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# Calmodulin Inhibitors Activate Glycogen Phosphorylase B to A Conversion in C6 Glioma Cells

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SUMMARY

Some neuroleptic drugs have been described as calmodulin antagonists *in vitro*, yet their usefulness as inhibitors of calmodulin in intact cell systems remains unclear. C6 glioma cells were preincubated with these drugs (0.1–0.5mM) in an attempt to determine whether they could block $Ca^{2+}$ ionophore (A23187)-induced conversion of glycogen phosphorylase from the B to the A form. Surprisingly, 7 of the 10 neuroleptic drugs tested were found to convert 36% of the phosphorylase from the B to the A form by themselves, and this activation could not be enhanced by the addition of A23187. The cyclic AMP phosphodiesterase inhibitors 3-isobutyl-1-methylxanthine (IBMX) and Ro 20-1724 at concentrations of 0.1 mM converted 25% and 33% of the phosphorylase to the A form, respectively; however, this activation could be further stimulated by A23187 in a partially additive manner. In C6 cell homogenates the phosphodiesterase inhibitors IBMX and Ro 20-1724 inhibit both $Ca^{2+}$-dependent and $Ca^{2+}$-independent phosphodiesterase activity, with $IC_{50}$ values of 0.1–0.3 mM, whereas the neuroleptic drugs inhibit only the $Ca^{2+}$-dependent form of phosphodiesterase, with $IC_{50}$ values of 0.09–0.13 mM. Despite this, in intact C6 cells the neuroleptic drugs were found to decrease the cyclic AMP concentration whereas both of the phosphodiesterase inhibitors (IBMX and Ro 20-1724) increased the cyclic AMP concentration. It is concluded that the neuroleptic drugs, unlike the phosphodiesterase inhibitors, activate phosphorylase conversion through a mechanism that does not involve cyclic AMP. The limitations of these drugs as probes for calmodulin are discussed.

## INTRODUCTION

C6 glioma cells are a favorable model system for studying *beta*-adrenergic responses because these cells are enriched in *beta*-adrenergic receptors linked to adenylate cyclase, and subsequent cyclic AMP-mediated responses can be monitored in a controlled medium. Previously, C6 glioma cells have been characterized with respect to a *beta*-adrenoceptor-stimulated phosphorylase B to A conversion which is mediated by cyclic AMP (1, 2). The conversion of phosphorylase B to A is carried out by the activation of phosphorylase kinase, which was initially described to be a cyclic AMP-dependent activation in liver (3) but was also found to be activated in other tissues by a non-cyclic AMP-mediated mechanism (4, 5). In the study by Drummond *et al.* (1) it was observed that *beta*-adrenergic agonists could stimulate the conversion of phosphorylase B to A through a cyclic AMP-mediated pathway in C6 glioma cells and that the $Ca^{2+}$ ionophore A23187 could also stimulate this conversion through a pathway independent of cyclic AMP. Although phosphorylase kinase has long been known to depend on $Ca^{2+}$ for activity (6), it is only recently that the $Ca^{2+}$-binding protein calmodulin has been shown to be a subunit of phosphorylase kinase (7). The activation of phosphorylase B to A conversion in C6 cells by the $Ca^{2+}$ ionophore A23187 and the presence of calmodulin as a subunit of phosphorylase kinase suggest that calmodulin may serve as a link between $Ca^{2+}$ availability in C6 cells and the activation of phosphorylase kinase.

At present there are no reported cell lines that are mutants for calmodulin and that can be used to test the role of calmodulin *in vivo*. However, an alternative approach is to use known *in vitro* inhibitors of calmodulin activity in a cell line which exhibits some $Ca^{2+}$-dependent physiological event. Compounds with an intracellular site of inhibition, such as the cyclic AMP phosphodiesterase inhibitor IBMX,[2] have been used successfully to demonstrate the cyclic AMP-dependent activation of phosphorylase kinase in C6 glioma cells (1); therefore, inhibitors of calmodulin activity should also be useful in this system. It was originally reported by Levin and Weiss (8) that some neuroleptic drugs could inhibit the calmodulin-dependent activation of phosphodiesterase and that this inhibition was due to the direct binding of these drugs to

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

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the $Ca^{2+}$-dependent conformation of calmodulin (9). Although the inhibition of calmodulin activity by neuroleptic drugs is nonstereospecific and unrelated to the clinical efficacy of these drugs (10), their ability to inhibit the activity of calmodulin *in vitro* makes them potentially useful tools for the inhibition of calmodulin *in vivo*. It was of interest to use these neuroleptic drugs in C6 glioma cells to evaluate their ability to block $Ca^{2+}$-activated phosphorylase kinase, which is presumed to be mediated by calmodulin.

In this report, a range of neuroleptic drugs was found to block the $Ca^{2+}$ ionophore (A23187)-induced activation of phosphorylase in C6 glioma cells. However, these drugs also caused an activation of phosphorylase by themselves. It is concluded that the activation of phosphorylase by neuroleptic drugs is not due to a cyclic AMP-mediated pathway but might be due to their promotion of internal $Ca^{2+}$ release in C6 cells.

