

MITOGEN-STIMULATED GLUCOSE TRANSPORT IN THYMOCYTES

Possible Role of Ca^{++} and Antagonism by Adenosine 3':5'-Monophosphate

RICHARD R. WHITESELL, ROGER A. JOHNSON, HAROLD L. TARPLEY,
and DAVID M. REGEN

From the Department of Physiology, Vanderbilt University School of Medicine, Nashville,
Tennessee 37232

ABSTRACT

The plant lectin, concanavalin A (Con-A), and the ionophore, A-23187 (specific for divalent cations), stimulated glucose transport in rat thymocytes. Con-A stimulation developed more slowly and was somewhat less extensive than that of A-23187. Both responses showed saturation dose dependencies. The two responses were poorly additive, suggesting that A-23187 may saturate regulatory processes shared by the two stimulatory mechanisms.

Doses of methylisobutylxanthine (MIX) and prostaglandin E_2 which raised adenosine 3':5'-monophosphate (cAMP) levels in these cells also antagonized the Con-A stimulation of glucose transport but did not inhibit basal glucose transport or the A-23187 stimulation. Dibutyryl-cAMP and 8-bromo-cAMP also antagonized Con-A stimulation without inhibiting basal glucose transport. MIX antagonized high Con-A doses about as strongly as it did low Con-A doses, suggesting that MIX did not compete in the Con-A binding step or other process saturable by Con-A. [^3H]Con-A binding was not affected by MIX.

The stimulatory effects of Con-A and A-23187 were reduced by reduction of Ca^{++} in the medium. Both Con-A and A-23187 enhanced $^{45}\text{Ca}^{++}$ influx and cellular Ca^{++} content. The A-23187 dose, which was saturating for glucose transport stimulation, enhanced Ca^{++} influx and cellular Ca^{++} content more than did the Con-A dose which was saturating for glucose transport stimulation. The dose of MIX which specifically antagonized Con-A stimulation of glucose transport proved also to reduce Ca^{++} influx and cellular Ca^{++} in the presence of Con-A but not in the presence of A-23187. Thus, glucose transport correlates rather well with cellular Ca^{++} .

These results are compatible with the view that Ca^{++} in a cellular compartment can promote glucose transport, that Con-A's enhancement of Ca^{++} entry contributes to its stimulation of glucose transport, and that MIX antagonizes Con-A action at least partly by reducing Ca^{++} entry. The action of MIX is apparently mediated by cAMP.

Lymphocyte proliferation in response to phytohemagglutinins is of considerable interest as a model for the proliferative events in the immune response (2). The earliest effects of such mitogens include enhancement of Ca^{++} influx (5, 24) and of glucose transport (12). Lymphocytes from the mouse spleen become committed to mitosis during the 1st day of exposure to concanavalin A (Con-A), and DNA synthesis rises during the 2nd day (7). The commitment appears to involve microtubules (19). The proliferative response of rat thymocytes can be seen within an hour of Con-A exposure (23). The enhanced Ca^{++} influx has been implicated as mediator of the proliferative response by the fact that lymphocytic mitosis can be induced by elevation of medium Ca^{++} concentration (23) and by addition of A-23187, an ionophore for divalent cations (10). Adenosine 3':5'-monophosphate (cAMP) appears to be an antagonist of proliferation in many cell types including the lymphocyte (2, 8, 16, 25) and to antagonize Con-A's stimulation of glucose transport in this cell (21). Our work explores the relationship of cAMP and Ca^{++} flux to Con-A stimulation of glucose transport.

MATERIALS AND METHODS

Preparation of Cells

Male Sprague-Dawley rats, weighing about 200 g, were obtained from Harlan Industries (Indianapolis, Ind.) and fed Purina Lab Chow (Ralston Purina Co., St. Louis, Mo.). The rats were killed by decapitation, their thymus glands removed and placed in 20 ml of 0.9% saline at room temperature. The saline was then poured off and replaced by 2 ml of incubation buffer (buffered balanced salt solution [BBSS] described below) in which the glands were minced. The mince was transferred to a loose-fitting Dounce homogenizer (Kontes Co., Vineland, N. J.) with 20 ml of buffer in which the fragments were crushed. The resulting cell suspension was filtered through nylon net and layered on 5 ml of BBSS containing 7% Ficoll (Pharmacia, Uppsala, Sweden) in a 50-ml conical centrifuge tube. This was centrifuged for 45 s in a size 2 International centrifuge (International Equipment Co., Needham Heights, Mass.) set on full speed (approaching 7,000 g). The supernatant fluid was removed and the pellet resuspended in 5 ml of BBSS and transferred to a 100-ml beaker with 0.1 μCi L-[U- ^{14}C]isoleucine (2.5 mCi/mg). The above-mentioned steps were all taken at room temperature. After 10 min of incubation with shaking at 37°C, the cell suspension was layered again on 7% Ficoll and centrifuged. Supernatant fluid was removed and cells were resuspended in BBSS at

4–6% packed cell volume (PCV). A 0.5-ml portion was centrifuged for 30 min at 7,000 g in a Hopkins vaccine tube (Kimble Products Div., Owens-Illinois, Toledo, Ohio) for PCV determination, and a 100- μl portion was assayed for cell ^{14}C by the procedures of Fig. 1. Since cell ^{14}C was reasonably stable (Fig. 2 and Table I), the ^{14}C measured in subsequent cell samples enabled us to detect sampling errors or cell injury. The relation between cell volume and cell count was determined in several experiments and found to be about 5×10^6 cells per ml of packed cells.

Buffered Balanced Salt

Solution (BBSS)

BBSS was prepared from isotonic salt solutions (0.31 osmol/liter) in the proportions: 114 NaCl, 5 KCl, 2 CaCl_2 , 1 MgSO_4 , 2 NaPO_4 (pH 7.3), 4 Na-HEPES (*N*-2-hydroxy-ethylpiperazine-*N'*-2-ethane sulfonic acid) (pH 7.3). Na- β -hydroxybutyrate (1 mM) and Na-lactate (5 mM) were added with the aim of minimizing energy deprivation during incubations without the preferred fuel, glucose. Bovine serum albumin (0.2%) was added, and the pH of the final solution checked at 37°C (pH 7.2–7.3). The reported effects of Con-A and methylisobutylxanthine (MIX) on glucose transport were not obliterated by reductions or increases in the inorganic ions, HEPES, fuels, albumin, nor by alteration of pH between 7.1 and 7.4.

Agents Used

The carboxylic ionophorous antibiotic, A-23187, a gift of Dr. Roger Hamill at Eli Lilly and Co. (Indianapolis, Ind.) was used from stock solutions of 0.1 mg/ml prepared in 95% ethanol or 30% dimethylsulfoxide. Neither dimethylsulfoxide nor ethanol had an effect on basal transport at the final concentrations realized, which were under 3%.

MIX, obtained from G. D. Searle and Co. (Arlington Heights, Ill.), Con-A, grade V, and α -methylmannoside, both obtained from Sigma Chemical Co. (St. Louis, Mo.) and prostaglandin E_2 (PGE_2), a gift of Dr. John Pike, at Upjohn Co. (Kalamazoo, Mich.) were all used from freshly prepared aqueous solutions.

Radiochemicals, L-[U- ^{14}C]isoleucine, D-3-O-[methyl- ^3H]methylglucose (methylglucose) and [^3H]Con-A, were obtained from New England Nuclear (Boston, Mass.). Unlabeled methylglucose was prepared as described by Crane et al. (3). L-[4,5- ^3H]isoleucine (18 Ci/mmol) was obtained from Amersham/Searle Corp. (Arlington Heights, Ill.) and ^{45}Ca (13 Ci/g) from New England Nuclear.

