonstrated that in C-6 astrocytoma cells high concentrations of norepinephrine can induce the rapid conversion of glycogen phosphorylase from the inactive *b* form to the active *a* form, and that this is associated with the breakdown of cellular glycogen and the release from the cell of radioactive material derived from D-[^{14}C]glucose (7–9).

The relationship between the cellular cyclic AMP content and the percentage of glycogen phosphorylase in the active form has been investigated in detail for liver, and has resulted in the formulation of the cascade theory for cyclic AMP-dependent phosphorylase activation (10). Subsequent observations, however, indicate that in a variety of tissues glycogen phosphorylase can be activated by a mechanism that does not involve either cyclic AMP or a cyclic AMP-dependent protein kinase (11–14). In liver, for example, there is increasing evidence that adrenergic receptor agonists can activate phosphorylase by an *alpha* receptor-linked, cyclic AMP-independent mechanism in addition to a *beta* receptor-linked, cyclic AMP-dependent mechanism (10, 14, 15). Other hormones, such as angiotensin II and vasopressin, also seem to act without altering protein kinase activity (13, 16).

Our work has involved, first, pharmacological characterization of the receptors involved in the activation of glycogen phosphorylase and increase in cyclic AMP content in C-6 astrocytoma cells; second, an investigation into the role of cyclic AMP in controlling the ratio of the two forms of phosphorylase in these cells; and, third, the effect of *beta* adrenergic receptor desensitization on these processes.

MATERIALS AND METHODS

Materials. Dulbecco's modified Eagle's medium, fetal calf serum used in cell culture, and Earle's balanced salt solution (Ca^{++}- and Mg^{++}-free) were obtained from Gibco, Glasgow; Earle's balanced salt solution without sodium bicarbonate, from Flow Laboratories, Bonn; glucose 6-phosphate dehydrogenase (D-glucose-6-phosphate:NADP oxidoreductase, EC 1.1.1.49), phosphoglucomutase (α-D-glucose 1,6-diphosphate:α-D-glucose 1-phosphate phosphotransferase, EC 2.7.5.1), adenosine 5′-monophosphate, and adenosine, from Boehringer/Mannheim; dibutyryladenosine 3′,5′-cyclic monophosphate, (−)-isoproterenol hydrochloride, and serotonin creatinine sulfate, from Sigma, Kingston-upon-Thames, U. K.; dopamine hydrochloride and histamine (base), from Fluka, Buchs, Switzerland; NADP and cyclic AMP, from Serva, Heidelberg; glycogen (for biochemical research), from Merck, Darmstadt; 3-isobutyl-1-methylxanthine, from Aldrich-Europe, Beerse, Belgium; (±)-hydroxybenzylpindolol, from Sandoz, Basel; and prostaglandin E_1, (+)- and (−)-propranolol, and (±)-salbutamol, from Dr. H. Schroeter, Ciba-Geigy, Basel. A23187 (Lilly) was a gift from Dr. R. Neher.

Culture conditions. Rat C-6 astrocytoma cells (American Type Culture Collection) were grown at 37° in monolayer culture under an atmosphere of 5% CO_2 in air in 10 ml of Dulbecco's modified Eagle's medium supplemented with 5% fetal calf serum, 100 units/liter of penicillin, and 100 μg/liter of streptomycin. The cells were seeded at 10^5/ml in 100-mm plastic tissue culture plates (Corning) and grew to confluence in 4–5 days. Twenty hours prior to an experiment, the confluent cells received a change of medium. For subculturing, the medium was replaced with 5 ml of 0.02% (w/v) EDTA for 2 min at room temperature. This was aspirated, and the plates were returned to the incubator for 2 min at 37°. The cells were then suspended in culture medium.

Cell incubations. In experiments at room temperature (22–23°) plates were removed from the incubator and the medium was replaced with 5 ml of Earle's balanced salt solution (pH 7.0) buffered with diso-

dium hydrogen phosphate (3.3 mM) in place of sodium bicarbonate. The cells were then incubated for 1 hr at room temperature in the presence or absence of IBMX,[2] as indicated, before addition of test substances. Unless otherwise indicated, drugs or vehicle (0.05 ml) were incubated with the cells for 2 min. At the end of the incubation the medium was removed by aspiration, and 5 ml of ice-cold buffer [10 mM glycylglycine, 0.1% (w/v) bovine serum albumin, 3 mM EDTA, and 0.1 M sodium fluoride, pH 7.4] were added. This was removed after 5 sec and replaced with 1 ml of the same buffer (ice-cold) containing added dithiothreitol (1 mM). The cells were immediately scraped off, transferred to an ice-cold plastic test tube, and sonicated (Branson B12 Sonifier, 110 W for 30 sec). Half the sonicate was immediately transferred to a glass tube and frozen in Dry Ice–acetone for later cyclic AMP assay, and the other 0.5 ml was placed on ice for phosphorylase assay. Preliminary experiments indicated that control cells had identical cyclic AMP levels whether incubation was terminated as above or by direct addition of 5% (w/v) trichloracetic acid to the plate. In stimulated cells cyclic AMP values were less than 20% lower when the reaction was stopped with trichloracetic acid.