## EXPERIMENTAL PROCEDURES

*Materials.* Dulbecco's modified Eagle's medium, fetal calf serum, and Earle's balanced salt solution were obtained from GIBCO (Glasgow, United Kingdom). 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), 5′-AMP, and NADP were obtained from Boehringer Mannheim (Mannheim, Federal Republic of Germany). 5′-Nucleotidase (*Crotalus atrox*) (EC 3.1.3.5) and cyclic AMP were obtained from Sigma Chemical Company (St. Louis, Mo.). Cyclic [8-$^3$H]AMP and cyclic AMP radioimmunoassay kits were obtained from New England Nuclear Corporation (Boston, Mass.). AG1X2 resin (200–400 mesh) was obtained from Bio-Rad Laboratories (Richmond, Calif.), and IBMX from Aldrich-Europe (Beerse, Belgium). Drugs were donated by the following companies: Lundbeck A/S (Copenhagen-Valby, Denmark) (*cis*- and *trans*-flupenthixol, *cis*- and *trans*-chloroprothixene); Janssen Pharmaceutica (Beerse, Belgium) (spiroperidol, haloperidol, and pimozide); Wander Ltd. (Berne, Switzerland) (clozapine); Delagrange International (Paris, France) (sulpiride); and Smith Kline & French (Brussels, Belgium) (chlorpromazine). Ro 20-1724 was a gift from Dr. W. Burkhard, Hoffman-La Roche (Basel, Switzerland); A23187 (Lilly) was a gift from Dr. R. Neher, of the Friedrich Miescher Institut.

*Culture conditions.* Rat C6 glioma 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, penicillin (100 units/liter), and streptomycin (100 μg/liter). The cells were seeded at a density of $10^5$/ml in 100-mm plastic tissue culture plates (Corning) and grown to confluence in 4–5 days. For subculturing, the medium was replaced with 5 ml of 0.02% (w/v) EDTA for 2 min at room temperature. This solution was then aspirated, and the cells were 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 disodium hydrogen phosphate (3.3 mM). The cells were then incubated for 1 hr at room temperature in the presence or absence of indicated drug. A23187 was added 4 min before the end of the incubation period where indicated. At the end of the incubation the medium was removed by aspiration, and 5 ml of ice-cold buffer (0.01 M $NaH_2PO_4$, 0.003 M EDTA, 0.01 M NaF, pH 7.0) were added. This was removed after 5 sec and replaced with 1 ml of the same ice-cold buffer containing added dithiothreitol (1 mM). The cells were harvested by scraping them off the plate immediately after adding the above buffer, transferring them to an ice-cold plastic test tube, and sonicating them with a Branson B12 sonifier (110 W for 30 sec). This sonicate was used directly in the glycogen phosphorylase assay and the cyclic AMP phosphodiesterase assay. For cyclic AMP determinations, 0.70 ml of sonicate was adjusted to 5% trichloracetic acid by the addition of 30% trichloroacetic acid. Approximately 4000 cpm of cyclic [$^3$H]AMP recovery marker were added to each tube at this time and the tubes were centrifuged at 4° for 10 min at 2000 rpm in a Sorvall table-top centrifuge. The resulting supernatant was lyophilized to dryness for subsequent cyclic AMP radioimmunoassay.

*Cyclic AMP radioimmunoassay.* The lyophylized supernatant of C6 cell homogenates was dissolved in 1.0 ml of 0.01 M sodium acetate (pH 6.8), and subsequent dilutions of this solution were made in the same buffer. The cyclic AMP content was measured with a cyclic AMP radioimmunoassay kit, and recoveries of cyclic AMP were monitored by the cyclic [$^3$H]AMP tracer added to the homogenates.

*Glycogen phosphorylase assay.* Phosphorylase was assayed exactly as described by Drummond *et al.* (1). A portion (20 μl) of the sonicated extract was 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, phosphoglucomutase (5 mg/ml), and glucose-6-phosphate dehydrogenase (1 mg/ml)]. 5′-AMP was added to one cuvette (final concentration 1 mM), and the absorbances of the two solutions were measured at 340 nm for 20 min at 30° in a Beckman Model 25 spectrophotometer with a kinetic attachment.

*Phosphodiesterase assay.* Phosphodiesterase activity was measured by a modification of the procedure of Thompson and Appleman (11), as previously described by Norman *et al.* (10). All reactions were carried out in 0.4-ml volumes at pH 8.0 containing 5 mM $MgCl_2$, 40 mM Tris, 0.1 mM EGTA, 4.0 mM 2-mercaptoethanol, 0.01 mM cyclic AMP (including 200,000 cpm of cyclic [$^3$H]AMP), 40 μl of C6 cell sonicate, and drugs as indicated. Tubes containing $Ca^{2+}$, where indicated, contained 0.3 mM $CaCl_2$. Preincubation of the above was conducted for 10 min at 30° without cyclic AMP. Subsequently, incubations were conducted for 10 min at 30° and terminated by boiling for 45 sec. Conversion of 5′-AMP reaction product to adenosine was accomplished by the addition of 0.1 ml of 5′-nucleotidase (0.5 mg/ml) for an additional 10-min incubation at 30°. This reaction was terminated by the addition of 1.0 ml of methanol, and adenosine was separated from unhydrolyzed cyclic AMP on a 1.0-ml

column of Bio-Rad resin, AG1X2. All samples were counted by liquid scintillation and corrected for percentage recovery of adenosine, which ranged from 85% to 95%. Background values containing no enzyme ranged from 1.0 to 1.5% of total counts added.