Assay of Transport Activity

Aliquots of the cell suspension (200 μl each) were distributed among small counting vials (1.4 \times 5-cm inner

dimensions) containing 50 μ l of BBSS with or without the agents, the effects of which were to be tested. These were incubated with shaking in a water bath at 37°C. At the time of the transport test, 20 μ l of 15 mM [3 H]methylglucose (10 μ Ci/ μ mol) was added. After 0.6 min (or other times where indicated), a 0.2-ml sample was transferred quickly into 3 ml of ice cold 0.9% NaCl-2 mM CaCl₂ layered over 9 ml of ice cold 10% sucrose-0.2% NaCl-2 mM CaCl₂ in a 12-ml conical centrifuge tube (imbedded in ice almost to the lip).¹ These tubes were then centrifuged for 5 min at 5,000 g in an International PR-2 centrifuge (International Equipment Co.) refrigerated at 2°C. The upper 5 ml was aspirated and the upper walls rinsed for about 7 s (from the lip down to the aspiration level); then the next 1.5 cm of supernatant fluid was aspirated and the upper walls were again rinsed; then another 1.5 cm of supernatant fluid was removed and the walls above this level were rinsed. The rinse solution was saline containing 0.005% Triton X-100 (Rohm & Haas Co., Philadelphia, Pa.). A diagram of the apparatus is given in Fig. 1. The remaining 4 cm of supernatant fluid was then aspirated, and the pellet was transferred in 1 ml of water through a tube to a counting vial.² 10 ml of liquid scintillation counting fluid was added through this route to rinse in the sample. This was counted in a Packard Tri-Carb liquid scintillation spectrophotometer (Packard Instrument Co., Inc., Downers Grove, Ill.) set for 3 H and 14 C determinations. The counting fluid was a 2:1 (vol:vol) mixture of toluene and Triton X-100 containing 0.5% 2,3-diphenyloxazole and 0.01% dimethyl-1,4-bis[2-(5-phenyloxazolyl)]benzene.

After removal from the incubation vial of the 200- μ l sample for determination of cell radioactivity, a 20- μ l sample was also transferred directly to a counting vial for determination of the total 3 H (cellular plus extracellular) per milliliter of incubating cell suspension. This was divided into the cellular 3 H (per milliliter cell suspension), yielding the fraction of [3 H]methylglucose which was inside cells ($\text{MeGlc}_i/\text{MeGlc}_t$)—an expression which is normalized with respect to variations in added [3 H]methylglucose. This expression was divided by PCV in the incubation vial. This procedure normalized the data with respect to day-to-day variations in PCV. Since more than 95% of the volume was extracellular, this

¹ The CaCl₂ was originally added in 45 Ca-uptake experiments in the hope of reducing the amount of quickly exchangeable 45 Ca associated with the cells. The CaCl₂ appeared to protect the cells, and we continued to add it routinely. Before the studies reported here, we successfully used 7% bovine serum albumin in 0.9% NaCl as a lower layer. This became too expensive.

² This system achieves thorough removal of extracellular radioactivity from cellular radioactivity. Studies of the temperature dependence of transport indicated that cooling stopped transport effectively and that addition of phloretin to these gradients was not necessary.

expression is essentially the same as distribution space (milliliters of medium containing the amount in a milliliter of cells), and its initial rate of rise is essentially the same as the influx coefficient (milliliters of medium containing the amount fluxing into a milliliter of cells per minute). In most experiments, methylglucose uptake after treatment with various agents was divided by that in controls to show directly the fold effect of the agent.

cAMP Determinations

From incubation vials containing 0.55 ml of cell suspensions, 0.3-ml samples were transferred to tubes containing 0.5 ml of 0.6 M perchloric acid and 8,000 cpm [G - 3 H]cAMP (24 Ci/mmol, Amersham/Searle Corp.) and mixed immediately. The extract was placed on a cation exchange column (0.7 \times 10 cm, Bio-Rad AG50-X8 (Bio-Rad Laboratories, Richmond, Calif.), 100–200 mesh, equilibrated with 0.1 N HCl) and eluted with 0.1 N HCl. The cAMP-containing eluent (13th–37th ml) was lyophilized and the residue was dissolved in 0.5 ml of 50 mM Na-acetate, pH 4.5. A 100- μ l portion was counted for determination of recovery, and portions were subjected to cAMP assay by the Gilman method (6). The material assaying as cAMP was more than 90% susceptible to destruction by cyclic nucleotide phosphodiesterase.

Calcium Flux

The uptake of 45 Ca⁺⁺ was measured essentially as was that of methylglucose, except that [3 H]isoleucine was used as a cell marker instead of [14 C]isoleucine, and samples were taken at times other than 0.6 min.

RESULTS

Adequacy of Procedures

Fig. 2 shows the accumulation of 14 C in cells during a 10-min exposure to [14 C]isoleucine and the maintenance of cell 14 C during a 30-min incubation after removal of extracellular 14 C. The fraction of 14 C taken up was several times the PCV (fraction of volume occupied by cells). By the 10th min of exposure, most of the cellular label was incorporated into acid-precipitable material, presumably macromolecules. Most of the acid-precipitable and acid-soluble radioactivity remained in the cells during subsequent incubation without external 14 C-amino acid, except perhaps when cycloheximide was added.

In other experiments, cell 14 C appeared to be perfectly stable for more than an hour in control cells and cells incubated with 30 μ g/ml Con-A or 0.1 mM MIX. Table I confirms the failure of Con-A and MIX to affect 14 C retention. The ionophore at 1.4 μ M reduced cell 14 C by about 7%. We don't

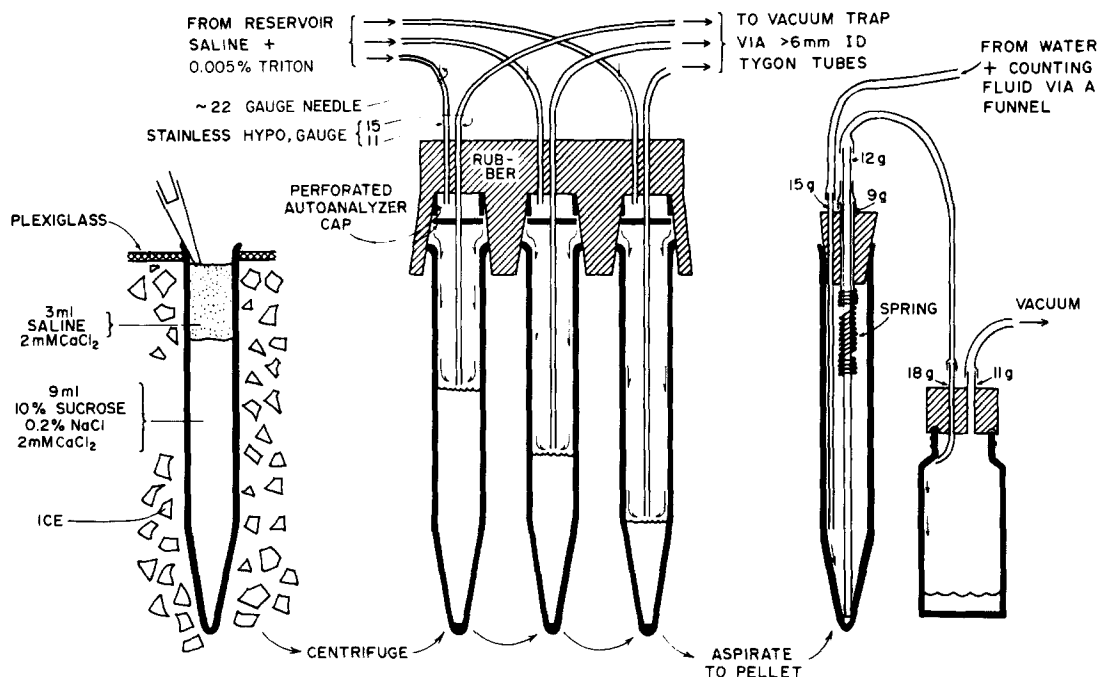


FIGURE 1 Apparatus used in assay of cellular radioactivity. The symbol *g* stands for gauge.