Cyclic AMP assay. After boiling in a water bath for 10 min and centrifugation to remove precipitated proteins, cyclic AMP in the supernatant was assayed by a protein binding method (17), using kits obtained from the Radiochemical Centre, Amersham.

Glycogen phosphorylase assay. Phosphorylase was assayed essentially by the method of Passonneau *et al.* (18) as modified by Browning *et al.* (8). Portions (25 μl) of the sonicated extract were added to each of two cuvettes containing 0.5 ml of reaction solution [50 mM imidazole, 1 mM EGTA, 0.5 mM $MgCl_2$, 0.5 mM NADP, 0.01% (w/v) glycogen, 5 mM sodium phosphate, 5 μg/ml of phosphoglucomutase, and 1 μg/ml of glucose 6-phosphate dehydrogenase]. AMP (final concentration, 1 mM) was added to one cuvette, and the absorbances of the two solutions were measured at 340 nm for 20 min at 30° in a Beckman model 25 spectrophotometer with kinetic attachment. Under these conditions pure phosphorylase *a* (rabbit muscle) was equally active in the presence and absence of AMP, whereas phosphorylase *b* (rabbit muscle) was virtually inactive in the absence of AMP. The reactions were completely dependent on the addition of glycogen, NADP, phosphoglucomutase, and glucose 6-phosphate dehydrogenase.

Protein assay. Protein was determined by the method of Lowry *et al.* (19), with bovine serum albumin (Calbiochem) as standard.

RESULTS

Effect of beta adrenergic agonists. The sensitivity of C-6 astrocytoma cells was first assayed at 37° by measuring phosphorylase activity between 1 and 10 min. Within 3–4 min a norepinephrine concentration of 10 nM stimulated the full conversion of phosphorylase to a form that was active in the absence of added AMP. It was assumed, by analogy with results obtained in other laboratories, that the phosphorylase activity represented conversion from the *b* to the *a* form (8).

In order to provide a more convenient assay system, phosphorylase activation was studied in a phosphate-buffered medium at room temperature. Following medium replacement, 1 hr was allowed for the system to reach equilibrium (phosphorylase conversion was initially stimulated by the change of medium). This system was more sensitive to norepinephrine, which produced full conversion of phosphorylase *b* to the *a* form at a concentration of 1 nM; the use of a temperature of 10° did not further increase the sensitivity of the system.

Since isoproterenol is a more potent *beta* adrenergic agonist than norepinephrine at *beta* receptors, it was used in subsequent experiments. Low concentrations of (−)-isoproterenol caused a rapid elevation of cyclic AMP levels and conversion of glycogen phosphorylase from the *b* to the *a* form (Fig. 1). With 0.1 nM (−)-isoprotere-

[2] The abbreviations used are: IBMX, 3-isobutyl-1-methylxanthine; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*′-tetraacetic acid; PGE_1, prostaglandin E_1.

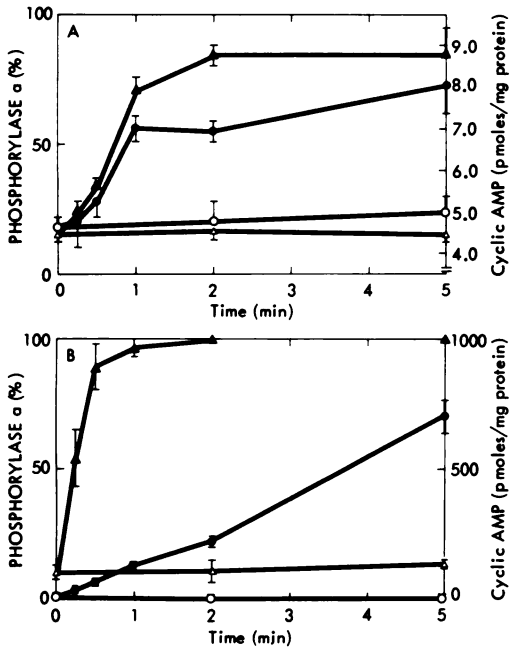

FIG. 1. *Effect of (−)-isoproterenol on phosphorylase a activity and cyclic AMP levels in C-6 astrocytoma cells*