*Protein assay.* Protein concentrations were determined with a Coomassie Blue staining kit (Bio-Rad), using γ-globulin as a standard (12).

## RESULTS

*Activation of glycogen phosphorylase.* The initial purpose of adding neuroleptic drugs to the C6 cell medium was to determine whether these calmodulin-inhibiting compounds could block the $Ca^{2+}$ ionophore A23187-induced activation of phosphorylase in these cells. When these neuroleptic drugs were added to the medium in the absence of A23187, a surprising activation of phosphorylase occurred in 7 of 10 of these drugs. In Fig. 1 a dose-dependent activation of phosphorylase is evident with both *cis*- and *trans*-chlorprothixene. These compounds caused an approximate 41% conversion of phosphorylase B to A over a background value of 20% for the resting phosphorylase A levels in these cells. This activation was nonstereospecific, and, since 5-hydroxytryptamine receptors and dopamine receptors can discriminate between the different isomers of neuroleptic drugs (13), this suggests that the response is not receptor-mediated. However, a lack of stereospecificity is a characteristic of calmodulin inhibition (10), and it is possible that the inhibition of a calmodulin-activated enzyme may be involved in this response. The data in Fig. 1 also show that IBMX and A23187 stimulate phosphorylase B to A conversion to 25% and 27% over background, respectively, and in a dose-dependent manner. The results observed for IBMX- and A23187-induced activation of phosphorylase conversion agree with the previous observations of Drummond *et al.* (1).

The data demonstrating that a number of neuroleptic drugs activate phosphorylase conversion are summarized in Table 1. All of the drugs listed in Table 1 were evaluated in a dose-dependent manner, and the activation of phosphorylase conversion could be observed at concentrations as low as 0.01 mM (Fig. 1). Although the concentration of drug required to activate the enzyme maximally varied from 0.1 mM to 0.5 mM, it was not possible to estimate the actual intracellular concentration of drug, and limitations on drug solubility prevented higher concentrations from being used. The extent to which these neuroleptic drugs activated phosphorylase conversion varied from 26% for chlorpromazine to 44% to *cis*-chlorprothixene. The ionophore A23187 was tested with each compound to determine whether the ionophore activation could be blocked by preincubation with the indicated drug. The seven compounds which did show stimulation of phosphorylase conversion also blocked the ability of A23187 to stimulate phosphorylase conversion further. The average stimulation of phosphorylase conversion shown for these seven neuroleptic drugs alone was 36% as compared with the average stimulation for these drugs plus A23187 which was 38%. These results indicate that these neuroleptics which activate phosphorylase conversion by themselves also block A23187 activity. The three neuroleptic drugs haloperidol, spiroperidol, and sulpiride did not significantly stimulate

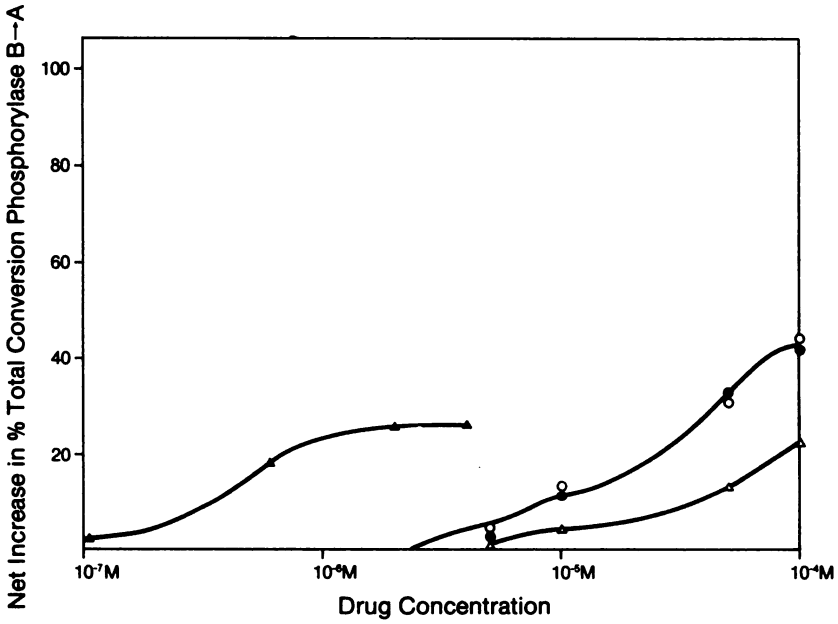


FIG. 1. *Dose-dependent activation of phosphorylase in C6 glioma cells by various drugs*

All drugs excepting A23187 were dissolved in Earle's balanced salt solution at the given concentrations and incubated with the cells for 1 hr at room temperature prior to harvest. Control cells were preincubated with Earle's balanced salt solution for 1 hr prior to harvest, and these control cells consistently existed in a 20% conversion state. All conversion values shown in Table 1 represent the net increase in conversion of phosphorylase B to A above these control values of 20%. A23187 was dissolved in ethanol at a concentration of 2 mM, and 20 μl of this solution were added to the preincubated cells where indicated 4 min prior to harvest. Ethanol alone had no effect on phosphorylase B to A conversion. ▲, A23187; △, IBMX; ○, *cis*-chlorprothixene; ●, *trans*-chlorprothixene.