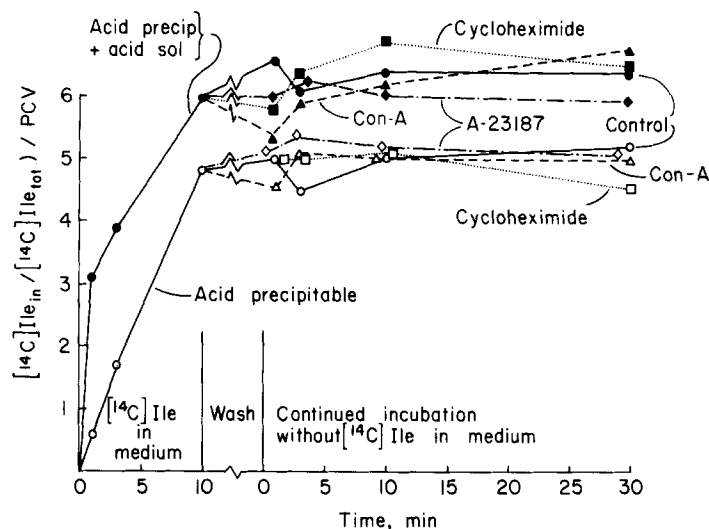


FIGURE 2 Labeling of cells with [¹⁴C]isoleucine. Cells were incubated with [¹⁴C]Ile, washed in Ficoll, resuspended and incubated as described in Materials and Methods. Con-A (20 μg/ml), A-23187 (0.93 μM), and cycloheximide (8 μg/ml) were present during the second incubation where indicated. Samples were then centrifuged through sucrose gradients, and extracellular fluid was removed in the usual way. Cell pellets were brought up in 0.5 ml of water, and 1 ml of 0.6 M perchloric acid was added. A portion of supernate was neutralized with K₂CO₃ and 1 ml of neutral extract was counted. After removal of residual acid supernate, the pellet was taken up in 1 ml of 0.2 M NH₄OH and transferred to a counting vial.

know whether this represents destruction of 7% of the cells or 7% leakage from all cells. We assumed the former and used correspondingly lower PCVs

in the uptake calculations. If this assumption is wrong, then we have overestimated rates in the presence of 1.4 μM A-23187 by about 8%.

TABLE I
Effects of Various Agents on Retention of [¹⁴C]Isoleucine Label by Thymocytes Incubated for 1 h without External [¹⁴C]Isoleucine

Agent	¹⁴ C in Cells, exptl/control
Con-A, 30 μg/ml	0.99 ± 0.01 (20)*
A-23187, 0.75 μM	0.96 ± 0.03 (10)
A-23187, 1.4 μM	0.93 ± 0.02 (8)
MIX, 0.1 mM	1.00 ± 0.02 (17)

* Ratios are given ± standard errors with numbers of observations in parentheses.

The time-course of methylglucose uptake (Fig. 3) justifies some of our routine procedures. It is seen that methylglucose accumulated steadily during the first 2 min. Accumulation was nearly linear before 1 min; and 0.6-min samples should, therefore, faithfully reflect initial accumulation rates. Extrapolation indicates nearly zero cellular methylglucose at 0 min, which attests to the effectiveness of the gradient system (Fig. 1) in removing extracellular radioactivity from the cells. Inadequate washing would result in a positive y intercept.

The initial slope of the control line is about 0.085/min (i.e., 0.085 ml of the system was cleared by entry/min per ml cell volume, which is approximately the same as 0.085 μmol entering/min per ml cells per mM concentration). There is considerable day-to-day variation in uptake but little variation among triplicates on a given day. 10 substrate dependency studies (each with 0.1, 3, 9, and 15 mM extracellular methylglucose) indicated a V_{max} of 0.64 μmol/min per ml cell volume and a K_m of 7.7 mM. Accordingly, a typical rate with 0.2 mM concentration would be $(0.64 \mu\text{mol}/\text{min} \cdot \text{ml}) \cdot 0.2 \text{ mM} / (0.2 \text{ mM} + 7.7 \text{ mM}) = 0.0162 \mu\text{mol}/\text{min} \cdot \text{ml}$. The entry rate divided by concentration would be 0.081 μmol/min per ml cells per mM concentration. Thus, the control data of Fig. 3 (initial slope of 0.085/min) are typical. The uptake rates of Figs. 9 and 11 were slower than this, but were consistent within each series of experiments.

Dose-Response Relations

The dependence of methylglucose permeability on Con-A concentration (Fig. 4) appears to be one of uncomplicated saturation. For most of our work, we have chosen to use 20–30 μg/ml, the lowest doses which are nearly maximally effective. 80–100-μg/ml doses usually produced agglutina-

tion, without further effect on glucose transport, as judged from the extended plateau. The above-mentioned features of the dose dependency were reproducible, though considerable day-to-day variation in the degree of response was experienced (cf., for example, Figs. 6, 7, etc.). An experiment to determine the maximal response to A-23187 and the doses required for near maximal response is shown in Fig. 5. The threefold stimulation is typical of this agent (cf. Figs. 6, 7, etc.), and a dose of 1.4 μM was nearly maximal. This dose was used for several studies in which A-23187 dose was not a variable. In other studies in which A-23187 doses below 1.4 μM were used, it appeared that 0.6 μM was maximally effective (Figs. 7 and 11) and that doses below 0.2 μM were ineffective (Fig. 11).

Time-Courses

As seen in Fig. 6, stimulation by A-23187 was nearly complete by 20 min of exposure, after which little further stimulation occurred. Con-A stimulation was typically slower in onset, as seen in this experiment and Figs. 10 and 12. In further studies in which time was not a variable, 1-h exposure was used, a time when A-23187 action was complete but Con-A action may not have been.

Additivity of Stimulation

In the experiment of Fig. 7, methylglucose entry was about doubled by 1 h exposure to Con-A and

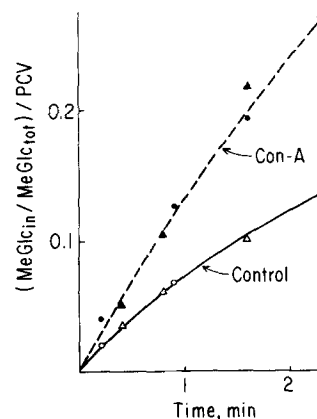


FIGURE 3 Time-course of methylglucose uptake. Where indicated, the cells were exposed to Con-A (30 μg/ml) for 1 h before addition of 0.2 mM [³H]-methylglucose (MeGlc). Controls were also incubated 1 h before [³H]-methylglucose addition. Two separate experiments are shown, one represented by circles (○, ●), the other by triangles (△, ▲).

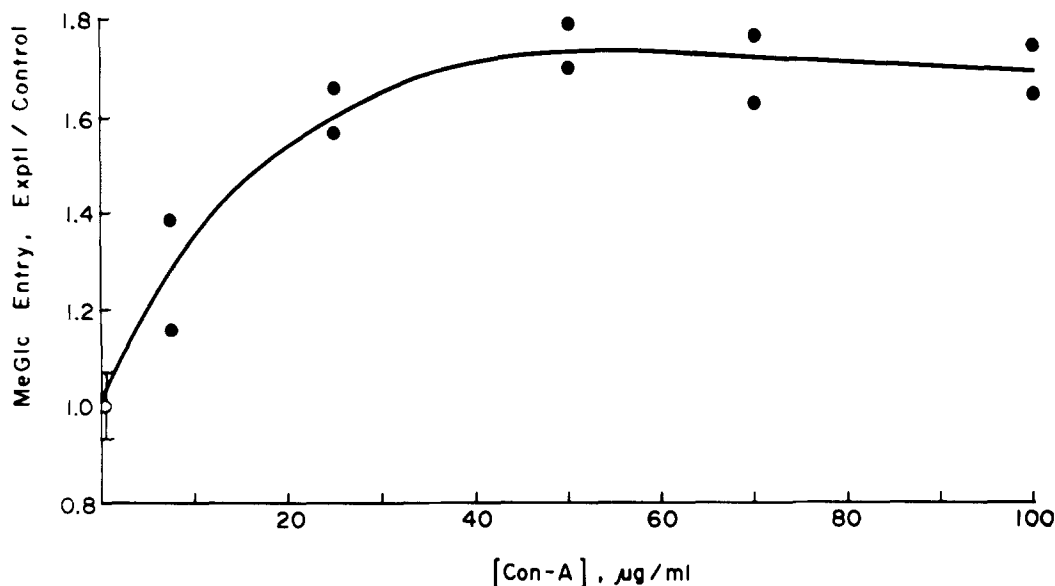


FIGURE 4 Dependence of methylglucose transport on Con-A concentration. In duplicate incubations, cells were exposed to Con-A for 1 h before transport tests. As shown on the ordinate, the 0.6-min ^3H methylglucose uptake in each vial was divided by the mean ^3H methylglucose uptake in triplicate controls (O, standard error). The controls showed a mean 0.6-min $(\text{MeGlc}_m/\text{MeGlc}_{tot})/\text{PCV}$ of 0.042.