Cells, treated as described in MATERIALS AND METHODS, were incubated for the times indicated with 0.1 nM (A) or 10 nM (B) (−)-isoproterenol [50 μl in 0.2% (w/v) ascorbic acid] or an equal volume of ascorbic acid. Phosphorylase *a*: △——△, ascorbic acid; ▲——▲, (−)-isoproterenol. Cyclic AMP: ○——○, ascorbic acid; ●——●, (−)-isoproterenol. Values shown are means ± standard errors for three plates.

nol increases in both parameters were apparent within 30 sec and were approximately parallel over the 5-min period of the experiment. With 10 nM (−)-isoproterenol both responses were more extensive, 90% of phosphorylase being in the *a* form within 30 sec. All experiments described below were carried out using a 2-min incubation period unless otherwise indicated.

In the presence of 0.1 mM IBMX, picomolar concentrations of (−)-isoproterenol caused maximal activation of phosphorylase *a*, accompanied by a small but significant increase in cyclic AMP (Fig. 2). The EC_{50} for phosphorylase activation by (−)-isoproterenol was 0.02 nM, while for stimulation of cyclic AMP production it was 1 nM. The *beta* adrenergic partial agonists (±)-salbutamol and (±)-hydroxybenzylpindolol also stimulated complete phosphorylase *b* to *a* conversion at low concentrations, despite the fact that (±)-hydroxybenzylpindolol, for example, had only 17.5% of the relative intrinsic activity of (−)-isoproterenol as regards cyclic AMP production.

In the presence of low concentrations of (−)-isoproterenol (less than 0.3 nM) there was parallel, stereoselective inhibition of cyclic AMP production and phosphorylase conversion by the *beta* adrenergic antagonist (−)-propranolol (Fig. 3). With higher (−)-isoproterenol concentrations this relationship no longer held, and a decrease in cyclic AMP production was no longer paralleled by a decrease in phosphorylase

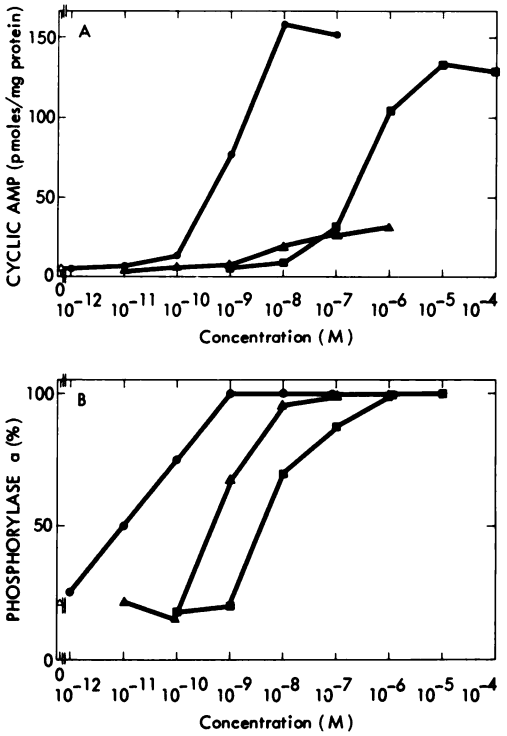

FIG. 2. *Effects of beta adrenergic agonists on phosphorylase a activity and cyclic AMP levels in C-6 astrocytoma cells*

Incubation conditions were the same as in MATERIALS AND METHODS, except that 0.1 mM IBMX was present during the preliminary incubation (60 min) and drug incubation (2 min) at room temperature. ●——●, (−)-isoproterenol; ▲——▲, (±)-hydroxybenzylpindolol; ■——■, (±)-salbutamol; △——△, ascorbate.