TABLE 1

*Effect of various drugs on glycogen phosphorylase B to A conversion in C6 glioma cells*

All drugs excepting A23187 were dissolved in Earle's balanced salt solution at the given concentrations and incubated with the cells for 1 hr at room temperature prior to harvest. Control cells were preincubated with Earle's balanced salt solution for 1 hr prior to harvest, and these control cells consistently existed in a 20% conversion state. All conversion values shown represent the net increase in conversion of phosphorylase B to A above these control values. A23187 was dissolved in ethanol at a concentration of 2 mM, and 20 μl of this solution were added to the preincubated cells where indicated 4 min prior to harvest. Ethanol alone had no effect on phosphorylase B to A conversion. The values shown for phosphorylase B to A conversion represent the mean taken from the ranges shown in parentheses.

| Class of drug | Drug | Concentration in medium (mM) | Net increase in % total conversion of phosphorylase B to A | Range | No. of experiments performed in duplicate |
|---|---|---|---|---|---|
| $Ca^{2+}$ ionophore | 1. A23187 | 0.004 | 27 | (19–31) | 28 |
| Phenothiazine | 2. Chlorpromazine | 0.5 | 26 | (21–30) | 3 |
| | 3. Chlorpromazine +A23187 | 0.5<br>0.004 | 25 | (21–30) | 3 |
| Thioxanthene | 4. *cis*-Chlorprothixene | 0.2 | 44 | (39–49) | 2 |
| | 5. *cis*-Chlorprothixene +A23187 | 0.2<br>0.004 | 35 | (31–39) | 2 |
| | 6. *trans*-Chlorprothixene | 0.2 | 38 | (34–42) | 2 |
| | 7. *trans*-Chlorprothixene +A23187 | 0.2<br>0.004 | 41 | (37–45) | 2 |
| | 8. *cis*-Flupenthixol | 0.2 | 42 | (39–45) | 3 |
| | 9. *cis*-Flupenthixol +A23187 | 0.2<br>0.004 | 49 | (44–54) | 3 |
| | 10. *trans*-Flupenthixol | 0.2 | 44 | (42–46) | 3 |
| | 11. *trans*-Flupenthixol +A23187 | 0.2<br>0.004 | 42 | (40–44) | 3 |
| Diphenylbutylamine | 12. Pimozide | 0.1 | 33 | (30–36) | 2 |
| | 13. Pimozide +A23187 | 0.1<br>0.004 | 39 | (35–43) | 2 |
| Dibenzodiazepine | 14. Clozapine | 0.1 | 28 | (26–30) | 2 |
| | 15. Clozapine +A23187 | 0.1<br>0.004 | 34 | (31–37) | 2 |
| Butyrophenone | 16. Haloperidol | 0.2 | 1 | (0–2) | 2 |
| | 17. Haloperidol +A23187 | 0.2<br>0.004 | 27 | (24–29) | 2 |
| | 18. Spiroperidol | 0.2 | 3 | (2–4) | 2 |
| | 19. Spiroperidol +A23187 | 0.2<br>0.004 | 27 | (26–28) | 2 |
| Benzamide | 20. Sulpiride | 0.5 | 1 | (1) | 2 |
| | 21. Sulpiride +A23187 | 0.5<br>0.004 | 21 | (21–22) | 2 |
| Methyl xanthine | 22. IBMX | 0.1 | 25 | (18–32) | 10 |
| | 23. IBMX +A23187 | 0.1<br>0.004 | 37 | (31–43) | 12 |
| 3-Butoxy-4-Methoxy benzyl-2-imidazolidinone | 24. Ro 20-1724 | 0.1 | 33 | (30–35) | 2 |
| | 25. Ro 20-1724 +A23187 | 0.1<br>0.004 | 53 | (50–56) | 2 |
| | 26. IBMX +*trans*-Flupenthixol | 0.1<br>0.5 | 59 | (54–64) | 2 |
| | 27. IBMX +*trans*-Flupenthixol +A23187 | 0.1<br>0.5<br>0.004 | 58 | (54–62) | 2 |

phosphorylase conversion. Haloperidol and spiroperidol are moderately potent inhibitors of calmodulin activity (10) and hence the fact that they had no stimulating ability in the phosphorylase conversion assay was not unexpected. Sulpiride is not an inhibitor of calmodulin activity (10) and showed no stimulation of phosphorylase activity in the C6 glioma cells. Additionally, these three compounds did not block the activation of phosphorylase