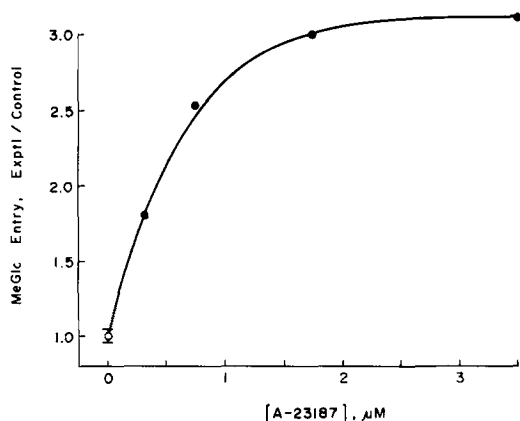


FIGURE 5 Dependence of methylglucose transport on A-23187 concentration. Cells were exposed to the agent for 1 h before transport tests. Data are expressed as in Fig. 2. A-23187 was dissolved in 30% dimethylsulfoxide, giving final concentrations less than 3%, a concentration which did not affect transport in the absence of A-23187. Similar results were obtained with A-23187 dissolved in 95% ethanol (final concentration < 3%). This solvent was, by itself, also without effect on glucose transport. The controls showed a mean 0.6-min $(\text{MeGlc}_m/\text{MeGlc}_{tot})/\text{PCV}$ of 0.050.

was nearly tripled by A-23187 doses as low as $0.56 \mu\text{M}$. The two agents together were not perceptibly more stimulatory than A-23187 alone.

The simplest interpretation of nonadditivity is that the stronger stimulus nearly saturated a response mechanism common to the two stimuli. Less parsimonious would be the view that one agent interfered with the other by a mechanism unrelated to its action as a transport stimulus. The experiment rules out completely independent actions of the two agents along with certain relations that would lead to synergism.

cAMP-Related Agents

We have observed that several agents which raise cAMP also antagonize Con-A stimulation of sugar permeability. Fig. 8a shows that MIX concentrations between 30 and $200 \mu\text{M}$ markedly reduce Con-A stimulation without affecting basal or A-23187-stimulated transport. The peculiar sensitivity of Con-A-stimulated transport is not due to enhancement of MIX's action to raise cAMP, as shown in Fig. 8b. It appears, then, that cAMP may antagonize the stimulatory action of Con-A but does not interfere with the function of the glucose carrier (confirmed also in Figs. 9 and 10). If, as suggested above (and below), the stimulatory sequences of Con-A and A-23187 converge to a common final mechanism, the step antagonized by MIX (or cAMP) is likely before the convergence.

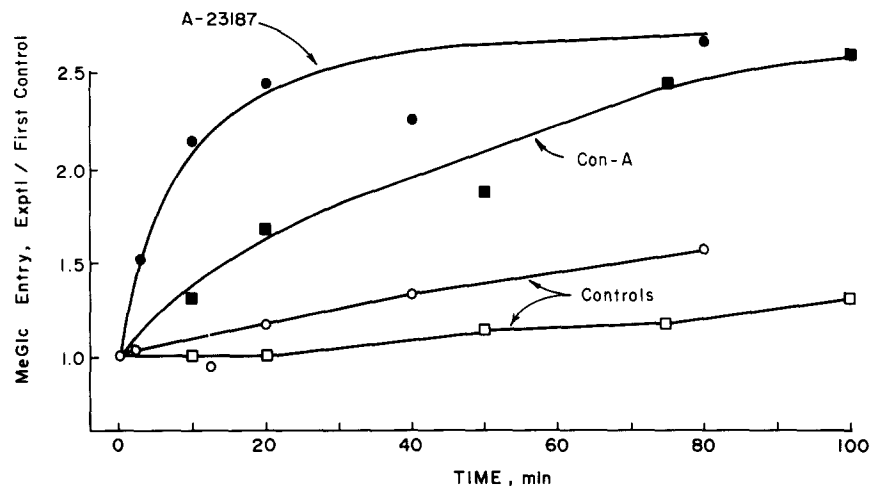


FIGURE 6 Time-course of A-23187 (●) and Con-A (■) stimulation. Two separate experiments are shown, 1.4 μ M A-23187 and its control (● and ○), and 20 μ g/ml Con-A and its control (■ and □). They are plotted together for comparison. Single vials were assayed for [3 H]methylglucose uptake at the times indicated. Uptake in each vial was divided by that in the earliest control. Note that Fig. 10 confirms the distinction between time-courses of A-23187 and Con-A stimulation. The first control for the A-23187 time-course showed a 0.6-min ($\text{MeGlc}_{\text{in}}/\text{MeGlc}_{\text{tot}}$)/PCV of 0.040. The first control for the Con-A time-course was 0.045.

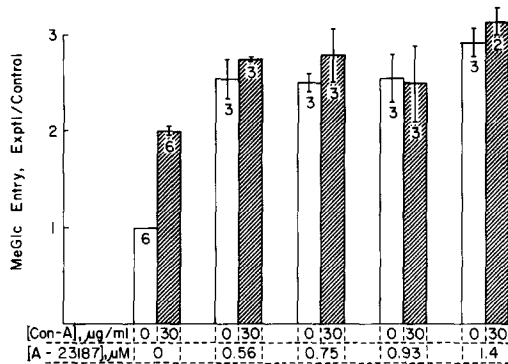


FIGURE 7 Test for additivity of Con-A and A-23187. The agents were added at the concentrations indicated below the bars. Data are expressed as in Fig. 2. Number of vials and standard errors are shown at the top of each bar. The controls showed a mean 0.6-min ($\text{MeGlc}_{\text{in}}/\text{MeGlc}_{\text{tot}}$)/PCV of 0.042.

To affirm that the phosphodiesterase inhibitor, MIX, antagonized Con-A stimulation by raising cAMP, we also studied the effects of PGE_2 which raises cAMP by stimulating adenylate cyclase (1). Concentrations in the range of 10^{-7} – 10^{-5} M PGE_2 reduced Con-A stimulation by 30–40% without affecting basal methylglucose permeability. This range also increased cAMP from control values of

1.9 ± 0.2 nmol/ml cell volume up to 5.3 nmol/ml cell volume. Dibutyl-cAMP at 0.4, 0.7, and 1.0 mM reduced Con-A stimulation by 30, 50, and 60%, respectively, but did not inhibit basal methylglucose transport. This appeared not to be due to butyrate released from the nucleotide, since butyrate at these concentrations was equally (though mildly) inhibitory to basal and Con-A-stimulated transport. The effects of 8-bromo-cAMP were indistinguishable from those of dibutyl-cAMP, while 8-bromo-AMP failed to antagonize Con-A stimulation. It is reasonable, then, to suppose that the MIX-induced rise in cAMP contributes to MIX's antagonism of Con-A stimulation.

Fig. 9 shows that MIX antagonized stimulation caused by a very high Con-A dose as effectively as it did the stimulation by a subsaturating Con-A dose, a result which we have confirmed in other experiments of similar design. Thus, MIX antagonism seems noncompetitive.

