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CALCIUM IONOPHORES A23187 AND X537A AFFECT CELL AGGLUTINATION BY LECTINS AND CAPPING OF LYMPHOCYTE SURFACE IMMUNOGLOBULINS

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SUMMARY

The microtubule-disruptive drugs colchicine and vinblastine alter ligand-induced redistribution of cell surface immunoglobulins and lectin receptors. These effects can be duplicated by treatment of cells with the divalent cation ionophores A23187 and X537A. Ionophore activity was dependent upon the presence of Ca^{2+} ($1.8 \cdot 10^{-3}$–$4 \cdot 10^{-4}$ M) in the culture medium. The K^+-selective ionophore valinomycin had no effect on ligand-induced redistribution of surface receptors. It is suggested that A23187 and X537A impair membrane-associated microtubules involved in transmembrane control of receptor mobility and topography. In contrast to the action of colchicine and vinblastine that bind directly to microtubules, it is proposed that ionophores indirectly affect microtubules by raising the concentration of Ca^{2+} in the cytoplasm to levels that favor microtubule depolymerization and inhibit microtubule assembly.

INTRODUCTION

Binding of multivalent ligands such as lectins [1–6] or antibodies [7–12] to the cell surface induces topographical redistribution of the appropriate receptors to form small "clusters", larger "patches" or large single "caps" of ligand-receptor complexes. Recent work in several laboratories suggests that the mobility and distribution of ligand-receptor complexes during patch and cap formation is controlled by cytoskeletal elements associated with the cytoplasmic face of the plasma membrane [13–18]. These membrane-associated elements appear to be microtubules or microfilaments, or components with similar pharmacological specificities [11, 12, 19–22]. This concept of transmembrane control of receptor topography by membrane-associated cytoskeletal elements has evolved from experimental observations on alterations in the mobility and distribution of ligand-receptor complexes by drugs that bind to microtubules and/or microfilaments. Microtubule-disruptive drugs such

as colchicine and the *Vinca* alkaloids inhibit cell agglutination by Concanavalin A [19, 20, 22] and also modify lectin-induced redistribution of membrane receptors [5, 21–23]. The recently reported effects of tertiary amine local anesthetics in enhancing concanavalin A-mediated agglutination of 3T3 cells [22, 23] and in preventing antibody-induced capping of surface immunoglobulins in lymphocytes [24] are also proposed as resulting from their action on similar membrane-associated cytoskeletal elements [22].

Biochemical and ultrastructural studies on microtubules in vitro and in vivo have shown that the assembly and the continued structural integrity of these structures are sensitive to changes in the concentration of Ca^{2+}. Microtubules undergo depolymerization with addition of Ca^{2+} [25, 26] and the polymerization of tubulin subunits to form microtubules is also inhibited by increasing Ca^{2+} concentration [27–29]. These findings have prompted proposals that microtubule function may be controlled by the local concentration of Ca^{2+} in the cell cytoplasm [27, 30–31].

In this communication we have examined whether the membrane-associated microtubules involved in transmembrane control of cell surface receptor topography might be sensitive to changes in intracellular Ca^{2+} concentration created by treating cells with the carboxylic ionophores A23187 and X537A. These drugs selectively transport divalent cations, and Ca^{2+} in particular, across membranes and have been shown to increase cytoplasmic Ca^{2+} concentrations [32–34], leading to activation of a wide variety of Ca^{2+}-dependent intracellular activities [33–38]. Our findings indicate that A23187 and X537A produce alterations in cellular responses to external ligands identical to those caused by colchicine and vinblastine. We interpret these findings as indicating that ionophores can impair microtubule function, possibly as an indirect result of their action in raising intracellular Ca^{2+} to levels that would favor depolymerization of microtubules and also inhibit assembly of new microtubules.

MATERIALS AND METHODS

Cell cultures. Mouse BALB/c 3T3 cells (3T3) and similar 3T3 cells transformed by SV40 virus (SV3T3) were grown in Dulbecco's modified Eagle's medium supplemented with 10 % calf serum as described previously [1, 23]. Spleen cells were harvested from 12-week-old BALB/c mice, purified on a Ficoll-Hypaque gradient [39] and maintained in RPMI 1640 medium supplemented with 0.5 % bovine serum albumin. Culture media were purchased from Gibco, Grand Island, N.Y.