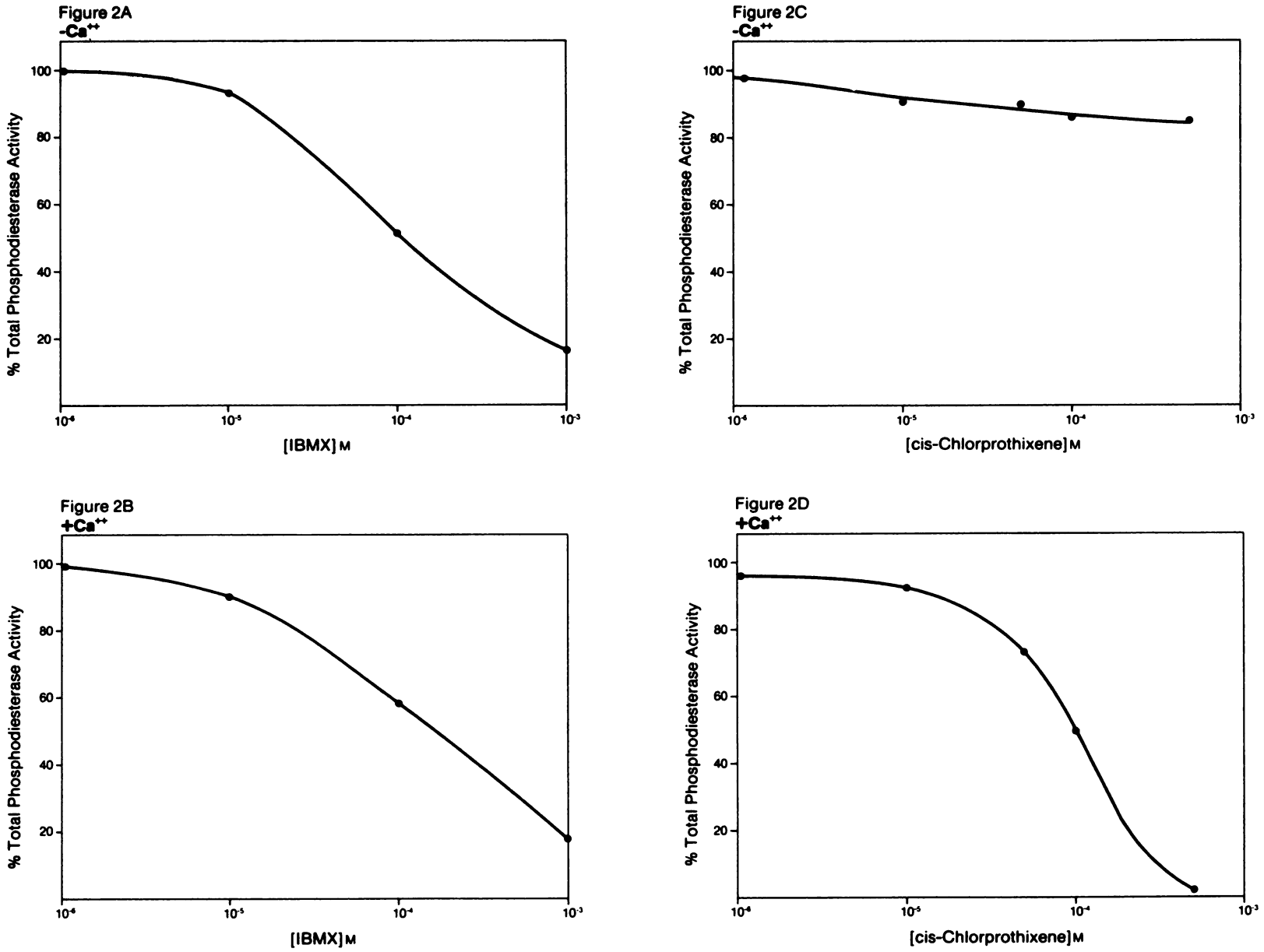


FIG. 2. *Inhibition of $Ca^{2+}$-independent and $Ca^{2+}$-dependent phosphodiesterase activity in C6 cell sonicates by IBMX and cis-chlorprothixene*

Ten confluent plates of C6 cells were harvested and sonicated in 6 ml of Buffer A without preincubation in Earle's balanced salt solution. This sonicate was assayed for cyclic AMP phosphodiesterase activity in the presence of increasing concentrations of IBMX or *cis*-chlorprothixene in parallel experiments with and without $Ca^{2+}$ in the assay buffer. In the absence of $Ca^{2+}$ the C6 cell sonicate had a specific activity of 150 pmoles of cyclic AMP hydrolyzed per minute per milligram of protein, and this specific activity is expressed as 100% total phosphodiesterase activity in A and C. In the presence of $Ca^{2+}$ the C6 cell sonicate had a specific activity of 288 pmoles of cyclic AMP hydrolyzed per minute per milligram of protein, and this value minus the specific activity in the absence of $Ca^{2+}$ represents the $Ca^{2+}$-activated phosphodiesterase activity; this $Ca^{2+}$-activated phosphodiesterase activity is represented as 100% total phosphodiesterase activity in B and D.

conversion by A23187 as did the other seven compounds studied.

The two phosphodiesterase inhibitors studied, IBMX and Ro 20-1724, caused 25% and 33% conversion of phosphorylase into the A form, respectively, and, unlike the neuroleptics, both of these effects could be stimulated further by the addition of A23187 (Table 1). This effect of phosphodiesterase inhibitors plus $Ca^{2+}$ ionophore is in accord with the previous results obtained by Drummond *et al.* (1), although, with IBMX in particular, these two effects are only partially additive.

The effects of IBMX and *cis*-flupenthixol in combination were additive, with a 59% net conversion of phosphorylase B to A, but these combined activities could not be further stimulated by A23187. These two agents are additive in their ability to stimulate phosphorylase conversion, suggesting that they might have different sites of action. Possibly the two different compounds inhibit different forms of phosphodiesterase. For this reason further studies with these drugs were carried out on $Ca^{2+}$-sensitive and $Ca^{2+}$-insensitive forms of phosphodiesterase.