The time-courses of Con-A and A-23187 stimulation in the presence of MIX illustrate certain points (Fig. 10). In this experiment, MIX exerted a strong and durable block to Con-A stimulation which was more obvious late than early. On the other hand, A-23187 stimulation was little affected by MIX at any time. Were MIX capable of

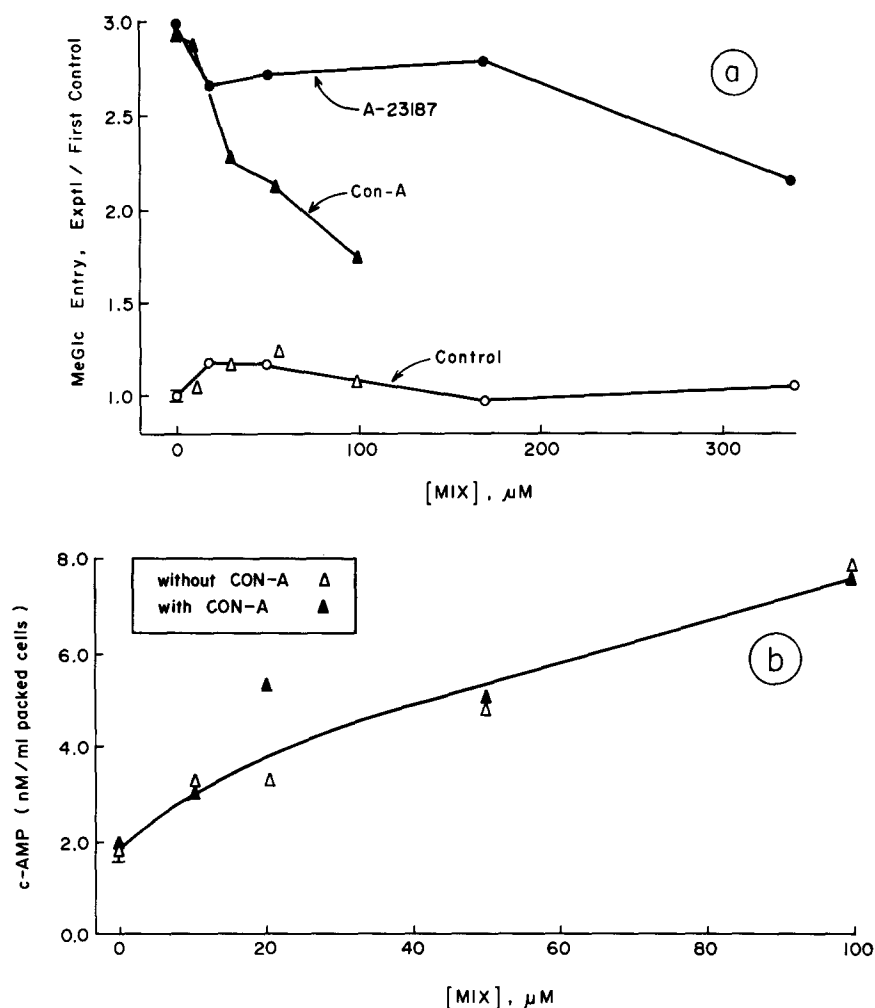


FIGURE 8 (a) MIX antagonism of Con-A stimulation and A-23187 stimulation. All agents were present for 1 h before transport test. Transport activity stimulated by $1.4 \mu\text{M}$ A-23187 (\bullet) was determined in the absence of MIX and in the presence of various concentrations of MIX. The same procedure was followed with $20 \mu\text{g/ml}$ Con-A (\blacktriangle). Unstimulated cells were also exposed to MIX (\circ , \triangle). Controls for the Con-A experiment (without Con-A, without MIX) showed a mean 0.6-min ($\text{MeGlc}_{\text{in}}/\text{MeGlc}_{\text{out}}$)/PCV of 0.046. Controls for the ionophore experiment (without A-23187, without MIX) showed a mean 0.6-min ($\text{MeGlc}_{\text{in}}/\text{MeGlc}_{\text{out}}$)/PCV of 0.043. (b) Effect of MIX on cAMP levels in absence (\triangle) and presence of Con-A (\blacktriangle). Samples for cAMP determination were taken at 10 min from the untreated and Con-A-treated incubations whose transport activity at 1 h is graphed in a. The control point ([MIX] = 0, without Con-A) shows the mean and standard error of six determinations, 1.9 ± 0.2 nmol/ml cells. This is equivalent to $38 \text{ pmol}/10^6$ cells, which is about 60% higher than reported elsewhere (23).

antagonizing any step in the A-23187 stimulatory sequence, it might have reduced the early, weak stimulus (at 10 min) even if not the late, stronger stimulus.

Fig. 11 shows A-23187 dose dependences in the absence and presence of MIX. It is seen that MIX

failed to antagonize the stimulatory effect of A-23187 at any dose and enhanced the effect of the "half-maximal" dose. This rules out the suspicion that the distinction between Con-A and A-23187, with respect to MIX antagonism (Figs. 8 and 10), was simply due to the greater strength of the A-

23187 stimulation. It appears, then, that sensitivity to antagonism by MIX defines a step in Con-A stimulation not shared by A-23187 stimulation.

Reversibility of Stimulation

When Con-A binding to cells was interrupted by addition of α -methylmannoside (7), the stimu-

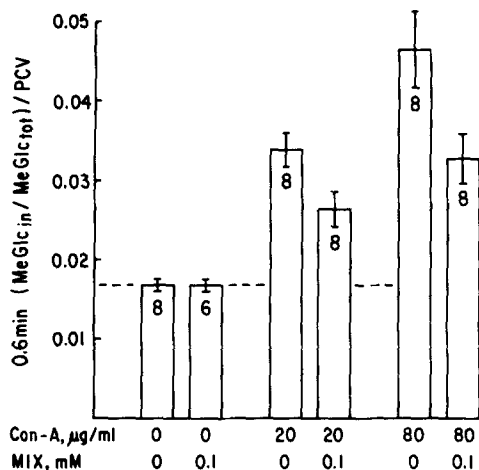


FIGURE 9 Antagonism by 0.1 mM MIX of stimulation by subsaturating and high saturating doses of Con-A. Data are expressed as the fraction of methylglucose in cells at 0.6 min divided by the fraction of the system which was cell volume (PCV). Cells were exposed to agents 1 h before transport tests. The number of experiments and standard error are shown at the top of each bar.

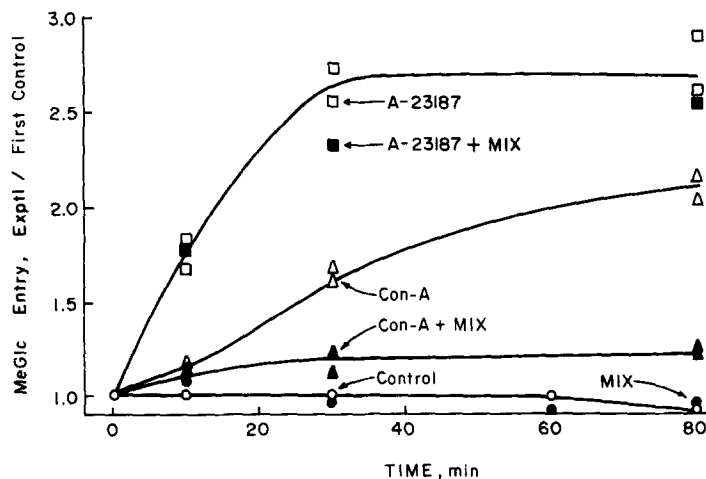


FIGURE 10 Time-course of transport elevation by 1.4 μM A-23187 (\square , \blacksquare) and 20 $\mu\text{g/ml}$ Con-A (Δ , \blacktriangle) after addition of the agents alone (open symbols) and simultaneous addition with 0.1 mM MIX (closed symbols). MIX alone (\bullet) did not affect transport at any time, nor did controls (\circ) change with time. The first control showed a 0.6-min (MeGlc_{in}/MeGlc_{tot})/PCV of 0.036.

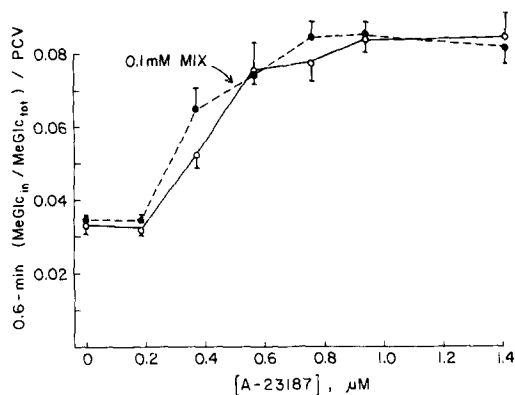


FIGURE 11 Effect of 0.1 mM MIX on methylglucose transport in presence of various concentrations of A-23187. Means and standard errors of four experiments are shown. Cells were exposed to agents for 1 h before transport tests. The stimulatory effect of MIX in the presence of 0.36 μM A-23187 was significant by pair analysis ($P = 0.01$). Symbols are: A-23187 alone (\circ), and A-23187 plus 0.1 mM MIX (\bullet).

lation declined (Fig. 12). Extrapolating slightly beyond the data, it appears that α -methylmannoside was about as effective when added at 55 min as when added at 30 or 15 min. This experiment, then, provides no evidence for a commitment to rapid transport within the 1st h that can endure after Con-A removal.