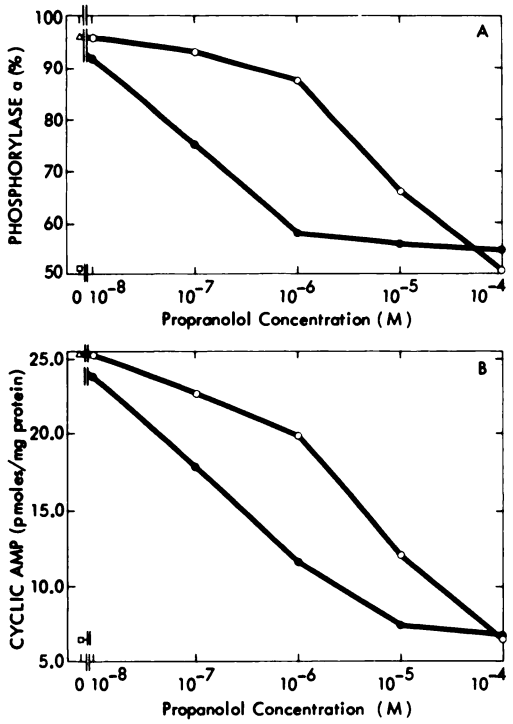

FIG. 3. *Effects of propranolol isomers on increase in phosphorylase a activity and cyclic AMP levels induced by (−)-isoproterenol in C-6 astrocytoma cells*

Incubation conditions were the same as in MATERIALS AND METHODS, except that 0.1 mM IBMX was present during the preliminary incubation (60 min) and drug incubation (2 min). Propranolol isomers were added simultaneously with (−)-isoproterenol (final concentration, 0.2 nM). □——□, ascorbic acid; △——△, (−)-isoproterenol; ●——●, (−)-isoproterenol plus (−)-propranolol; ○——○, (−)-isoproterenol plus (+)-propranolol. Neither (+)- nor (−)-propranolol added in the absence of (−)-isoproterenol altered C-6 cell phosphorylase *a* activity or cyclic AMP content (not shown).

activity (results not shown).

Effect of phosphodiesterase inhibition. The results in Fig. 4 illustrate the effect of prior treatment of the cells with IBMX on the dose-response curves for (−)-isoproterenol and (±)-hydroxybenzylpindolol against phosphorylase *a* activity and cell cyclic AMP levels. The presence of IBMX clearly shifted the agonist EC_{50} values for phosphorylase *a* conversion to lower concentrations. Simultaneously it enhanced the rise in cyclic AMP produced in the cell by these drug concentrations. Treatment with IBMX always caused a variable but significant increase in the basal levels of both parameters (Fig. 5). Phosphorylase *a* levels rose from 19.5% ± 3.1% in controls to 55.9% ± 4.9% in IBMX-treated cells, while cyclic AMP levels rose from 4.5 ± 0.4 pmoles/mg of protein in controls to 6.6 ± 0.5 pmoles/mg of protein in phosphodiesterase-inhibited cells. The data in Fig. 5 also emphasize the proportionality between these two parameters in this range of cellular cyclic AMP concentration. Linear regression analysis of the data gave an *r* value of 0.84.

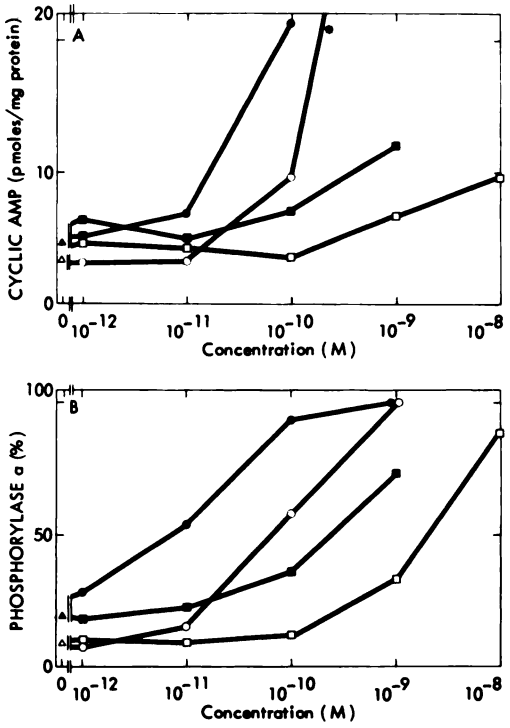

FIG. 4. *Effect of IBMX on increase in phosphorylase a activity and cyclic AMP levels induced by (−)-isoproterenol and (±)-hydroxybenzylpindolol in C-6 astrocytoma cells*

Incubation conditions were the same as described in MATERIALS AND METHODS, except for samples containing 0.1 mM IBMX (60 min). Drug incubations were carried out for 2 min at room temperature. Open symbols, no IBMX; solid symbols, with IBMX. ○——○ and ●——●, (−)-isoproterenol; □——□ and ■——■, (±)-hydroxybenzylpindolol.