*Inhibition of phosphodiesterase.* It has been previously demonstrated that the $Ca^{2+}$-induced stimulation of phosphodiesterase in C6 glioma cells is conferred by calmodulin (14) and this $Ca^{2+}$-dependent activity accounts for almost as much activity as the $Ca^{2+}$-independent form. It is conceivable that the seven neuroleptic drugs which were able to stimulate phosphorylase B to A conversion were acting by inhibiting calmodulin-induced stimulation of phosphodiesterase. The data in Fig. 2 illustrate the inhibition observed with IBMX, a classical phosphodiesterase inhibitor, and with *cis*-chlorprothixene, a neuroleptic calmodulin inhibitor. In Fig. 2A and B it is evident that IBMX inhibits both $Ca^{2+}$-independent and $Ca^{2+}$-dependent phosphodiesterases with about the same potency. Figure 2C shows the weak inhibitory action of *cis*-chlorprothixene on $Ca^{2+}$-independent phosphodiesterase, yet in Fig. 2D *cis*-chlorprothixene is as potent as IBMX as an inhibitor of $Ca^{2+}$-dependent phosphodiesterase. These results are summarized in Table 2, which lists the $IC_{50}$ values for inhibition of $Ca^{2+}$-independent and $Ca^{2+}$-dependent phosphodiesterase by some of the compounds which were active in stimulating phosphorylase B to A conversion. The $IC_{50}$ values obtained for $Ca^{2+}$-independent and $Ca^{2+}$-dependent phosphodiesterase inhibition by IBMX and Ro 20-1724 are approximately the same, showing that these compounds do not selectively inhibit different phosphodiesterase forms in C6 cells. Also, IBMX appears to be slightly more potent than Ro 20-1724 as a phosphodiesterase inhibitor. The four neuroleptic drugs listed in Table 2 showed very little inhibitory activity toward $Ca^{2+}$-independent phosphodiesterase activity at concentrations up to $10^{-2}$ M. The range of potency of these four compounds as inhibitors of $Ca^{2+}$-stimulated phosphodiesterase was $0.9 \times 10^{-4}$ M to $1.3 \times 10^{-4}$ M which is comparable with the $IC_{50}$ values observed for IBMX and Ro 20-1724 against both phosphodiesterase forms. The potencies of *cis*- and *trans*-chlorprothixene are identical; this lack of stereospecificity is a characteristic of this drug's inhibition of calmodulin (10). The $IC_{50}$ values for $Ca^{2+}$-stimulated phosphodiesterase inhibition are higher than those observed for inhibition of purified calmodulin and partially purified phosphodiesterase (8, 10), but this difference may be due to nonspecific binding of these drugs to components of the C6 cell homogenate. The drug concentrations necessary for phosphorylase activation range from $1.0 \times 10^{-4}$ M for pimozide to $5 \times 10^{-4}$ M for chlorpromazine. These values are in agreement with the observed $IC_{50}$ values for $Ca^{2+}$-dependent phosphodiesterase inhibition. Therefore, the possibility that phosphorylase is activated by a cyclic AMP-mediated process brought about by inhibition of $Ca^{2+}$-dependent phosphodiesterase remained to be established. For this reason, cyclic AMP determinations were made in C6 cells after drug treatment.

TABLE 2

*Inhibition of $Ca^{2+}$-independent and $Ca^{2+}$-dependent cyclic AMP phosphodiesterase activity in C6 cell sonicates by various drugs*

C6 cells were harvested, sonicated, and assayed for cyclic AMP phosphodiesterase activity as described in the legend to Fig. 2. $Ca^{2+}$-independent and $Ca^{2+}$-dependent cyclic AMP phosphodiesterase specific activities were the same as described in the legend to Fig. 2. The $IC_{50}$ values for each activity represent the concentrations of drug necessary for 50% inhibition of that activity.

| Drug | $Ca^{2+}$-Independent phosphodiesterase activity, $IC_{50}$ (M) | $Ca^{2+}$-Dependent phosphodiesterase activity, $IC_{50}$ (M) |
|---|---|---|
| 1. IBMX | $1.1 \times 10^{-4}$ | $1.6 \times 10^{-4}$ |
| 2. Ro 20-1724 | $3.0 \times 10^{-4}$ | $2.6 \times 10^{-4}$ |
| 3. *cis*-Chlorprothixene | $>10^{-2}$ | $1.0 \times 10^{-4}$ |
| 4. *trans*-Chlorprothixene | $>10^{-2}$ | $1.0 \times 10^{-4}$ |
| 5. *cis*-Flupenthixol | $>10^{-2}$ | $1.3 \times 10^{-4}$ |
| 6. Chlorpromazine | $>10^{-2}$ | $0.9 \times 10^{-4}$ |