Fig. 13 shows that MIX can at least partially reverse an established Con-A stimulation. The

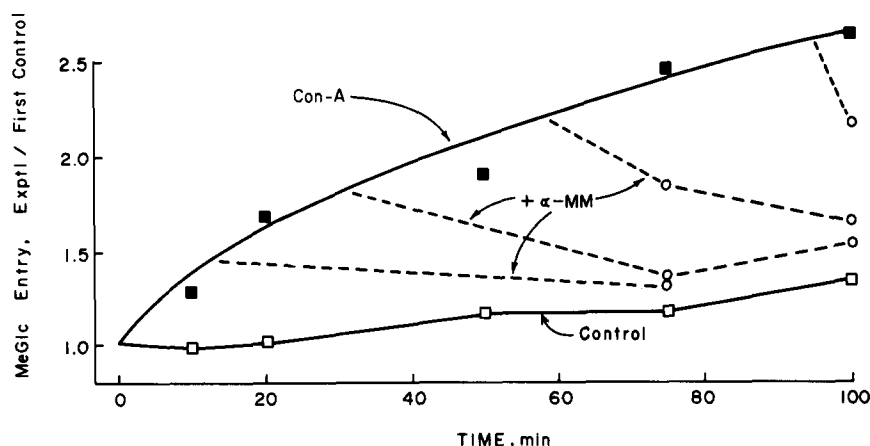


FIGURE 12 Effect of α -methylmannoside (α -MM) added at various times to Con-A-stimulated cells. Dashed lines diverge from the solid line at the times of α -MM additions (20 mM). Symbols are: control (\square), Con-A (\blacksquare), Con-A + α -MM (\circ). α -MM did not affect unstimulated transport. The first control showed a 0.6-min ($\text{MeGlc}_{\text{in}}/\text{MeGlc}_{\text{out}}$)/PCV of 0.045.

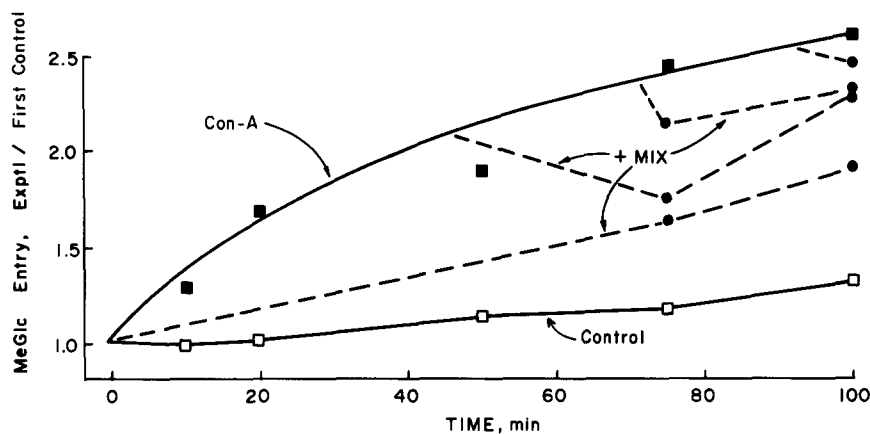


FIGURE 13 Effect of MIX added at various times to Con-A-treated cells. Symbols are: control (\square), 20 $\mu\text{g}/\text{ml}$ Con-A alone (\blacksquare), and Con-A + 0.1 mM MIX (\bullet). Dashed lines diverge from the solid line at the times of MIX addition. The first control showed a 0.6-min ($\text{MeGlc}_{\text{in}}/\text{MeGlc}_{\text{out}}$)/PCV of 0.045.

antagonistic effects of MIX added at 45 and 70 min did not progress between 75 and 100 min. These antagonistic effects, therefore, appeared complete within the period of observation and to be weaker than that of MIX added at the beginning of the incubation. The data suggest a growing recalcitrance to antagonism by MIX.

Ca⁺⁺ Influx

We have reported that omission of Ca^{++} from the medium together with addition of EDTA (equivalent to one-fourth the Mg^{++}) reduces the transport response to Con-A markedly (22). The

DNA-synthetic response is likewise dependent on extracellular Ca^{++} and is mimicked by high extracellular Ca^{++} (23). This together with the fact that A-23187 can mimic Con-A (10, 22) certainly suggests that Con-A's enhancement of Ca^{++} influx (24) contributes to the enhancements of glucose transport and mitosis. The data of Fig. 14 are compatible with this view. It is seen that A-23187 (at a concentration which saturates the glucose-transport response) increases Ca^{++} influx and cellular content by at least fourfold, whereas Con-A increases these values less than twofold. This relation correlates with the relative strengths of these

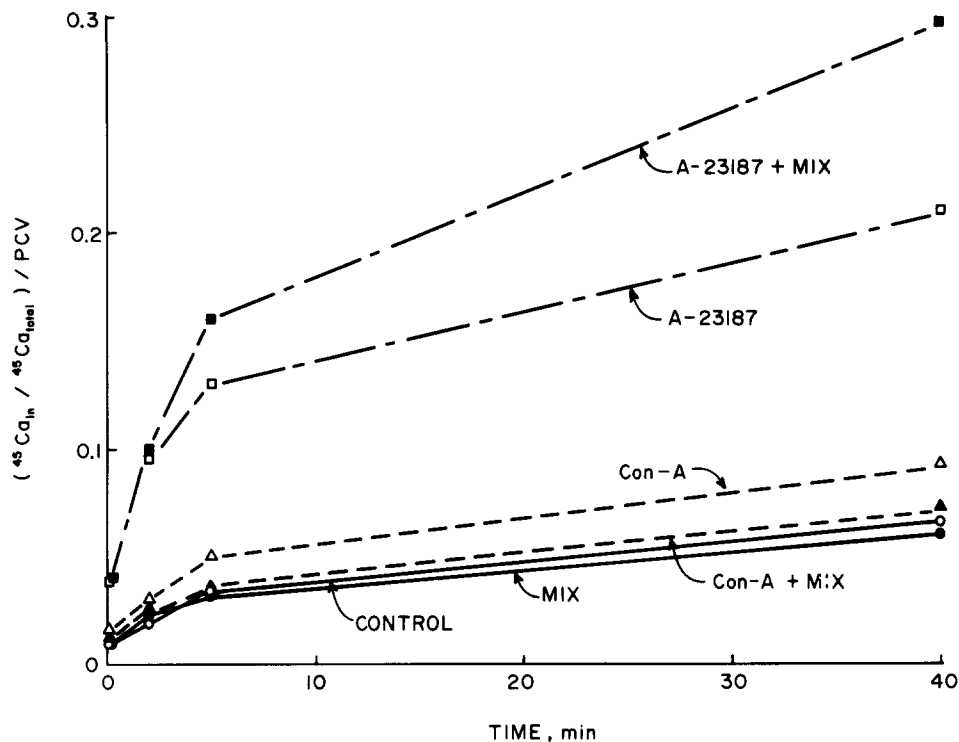


FIGURE 14 Uptake of $^{45}\text{Ca}^{++}$ into thymocytes treated with $1.4 \mu\text{M}$ A-23187, $20 \mu\text{g/ml}$ Con-A, and 0.1 mM MIX. The ordinate is fraction of $^{45}\text{Ca}^{++}$ in cells divided by the fraction of the system which was cell volume.

agents as glucose-transport stimulants. Moreover, MIX reduced Ca^{++} influx in the presence of Con-A but not in the presence of A-23187. This correlates with the susceptibility of Con-A-stimulated glucose transport to MIX antagonism and the non-susceptibility of A-23187-stimulated glucose transport. MIX actually enhanced the A-23187 stimulation of Ca^{++} influx. On the assumption that Ca^{++} influx mediates the A-23187 stimulation of glucose transport, this effect of MIX would not be expected to enhance glucose transport stimulation by A-23187 doses which are near saturation for glucose transport but may account for the enhancement by MIX of glucose transport with half-saturating A-23187 (Fig. 11).