* The value for cell cyclic AMP in the presence of 1 nM (−)-isoproterenol minus IBMX is 52.5 pmoles/mg of protein. Figure 4A is drawn to a scale that excludes this value to facilitate comparison with graph B.

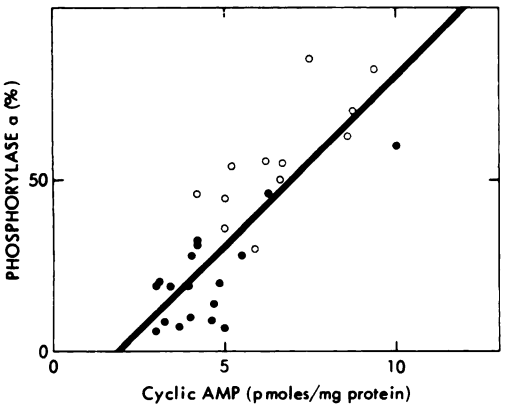

FIG. 5. *Effect of IBMX on phosphorylase a activity and cyclic AMP levels in C-6 astrocytoma cells*

Incubation conditions were the same as described in MATERIALS AND METHODS, except for samples containing 0.1 mM IBMX (60 min). All samples received 0.002% (w/v) ascorbic acid (2 min). ●——●, no IBMX; ○——○, with 0.1 mM IBMX. The line was drawn by linear regression analysis.

Effects of other agents. If phosphorylase *b* to *a* conversion is directly related to cell cyclic AMP levels in the range of 2–12 pmoles of cyclic AMP per milligram of protein, other agents causing a rise in cyclic AMP within this range should stimulate phosphorylase conversion. Serotonin, adenosine, and histamine had no effect on either parameter, while dopamine, at concentrations of 10 μM and greater, stimulated both (Table 1). This effect of dopamine appeared to be mediated via its weak *beta* agonist activity (20), since cyclic AMP accumulation in response to dopamine was inhibited stereoselectively by (−)-propranolol and only weakly blocked by spiroperidol (10 μM), a dopamine receptor blocker (21) (results not shown). PGE_1, which caused a maximal 1.6-fold rise in cyclic AMP levels, activated phosphorylase *a* to 70%. In the presence of IBMX (0.1 mM) dibutyryl cyclic AMP (0.1–1 mM) fully activated phosphorylase *b* to *a*. In the absence of the phosphodiesterase inhibitor its effects were much weaker. Cyclic AMP itself had little effect under either condition.

Effect of A23187. The divalent cation ionophore A23187 caused 50–60% conversion of phosphorylase *b* to *a* without significantly increasing the cellular levels of cyclic AMP (Table 1). The dose-response relationships for A23187 against both parameters (Fig. 6) indicate that when the system was depleted of divalent cations by previous incubation in divalent cation-free Earle's medium, the response of the phosphorylase system to A23187 was diminished. The responses of both parameters to (−)-isoproterenol (0.01–10 nM) were unaffected by this treatment (not shown). Table 2 summarizes the effects of A23187 (1 μg/ml) on cellular cyclic AMP levels and phosphorylase *a*, and indicates that in the absence of IBMX there was a small, but probably insignificant, drop in cyclic AMP levels. In IBMX-stimulated cells the inhibitory effect of A23187 was more marked. The phosphorylase-activating effect of A23187, however, was present under both conditions.

Effect of (−)-isoproterenol-induced desensitization. Repeated or long-term exposure of C-6 astrocytoma cells to high concentrations of norepinephrine causes a decrease in the ability of these cells to respond to a further norephinephrine stimulus (6, 8, 22, 23). When C-6 cells were desensitized to *beta* receptor stimulation by prior treatment with low concentrations of (−)-isoproterenol, a second addition of (−)-isoproterenol produced a much smaller increase in cyclic AMP. After the maximal cyclic AMP response had been decreased by 38% by treatment for 3 h with (−)-isoproterenol, phosphorylase could still be maximally converted to the *a* form by a subsequent addition of (−)-isoproterenol, although approximately 5-fold higher concentrations were required (Fig. 7A). In a more severely desensitized cell (62.7% desensitized, Fig. 7B) essentially maximal conversion of phosphorylase *b* to *a* could also be obtained, although only at 15-fold higher concentrations of (−)-isoproterenol than in controls.