TABLE 3

*Effect of various drugs on cyclic AMP concentrations in C6 glioma cells*

All drugs excepting A23187 were dissolved in Earle's balanced salt solution at the given concentrations and incubated with the cells for 1 hr at room temperature prior to harvest. Control cells were preincubated with only Earle's balanced salt solution. A23187 was dissolved in ethanol at a concentration of 2 mM, and 20 μl of this solution were added to the preincubated cells where indicated 4 min prior to harvest. After the harvested cells were sonicated, 0.75 ml of the sonicate was mixed with 0.15 ml of 30% trichloroacetic acid. Cyclic [$^3$H]AMP was added as a tracer and the sonicate was precipitated by centrifugation at 2000 rpm for 10 min in a Sorvall table-top centrifuge. The resulting supernatant was then assayed for cyclic AMP content by radioimmunoassay as described under Experimental Procedures. The values listed for cyclic AMP levels represent the mean taken from the range.

| Drug | Concentration in medium (mM) | Cyclic AMP (pmoles/mg protein) | Range | No. of experiments performed in triplicate |
|---|---|---|---|---|
| 1. Control | — | 9.49 | 9.28–9.70 | 2 |
| 2. Ethanol | 0.002% | 9.89 | 9.69–10.09 | 3 |
| 3. A23187 | 0.004 | 9.83 | 9.80–9.86 | 2 |
| 4. IBMX | 0.1 | 12.95 | 12.65–13.25 | 2 |
| 5. Ro 20-1724 | 0.1 | 14.80 | 14.86–15.02 | 2 |
| 6. Chlorpromazine | 0.5 | 8.59 | 8.30–8.88 | 2 |
| 7. *cis*-Chlorprothixene | 0.2 | 8.51 | 8.18–8.84 | 2 |
| 8. *trans*-Flupenthixol | 0.2 | 8.90 | 8.61–9.19 | 2 |
| 9. IBMX + A23187 | 0.1<br>0.004 | 12.05 | 11.85–12.25 | 2 |

*Cyclic AMP determinations.* All of the compounds listed in Table 3 were tested for their effect on intracellular cyclic AMP levels at the same concentration which produced an activation of phosphorylase conversion. Three of the seven active neuroleptic compounds were tested, and all three compounds lowered cyclic AMP levels in C6 cells relative to control or untreated cells (Table 3). The cyclic AMP concentration was lowered to 8.51–8.90 pmoles/mg of protein by these compounds relative to 9.49 pmoles/mg of protein for control cells. This result indicates that the activation of phosphorylase by these compounds cannot be mediated by a cyclic AMP dependent pathway. In contrast, both IBMX and Ro 20-1724 elevated cyclic AMP levels, to 12.95 and 14.80 pmoles/mg of protein, respectively, so it is not likely that the neuroleptics have the same mechanism of action as these phosphodiesterase inhibitors. The higher cyclic AMP concentration observed with Ro 20-1724 is compatible with the higher activation of phosphorylase conversion seen with this compound. A23187 and its corresponding solvent, ethanol, had no significant effect on cyclic AMP concentrations.

The above results are compatible with the earlier findings of Drummond *et al.* (1), who reported that a 1.4-fold increase in cyclic AMP converted 36% of the phosphorylase to the A form. Although these investigators found a slight lowering of cyclic AMP with A23187, no change in cyclic AMP levels were observed in the present experiments. This is only a minor discrepancy and may be due to the cyclic AMP determination method used. The use of IBMX and A23187 in combination resulted in an elevation of cyclic AMP comparable to that seen with IBMX alone, although the combined effect of these two compounds is a partially additive phosphorylase conversion from the B to the A form.

## DISCUSSION

Previous work using C6 glioma cells has shown that the conversion of phosphorylase B to A can progress by a cyclic AMP-mediated process and also, when the stimulant is the $Ca^{2+}$ ionophore A23187, by a non-cyclic AMP-dependent mechanism (1). The purpose of this study was to determine whether calmodulin-inhibiting compounds could block the $Ca^{2+}$-dependent activation by A23187 of phosphorylase conversion. Surprisingly, the calmodulin-inhibiting compounds were found to activate the conversion of phosphorylase B to A in C6 cells by themselves. This was surprising since calmodulin is a subunit of phosphorylase kinase and it would be expected that a calmodulin-inhibiting drug would inhibit rather than stimulate phosphorylase B to A conversion in intact cells. To examine the possibility that the activation of phosphorylase conversion by calmodulin inhibitors occurs through a cyclic AMP-mediated mechanism, the inhibition of $Ca^{2+}$/calmodulin-dependent phosphodiesterase activity by these drugs was measured. Although $Ca^{2+}$/calmodulin-stimulated phosphodiesterase activity was inhibited by these compounds at concentrations similar to those required for phosphorylase conversion, the levels of cyclic AMP in C6 cells were found to decrease after treatment with these drugs. This result indicates that the stimulation of phosphorylase conversion by calmodulin-inhibiting compounds is not due to a cyclic AMP-mediated mechanism.