Con-A Binding

Fig. 15 shows that, with Con-A added at $30 \mu\text{g/ml}$, normal cells absorbed and/or took up about $500 \mu\text{g}$ Con-A/ml of cells, reaching an equilibrium or steady state by 20 min. This was about one-third of the added Con-A (PCV being 2%), and a corresponding loss of one-third of the $[^3\text{H}]\text{Con-A}$

from the medium was observed. Neither A-23187 nor MIX influenced Con-A binding, whereas α -methylmannoside (α -MM) reduced it by 80%. These results indicate that MIX antagonism of Con-A stimulation is exerted at some step subsequent to Con-A binding. They also indicate that the inability of Con-A to stimulate glucose transport beyond that achieved by A-23187 (nonadditivity) was not due to lack of Con-A binding and was more likely due to saturation of the stimulatory mechanism by A-23187.

DISCUSSION

Associations of glucose-transport activity and glucose metabolism with anabolic activity (synthesis of protein, RNA, DNA, and growth and/or proliferation) are seen in such diverse systems as cardiac and skeletal muscle responding to insulin (13), uterine muscle responding to estrogen (15), cultured fibroblasts responding to optimization of pH (14), addition of serum (9) and viral transformation (18), and thymocytes responding to glucocorticoids (11). The glucose-transport response con-

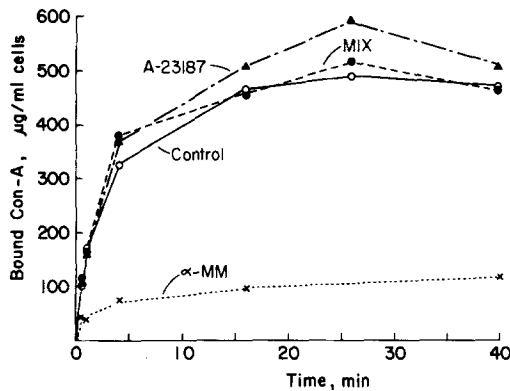


FIGURE 15 Con-A binding in presence of other agents. Binding of [^3H]Con-A was studied as a function of time without and with the following agents: 20 mM α -MM, 0.1 mM MIX, and 0.9 μM A-23187. Incubation vials were prepared with 100 μl of BBSS containing the above agents in amounts to achieve the indicated final concentrations (after addition of components described below). The nonpermeant, [^{14}C]mannitol (final concentration 10 mM, 0.1 $\mu\text{Ci/ml}$) was present as an extracellular marker. The prepared vials in a 37°C water bath received 200 μl of cell suspension in BBSS (final PCV about 2%). Binding was initiated by addition of 50 μl of BBSS containing [^3H]Con-A (0.6 $\mu\text{Ci}/\mu\text{g}$, final concentration 0.25 $\mu\text{Ci/ml}$) and unlabeled Con-A (final concentration 30 $\mu\text{g/ml}$). At the indicated times, 300- μl samples were centrifuged for 3 min in 0.4-ml polyethylene centrifuge tubes in the quick-starting Beckman Microfuge (Beckman Instruments, Inc. Spinco Div., Palo Alto, Calif.). A 50- μl sample of supernate was counted (^{14}C and ^3H) in 4 ml of counting fluid with 0.4 ml of saline. Residual supernate was aspirated. Saline was carefully added to fill the tube above the cells and aspirated, and this rinsing procedure was repeated. Cells were then suspended in 0.2 ml of saline in which they were transferred to a counting vial. A second 0.2 ml was used to rinse residual cells from centrifuge tube to counting vial, to which 4 ml of counting fluid was added in preparation for ^3H and ^{14}C counting. Bound ^3H was taken to be total pellet ^3H minus that ^3H associated with extracellular fluid contaminating the pellet. The latter was calculated as $[\text{H}_{\text{medium}}] \cdot \text{C}_{\text{pellet}} / [\text{C}_{\text{medium}}]$. This was a minor correction, owing to the avidity of Con-A binding. Bound ^3H was divided by pellet volume (PCV \cdot 0.3 ml) and by specific activity (counts per minute per microgram) to give microgram bound per milliliter cell volume. Each point in each time-course was taken from a separate incubation vial, and the whole experiment was carried out twice (separate days, means shown) with concurring results. There is, therefore, considerable redundancy in the evidence against reduction of Con-A binding by MIX and A-23187.

tributes to the anabolic response in the thymocyte (11) and cultured fibroblast as judged from the glucose dependency of growth (17) and the fact that transport limits cellular glucose concentration (18). It is attractive to suspect mechanistic analogies among these associations. The present studies were undertaken in hopes of disclosing some features of the regulatory signals leading from Con-A binding to enhanced glucose transport.

Full development of the glucose-transport response to Con-A takes at least 1 h, while the DNA-synthetic response in thymocytes is complete by 1 h. This system differs from other systems such as the fibroblast responding to anabolic stimuli (9, 14, 18), where the glucose-transport response precedes the DNA synthetic response by several hours.

The dependences of glucose transport on Con-A and A-23187 doses differ from those of DNA synthesis. Both agents produce optimal stimulation of DNA synthesis at doses which stimulate glucose transport submaximally. At higher doses, DNA stimulation is suppressed (10, 20, 23) while glucose transport is further stimulated (Figs. 4 and 5). This suggests that the glucose-transport response is not a direct consequence of the DNA response. It is reasonable to suppose that the mechanism of transport stimulation is the same at high as at low doses of stimulants and that DNA synthesis is more fastidious with respect to ionic environment, energy charge, or other features of overstimulation.

Evidence that Con-A and A-23187 stimulate DNA synthesis and mitosis by promoting Ca^{++} influx has been summarized in the introduction (5, 10, 23, 24). The present data are explicable in terms of a similar role of Ca^{++} in the glucose-transport responses to the two agents. The glucose-transport response to each agent is dependent on Ca^{++} in the medium (data not shown). Saturating A-23187 doses enhance Ca^{++} influx more than do saturating doses of Con-A, which correlates with the fact that A-23187 is the stronger glucose-transport stimulus.

The correlation extends also to the effects of MIX. This agent alone (at 0.1 mM) had no perceptible effect on glucose transport or Ca^{++} influx. It antagonized the stimulatory effects of Con-A on both glucose transport and Ca^{++} influx, as expected if the latter mediates the former. The effects of MIX in the presence of A-23187 are somewhat surprising but present no paradoxes

with respect to the view that increased Ca^{++} influx mediates glucose transport stimulation by the ionophore. We have no explanation for MIX's enhancement of Ca^{++} influx in the presence of A-23187. However, given this observation, the failure of MIX to antagonize A-23187 stimulation of glucose transport is obviously compatible with the view that Ca^{++} influx mediates the stimulation. Failure of MIX to enhance glucose transport stimulation by near saturating A-23187 doses is also compatible with a mediating role of Ca^{++} , since the presumed Ca^{++} -responsive process should be already saturated. Conceivably the enhancement by MIX of glucose-transport stimulation by the half-maximal A-23187 dose is related to enhancement of the ionophore-mediated Ca^{++} flux.

The failure of Con-A binding to stimulate glucose transport in the presence of nearly saturating doses of A-23187 indicates either that A-23187 alone can saturate some step in the Con-A-responsive system (the classical interpretation that the two stimuli have a common final mechanism) or that A-23187 blocks an event in the Con-A-responsive system regardless of relations between the two stimulatory mechanisms. The result is certainly compatible with the suggested role of Ca^{++} as mediator of both responses. Additivity or synergism would have been difficult to reconcile with such a role; for, if additional ionophore could not increase the response, additional Ca^{++} influx by Con-A would not be expected to do so. Thus, the additivity experiment might have given evidence against a role of Ca^{++} but did not.