DISCUSSION

Like the rise in cell cyclic AMP content (4), the stimulation of phosphorylase *a* activity by adrenergic agents in C-6 astrocytoma cells appears to be mediated by an action of *beta* adrenergic receptors. Both effects are stereoselectively blocked by the

TABLE 1

Effects of various agents on phosphorylase activation and cyclic AMP accumulation in C-6 astrocytoma cells

Incubation conditions were the same as in Fig. 1, except when IBMX (0.1 mM) was present during the preliminary incubation (60 min) and drug incubation. All drug incubations were carried out for 2 min at room temperature, except for the experiments with cyclic AMP and dibutyryl cyclic AMP, which were conducted for 5 min. A23187 was dissolved in acetone (1%). Cyclic AMP values are means ± standard errors for 3–5 plates.

Expt.	Agent	Concentration	IBMX (0.1 mM)	Phosphorylase *a*	Cyclic AMP
				%	*pmoles/mg protein*
1	None		−	16	4.8 ± 0.4
	Dopamine	10 μM	−	84	8.1 ± 1.0
		100 μM	−	100	85.7 ± 8.3
	Histamine	10 μM	−	23	4.5 ± 0.8
		100 μM	−	22	4.7 ± 0.2
	Serotonin	100 μM	−	13	4.8 ± 0.2
	Adenosine	10 μM	−	23	4.8 ± 0.6
	PGE_1	0.1 μM	−	71	6.5 ± 1.0
		1 μM	−	68	7.7 ± 1.0
2	Acetone	1%	−	15	5.7 ± 0.3
	A23187	1 μg/ml	−	57	5.0 ± 0.2
3	None		−	4	
	Cyclic AMP	0.1 mM	−	8	
		1 mM	−	1	
	Dibutyryl cyclic AMP	0.1 mM	−	7	
		1 mM	−	23	
	None		+	28	
	Cyclic AMP	0.1 mM	+	37	
		1 mM	+	15	
	Dibutyryl cyclic AMP	0.1 mM	+	77	
		1 mM	+	93	

beta adrenergic antagonist (−)-propranolol, which is 20–40 times more effective than its (+) isomer. Moreover, the proportion of glycogen phosphorylase in its active form is increased by incubation with very low concentrations of the *beta* agonist (−)-isoproterenol. In the presence of the phosphodiesterase inhibitor IBMX, 1 pM(−)-isoproterenol causes a slight conversion of phosphorylase to the *a* form, and the effect is maximal at a concentration of 1 nM. (±)-Salbutamol and (±)-hydroxybenzylpindolol, which are *beta* adrenergic receptor partial agonists on other tissues (24, 25), also convert phosphorylase to the *a* form and increase C-6 cell cyclic AMP content.

In the experiments with *beta* adrenergic receptor agonists, every instance of an increase in the proportion of phosphorylase in the active form was associated with a small but significant rise in cell cyclic AMP content. For a number of reasons it seems probable that the activation of phosphorylase results from the rise in cyclic AMP. (a) Cellular cyclic AMP levels rise at least as fast, in response to *beta* agonists, as phosphorylase *a* activity. (b) IBMX, which has been shown to inhibit cyclic AMP catabolism in this cell type (6), causes a 1.4-fold rise in cell cyclic AMP content and increases the percentage of phosphorylase in the *a* form from 19.5% to 55.8%. (c) In the presence of IBMX the threshold concentrations of *beta* agonists required to activate both parameters are 10 times lower. (d) High concentrations of N^6-2′-*O*-dibutyryl cyclic AMP activate

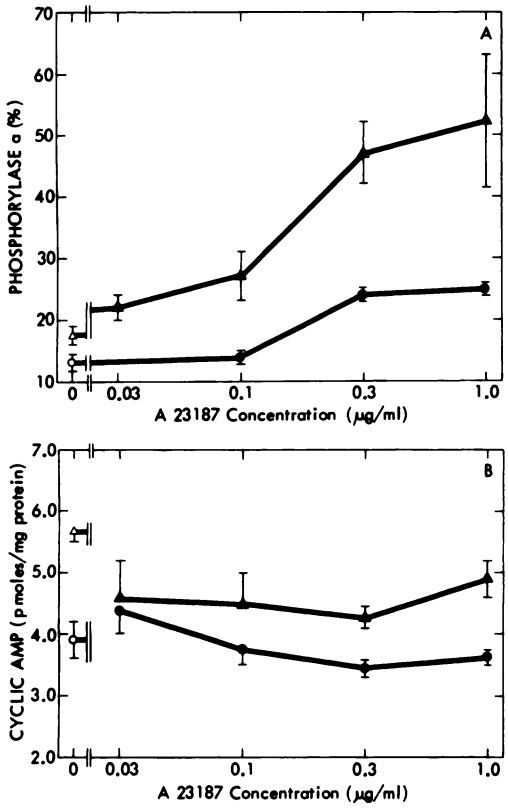

FIG. 6. *Effect of A23187 on phosphorylase a activity and cyclic AMP levels in C-6 astrocytoma cells*