The $Ca^{2+}$ ionophore A23187 allows the entry of $Ca^{2+}$ into the cell and is presumed to activate phosphorylase kinase by increasing cytoplasmic $Ca^{2+}$ concentrations (1). The effects of the calmodulin inhibitors and A23187 on phosphorylase conversion are not additive, and this suggests that both may have the effect of increasing cytoplasmic $Ca^{2+}$ concentrations. Several reports exist which indicate that local anesthetic amines such as chlorpromazine adsorb to biomembranes and displace $Ca^{2+}$ from negatively charged sites of the membrane at concentrations of $10^{-5}$ M–$10^{-4}$ M (15). This might be a mechanism whereby chlorpromazine and other phenothiazine compounds could elevate cytoplasmic $Ca^{2+}$ concentrations. Also, chlorpromazine and perphenazine can inhibit $Ca^{2+}$ uptake by isolated brain mitochondria at concentrations of $10^{-5}$ M–$10^{-4}$ M (16). This might be another mechanism by which calmodulin-inhibiting compounds could increase cytoplasmic $Ca^{2+}$ concentrations. Another mechanism whereby these neuroleptic drugs could elevate the intracellular $Ca^{2+}$ concentration is through the selective inhibition of a membrane-associated calmodulin-stimulated $Ca^{2+}/Mg^{2+}$-ATPase similar to that described in erythrocytes (17, 18). Calmodulin inhibition by these drugs should result in the blockade of ATPase as well as phosphorylase kinase. Since phosphorylase B to A conversion can be stimulated by these calmodulin antagonists in C6 cells, it is unlikely that phosphorylase kinase is inhibited at the concentration of drug used in the medium. Therefore, only the selective inhibition of membrane-associated calmodulin could explain an increase in the cytoplasmic $Ca^{2+}$ concentration without inhibiting phosphorylase kinase.

The finding that three calmodulin-inhibiting drugs cause a reduction in cyclic AMP levels in C6 cells has interesting implications for the regulation of phosphodiesterase in intact cells. At least one form of adenylate cyclase and cyclic AMP phosphodiesterase in C6 glioma cells are activated by calmodulin *in vitro* (14, 19). Therefore, the inhibition of calmodulin by neuroleptic drugs should reduce the activity of both enzymes *in vitro* as well as in intact C6 glioma cells. In these experiments cyclic AMP levels were reduced after treatment with calmodulin-inhibiting compounds, suggesting that adenylate cyclase is regulated by calmodulin under physiological conditions. The form of phosphodiesterase which is activated by $Ca^{2+}$-calmodulin accounts for almost one-half of the total specific activity of phosphodiesterase in C6 cells and is inhibited by calmodulin inhibitors *in vitro*, yet the reduced cyclic AMP levels observed in C6 cells after drug treatment indicate that this form of phosphodiesterase may not be physiologically important. Our findings agree with the results of Brostrom *et al.* (20), who found that cyclic AMP concentrations are reduced in C6 cells after treatment with trifluoperazine. However, in rabbit ileum, $10^{-4}$ M trifluoperazine was found to have no effect on cyclic AMP concentrations (21), but calmodulin has not been described as regulating adenylate cyclase in this tissue.

This study provides evidence that calmodulin-inhibiting compounds can activate phosphorylase B to A con-

version in intact cells and illustrates some of the problems involved with the use of neuroleptic compounds as inhibitors of calmodulin in tissue culture cells. Care should be taken not to implicate calmodulin in a defined physiological process simply because this process is perturbed by one of these neuroleptic compounds, such as trifluoperazine or chlorpromazine. Some $Ca^{2+}$-dependent secretory processes are inhibited by trifluoperazine (21, 22) as well as vasopressin-stimulated water permeability in bladder (23, 24). Other investigators have demonstrated that trifluoperazine can produce changes in platelet shape in the absence of platelet-aggregating agents (25) and can block responses of Hela cells to tumor promoters (26). Many of these reports do not consider the toxic effects of phenothiazines which may be related to their $Ca^{2+}$ displacement and uptake inhibition effects. Recently Tsao *et al.* (27) examined the effect of chlorpromazine on isolated hepatocytes and found that 5 μM chlorpromazine can produce significant changes in plasma membrane permeability whereas concentrations of 100 μM can cause the release of cytoplasmic and mitochondrial enzymes. These toxic effects are greater at concentrations above 100 μM and may account for the inability of the C6 cells used in these experiments to be successfully passaged after treatment with neuroleptic drugs at concentrations greater than 100 μM. In spite of the toxic effects of phenothiazines and a report that they cause a reduction in intracellular ATP concentration (28), the C6 cells in these experiments remained sufficiently intact to allow the activation of phosphorylase B to A conversion after drug treatment. Although viability is reduced at drug concentrations greater than 100 μM, an activation of phosphorylase B to A conversion can occur at drug concentrations as low as 10 μM, where some toxic effects can begin. It is concluded that neuroleptic drugs are unsuitable as probes for calmodulin function in cell culture because of their potential for perturbing intracellular $Ca^{2+}$ concentrations and exerting cytotoxic effects. Recently Landry *et al.* (29) have suggested that the membrane-stabilization effects observed with neuroleptic calmodulin inhibitors may account for many of the diverse biological phenomena in which calmodulin has been implicated. Another approach to ellucidating the physiological role of calmodulin may come from the introduction of calmodulin-neutralizing antibody into cells. This has been attempted by Hall *et al.* (30), who used liposomes for the introduction of calmodulin antibody into mouse adrenal tumor cells. The development of a potent calmodulin-inhibiting compound which does not have the side effects of $Ca^{2+}$ displacement, $Ca^{2+}$ uptake inhibition, and membrane stabilization may provide a valuable probe of calmodulin's physiological function as well as a useful therapeutic agent. However, such a compound is not presently available.

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