In general, then, the evidence favoring a role of Ca^{++} in Con-A stimulation of glucose transport is very much like that favoring a role of Ca^{++} in the mitogenic response to Con-A. Participation of Ca^{++} in glucose-transport regulation has been suggested by others (4).

In the context of this work, MIX (at 0.1 mM) antagonized only Con-A action. It did not interfere with basal glucose transport, basal Ca^{++} influx, or these processes stimulated by A-23187. Since Con-A did not enhance the cAMP-raising action of MIX, we suppose that Con-A's stimulatory mechanism involves a step(s) which is peculiarly sensitive to antagonism by MIX or cyclic nucleotide. The sensitive step(s) appears to be subsequent to Con-A binding but early in the sequence of events leading from Con-A binding to glucose-transport stimulation. If, as argued above, Ca^{++} influx is in the sequence, the MIX-sensitive step is between Con-A binding and Ca^{++} influx,

since MIX antagonized Con-A stimulation of Ca^{++} influx. Also, if, as suggested above, the final events of Con-A stimulation and A-23187 stimulation are the same, then failure of MIX to antagonize A-23187 stimulation tends to place the MIX-sensitive step(s) of Con-A stimulation before the steps (probably beginning with Ca^{++} influx) which are common to the two stimulatory sequences.

The action of MIX, a cyclic nucleotide phosphodiesterase inhibitor, on the sensitive step(s) of Con-A stimulation might be direct, mediated by cAMP, mediated by cGMP, or mediated by some other signal. At present, cAMP mediation appears likely. The effective MIX doses did increase cAMP. Moreover, PGE_2 , an adenylate cyclase stimulant (1), also increased cAMP and antagonized Con-A stimulation of glucose transport. Inasmuch as PGE_2 and MIX differ greatly in chemical and physical properties and in their primary sites of action, it is likely that their common property of raising cAMP levels accounts for their antagonism of Con-A stimulation. This view is strengthened by the actions of dibutyryl-cAMP and 8-bromo-cAMP, both of which antagonize Con-A stimulation of glucose transport but do not inhibit basal glucose transport.

We wish to thank Ms. Janette Welden for assistance in the cAMP assays.

This work was supported by Diabetes Center grant U. S. Public Health Service (USPHS) 1 P17 AM 17026, Physiology Training Program grant USPHS 5 T01 GM 00423, Physiology Program grant USPHS 2 P01 AM 07462, and USPHS 1 R01 AM 18733.

Received for publication 17 December 1975, and in revised form 13 September 1976.

REFERENCES

1. BOURNE, H. R., L. M. LICHTENSTEIN, and K. L. MELMON. 1972. Pharmacologic control of allergic histamine release in vitro: evidence for an inhibitory role of 3',5'-adenosine monophosphate in human leukocytes. *J. Immunol.* **108**:695-705.
2. BOURNE, H. R., L. M. LICHTENSTEIN, K. L. MELMON, C. S. HENNEY, Y. WEINSTEIN, and G. M. SHEARER. 1974. Modulation of inflammation and immunity by cyclic AMP. *Science (Wash. D. C.)* **184**:19-28.
3. CRANE, R. K., R. A. FIELD, and C. F. CORI. 1957. Studies of tissue permeability. I. The penetration of sugars into the Ehrlich ascites tumor cells. *J. Biol. Chem.* **224**:649-662.
4. ELBRINK, J., and I. BIHLER. 1975. Membrane

- transport: Its relation to cellular metabolic rates. *Science (Wash. D. C.)*. **188**:1177-1184.
5. FREDMAN, M. H., M. C. RAFF, and B. GOMPERTS. 1975. Induction of increased calcium uptake in mouse T lymphocytes by concanavalin A and its modulation by cyclic nucleotides. *Nature (Lond.)*. **255**:378-382.
 6. GILMAN, A. G. 1972. Protein binding assays for cyclic nucleotides. *Adv. Cyclic Nucleotide Res.* **2**:9-24.
 7. GUNTHER, G. R., J. L. WANG, and G. M. EDELMAN. 1974. The kinetics of cellular commitment during stimulation of lymphocytes by lectins. *J. Cell Biol.* **62**:366-377.
 8. KERSEY, J. H., A. SABAD, J. C. VANCE, J. G. WHITE, and A. N. NEELEY. 1975. Calcium ionophore as a mitogen: response of normal human T and B and immunodeficient lymphocytes. *Clin. Res.* **23**:411. (Abstr.)
 9. KLETZIEN, R. F., and J. F. PERDUE. 1974. Sugar transport in chick embryo fibroblasts: III. Evidence for post-transcriptional and post-translational regulation of transport following serum addition. *J. Biol. Chem.* **249**:3383-3387.
 10. LUCKASEN, J. R., J. G. WHITE, and J. H. KERSEY. 1974. Mitogenic properties of a calcium ionophore, A-23187. *Proc. Natl. Acad. Sci. U. S. A.* **71**:5088-5090.
 11. MUNCK, A. 1971. Glucocorticoid inhibition of glucose uptake by peripheral tissues: Old and new evidence, molecular mechanisms, and physiological significance. *Perspect. Biol. Med.* **14**:265-289.
 12. PETERS, J. H., and P. HAUSEN. 1971. Effect of phytohemagglutinin on lymphocyte membrane transport. II. Stimulation of "facilitated diffusion" of 3-O-methylglucose. *Eur. J. Biochem.* **19**:509-513.
 13. PILKIS, S. J., and C. R. PARK. 1974. Mechanism of action of insulin. *Annu. Rev. Pharmacol.* **14**:365-387.
 14. RUBIN, H. 1971. pH and population density in the regulation of animal cell multiplication. *J. Cell Biol.* **51**:686-702.
 15. SMITH, D. E., and J. GORSKI. 1968. Estrogen control of uterine glucose metabolism. An analysis based on the transport and phosphorylation of 2-deoxyglucose. *J. Biol. Chem.* **243**:4169-4174.
 16. SMITH, J. W., A. L. STEINER, and C. W. PARKER. 1971. Human lymphocyte metabolism. Effects of cyclic and non-cyclic nucleotides on stimulation by phytohemagglutinin. *J. Clin. Invest.* **50**:442-448.
 17. TEMIN, H. M. 1968. Studies on carcinogenesis by avian sarcoma viruses: VIII. Glycolysis and cell multiplication. *Int. J. Cancer.* **3**:273-282.
 18. VENUTA, S., and H. RUBIN. 1973. Sugar transport in normal and Rous sarcoma virus-transformed chick-embryo fibroblasts. *Proc. Natl. Acad. Sci. U. S. A.* **70**:653-657.
 19. WANG, J. L., G. R. GUNTHER, and G. M. EDELMAN. 1975. Inhibition by colchicine of the mitogenic stimulation of lymphocytes prior to the S phase. *J. Cell Biol.* **66**:128-144.
 20. WANG, J. L., D. A. McCLAIN, and G. M. EDELMAN. 1975. Modulation of lymphocyte mitogenesis. *Proc. Natl. Acad. Sci. U. S. A.* **72**:1917-1921.
 21. WHITESELL, R. R., and D. M. REGEN. 1975. Cyclic-nucleotide-phosphodiesterase inhibitors oppose concanavalin-A stimulation of glucose transport in thymocytes. *Fed. Proc.* **34**:300.
 22. WHITESELL, R. R., and D. M. REGEN. 1975. Role of Ca⁺⁺ in regulation of glucose transport. *Diabetes*. **2**(Suppl. 24):448.
 23. WHITFIELD, J. F., J. P. MACMANUS, A. L. BOYNTON, D. J. GILLAN, and R. J. ISAACS. 1974. Concanavalin A and the initiation of thymic lymphoblast DNA synthesis and proliferation by a calcium-dependent increase in cyclic GMP level. *J. Cell. Physiol.* **84**:445-457.
 24. WHITNEY, R. B., and R. M. SUTHERLAND. 1973. Kinetics of calcium transport in lymphocytes before and after stimulation by phytohemagglutinin. *Proc. Leucocyte Cult. Conf.* **7**:63-74.
 25. WRIGHT, J. A. 1973. Morphology and growth rate changes in Chinese hamster cells cultured in presence of sodium butyrate. *Exp. Cell Res.* **78**:456-460.