Incubation conditions were the same as described in MATERIALS AND METHODS, except for samples containing Earle's balanced salt solution minus calcium and magnesium (60 min). Drug incubations were carried out for 2 min at room temperature. Values shown are means ± standard errors for three plates. Circles, Ca^{++}- and Mg^{++}-free medium; triangles, normal medium. ○——○ and △——△, acetone (1%); ●——● and ▲——▲, A23187 plus acetone (1%).

phosphorylase conversion, and this effect is markedly potentiated by prior treatment with IBMX. (e) PGE_1, which causes a 1.6-fold increase in the cell cyclic AMP content, activates phorphorylase *a* from 16% to 71%. (f) A number of other agents, such as histamine, serotonin, and adenosine, which do not alter cell cyclic AMP levels, are without effect on phosphorylase *a* activity. In this respect the divalent cation ionophore A23187 is an exception (see below).

The results in Fig. 5 (compiled from data in control and IBMX-treated cells) demonstrate a correlation between the percentage of phosphorylase in the *a* form and the cell cyclic AMP concentration within the range of 2–12 pmoles of cyclic AMP per milligram of protein. A similar relationship was found when the data from the other experiments were plotted in this way (not shown). With all cyclic AMP concentrations greater than 12 pmoles/mg, phosphorylase remains fully in the active form. Under our routine experimental conditions (2 min at 23°) *beta* adrenergic agonists can increase cell cyclic AMP levels to around 200 pmoles/mg of protein; 6% of this concentration suffices to activate phosphorylase *a* fully. It seems possible that this relationship may be of paramount importance when the cell is in a desensitized state. Although the mechanism responsible for this refractoriness remains unresolved, contributing factors appear to be a cyclic AMP-dependent induction of phosphodiesterase (26) and, as in other cells (27, 28), a decrease in the number of membrane *beta* adrenoceptors

TABLE 2

Effect of A23187 (1.0 μg/ml) on phosphorylase conversion and cyclic AMP accumulation in C-6 astrocytoma cells

Incubation conditions were the same as in Fig. 1, except when IBMX (0.1 mM) was present during the preliminary incubation (60 min) and incubation with acetone or A23187 (plus acetone) (2 min). Values are the means and ranges (in parentheses) from two or three experiments.

Acetone	A23187 (1.0 μg/ml)	IBMX (0.1 mM)	Phosphorylase *a*	Cyclic AMP
			%	*pmoles/mg protein*
+	–	–	9.5 (6.5–15.0)	4.5 (3.4–5.7)
+	+	–	47.8 (34.0–57.0)	3.6 (3.0–4.5)
+	–	+	60.0 (55.2–64.8)	8.8 (7.5–10.1)
+	+	+	98.0 (96.0–100.0)	5.4 (4.5–6.3)

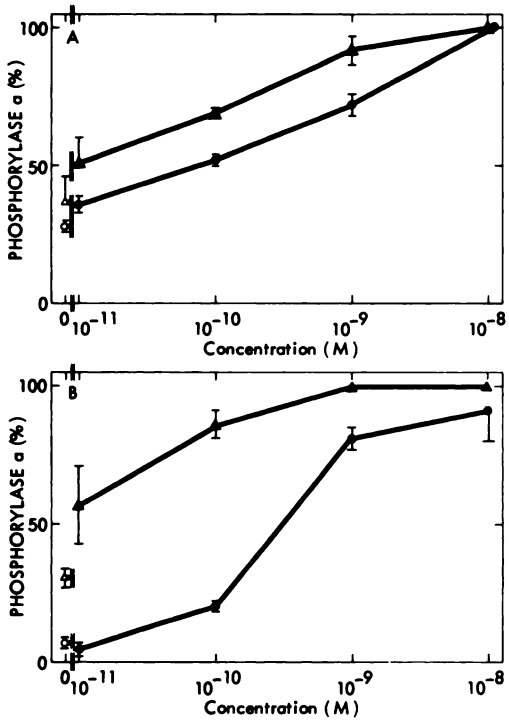

FIG. 7. *Effect of beta receptor desensitization on increase in phosphorylase a activity by (−)-isoproterenol in C-6 astrocytoma cells*

Preliminary incubation conditions were the same as in MATERIALS AND METHODS. Three hours before the medium (containing serum) was replaced by 5 ml of Earle's balanced salt solution, 50 μl of 0.2% (w/v) ascorbic acid or 50 μl of (−)-isoproterenol were added to the tissue culture dishes, and samples were again placed in the incubator (37°). *A*. The final concentration of (−)-isoproterenol was 1 nM. B. The final concentration of (−)-isoproterenol was 5 nM. After 3 hr the medium was removed by aspiration, and the cells were washed twice with 5 ml of Earle's solution to remove free (−)-isoproterenol. Then 5 ml more of Earle's salt solution were added, and the cells were left at room temperature for 60 min. After this preliminary incubation the desensitized and nondesensitized cells were further incubated with ascorbate or (−)-isoproterenol (2 min). The concentration of (−)-isoproterenol used for the second incubation is shown on the abscissae. Methods of preparing samples for phosphorylase and cyclic AMP assay are described in the text. A. The cyclic AMP values with 10 nM (−)-isoproterenol as the stimulatory agent were 191.3 pmoles/mg of protein for control (ascorbate-incubated, 3 hr) cells and 119.6 pmoles/mg of protein for (−)-isoproterenol-incubated (3 hr) cells. B. Corresponding values were 188.2 pmoles/mg of protein for controls and 51.5 pmoles/mg of protein for (−)-isoproterenol-incubated cells. Values shown are means ± standard errors for three plates. O——O, 3 hr with (−)-isoproterenol, 2 min with ascorbate; ●——●, 3 hr with (−)-isoproterenol, 2 min with (−)-isoproterenol; △——△, 3 hr with ascorbate, 2 min with ascorbate; ▲——▲, 3 hr with ascorbate, 2 min with (−)-isoproterenol.

as measured by [^{3}H]dihydroalprenolol or [^{125}I]hydroxybenzylpindolol binding.[3]

In confirmation of the work of Franklin and Twose (29), we could find no change in the EC_{50} value for (−)-isoproterenol in increasing cell cyclic AMP levels between control and desensitized cells, but rather a decrease in the maximum obtainable cyclic AMP level. However, when the cyclic AMP-controlled response, in this case phosphorylase conversion, is considered, the dominant effect of desensitization is not to change the ability of glycogen phosphorylase to exist fully in the active form but to increase the concentration of *beta* agonist necessary to produce this effect. The results presented in Fig. 7 indicate that in two experiments in which the system was desensitized (as far as cyclic AMP production is concerned) by 38% and 63% following incubation with 1 and 5 nM (−)-isoproterenol, respectively, the dose-response relationships for (−)-isoproterenol-induced phosphorylase conversion shifted to 5- and 15-fold higher concentrations, respectively; in both cases, however, maximum conversion could be elicited. The almost ubiquitous ability of cells and tissues to regulate their cyclic AMP concentration over a much wider range than that controlling a physiological response (25, 30–32) may therefore represent a subtle mechanism whereby the cell can regulate the sensitivity of its particular physiological response to agonist concentration.

The divalent cation ionophore A23187 represents an example of an agent that appears to activate glycogen phosphorylase by an action independent of cyclic AMP. Under the conditions used, A23187 can convert approximately 50% of the glycogen phosphorylase to the *a* form. This effect was not associated with a significant

[3] M. Staehelin and P. Müller, manuscript in preparation.

rise in cell cyclic AMP content; in fact, a slight fall in cyclic AMP resulted, which was emphasized in cells previously treated with IBMX. While it is still possible that a rise in cyclic AMP in some small subcellular compartment mediates the stimulatory action of A23187 on phosphorylase conversion, this seems unlikely; the effects of A23187 on cyclic AMP levels on other tissues are, as here, either insignificant or, in stimulated systems, inhibitory (33–36). Our findings with A23187 are very similar to those reported by Pointer *et al.* (36) and Keppens *et al.* (37), showing that the ionophore stimulates glycogenolysis and glycogen phosphorylase activity, respectively, in rat liver cells without altering cyclic AMP levels or protein kinase activity.

It seems likely that A23187, by increasing cytoplasmic Ca^{++} levels, activates phosphorylase *b* kinase and that this, in turn, allows the conversion of phosphorylase to the active form. Preliminary results[4] indicate that C-6 cell phosphorylase *b* kinase, like the liver and muscle enzyme (38, 39), can be stimulated by low concentrations of Ca^{++} ions. Another possible mechanism of A23187-induced phosphorylase conversion, which cannot be excluded, is inhibition of energy production within the cell (40), resulting either in accumulation of the allosteric effector AMP (41) or in a decrease in cell glucose, which would favor glycogenolysis (9).

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