Lectins. Concanavalin A was purchased as a twice crystallized preparation (Miles, Elkhart, Ind.) and purified further by affinity chromatography as described previously [5]. [^{3}H]Concanavalin A (specific activity $3.8 \cdot 10^6$ cpm/mg) was prepared from affinity-purified concanavalin A [5] and specific binding of [^{3}H]concanavalin A (50 μg/ml) to cells measured at 0 °C as described before [5]. Cell agglutination by concanavalin A was determined by microscopic examination of cells after incubation with concanavalin A for 20 min at room temperature as described previously [40] and scored as 0, +, ++, +++ or ++++ for 0, 25, 50, 75 or > 95 % of cells agglutinated, respectively.

Effects of ionophores and drugs on cell agglutination and distribution of surface receptors on lymphocytes. 3T3 and SV3T3 cells ($2 \cdot 10^7$/ml) were incubated

in suspension in serum-free Dulbecco's modified Eagles medium for 30 min at 37 °C with or without ionophores or the various drugs listed in the results, then washed three times and finally assayed for their susceptibility to agglutination by different concentrations of affinity-purified concanavalin A. In experiments involving two drugs, cells were incubated for two successive 30-min periods at 37 °C with each drug and then treated as above.

Suspension cultures of mouse splenic lymphocytes ($1 \cdot 10^7$ cells/ml) were incubated for 15 min at 37 °C in 1.0 ml serum-free RPMI 1640 medium with or without the ionophores or the various drugs listed in the text and then incubated for an additional 60 min at room temperature, still in the presence or absence of drugs, with 50 μg fluorescein-conjugated rabbit anti-mouse immunoglobulin prepared by the method of Cebra and Goldstein [41]. Cells were then centrifuged onto glass coverslips using a cytocentrifuge (Shandon Instruments, Pittsburgh), fixed with 2.5 % glutaraldehyde for 15 min at 20 °C and the attached cells examined for specific immunofluorescence on a Leitz Ortholux microscope under ultraviolet light using UG-1 excitation and K-430 barrier filters. At least 100 stained non-aggregated cells were examined at 1000 × magnification and the number of such cells displaying caps of immunoglobulin receptors determined. In experiments on the effect of concanavalin A on capping of immunoglobulin receptors similar lymphocyte populations were incubated for 30 min at room temperature with either concanavalin A alone or concanavalin A together with A23187, colchicine or valinomycin before final incubation with fluorescein-conjugated rabbit anti-immunoglobulin and measurement of the number of cells with capped immunoglobulin receptors.

Reagents. A23187 (Lot No. 361-D66-282) was generously provided by Dr. R. J. Hosley of Lilly Research Laboratories and X537A (Lot No. 2938-94) by Dr. W. E. Scott of Hoffmann-La Roche. Both ionophores were dissolved in Me_2SO. Colchicine, glutaraldehyde and valinomycin were obtained from Sigma, St. Louis, cytochalasin B from Aldrich Chemical Co., Milwaukee and fluorescein isothiocyanate from BBL Division of Bioquest, Cockeysville, Md.

RESULTS

Previous studies have shown that agglutination of simian virus 40-transformed mouse 3T3 cells (SV3T3) by concanavalin A is inhibited by pretreatment with colchicine or the *Vinca* alkaloids [20, 22]. To test whether ionophore-induced increases in cytoplasmic Ca^{2+} levels in SV3T3 cells might impair membrane-associated microtubules and modify cell agglutination in similar fashion to colchicine, SV3T3 cells were incubated with A23187 or X537A before treatment with concanavalin A and their agglutinability compared with control SV3T3 cell populations treated with concanavalin A alone. The results (Table I) indicate that Ca^{2+} ionophores cause a significant reduction in cell agglutinability without altering cellular binding of concanavalin A. The ionophores at the indicated concentrations had no significant effect on cell viability. Table I further shows that the inhibition of cell agglutination caused by the ionophores was similar to that produced by pretreatment with colchicine or vinblastine. As shown in Table I, treatment of A23187-treated SV3T3 cells with colchicine (or vice versa) before final incubation with concanavalin A did not further increase the inhibition of agglutination, suggesting that A23187

TABLE I

EFFECT OF IONOPHORES AND MICROTUBULE-DISRUPTIVE DRUGS ON CONCANAVALIN A-MEDIATED AGGLUTINATION AND BINDING TO SV3T3 CELLS

The results represent mean values from five separate experiments.

	Treatment	Concentration	Agglutination by concanavalin A (150 μg/ml)*	Specific binding of [^{3}H]concanavalin A (cpm $\times$ 10^7 cells)**
(1)	Untreated	–	++++	5175
(2)	A23187***	10 μg/ml	+	4983
		5 μg/ml	+	5239
		2 μg/ml	+	5386
(3)	X537A***	10 μg/ml	+	5111
(4)	Colchicine	$1 \cdot 10^{-5}$ M	+	4795
(5)	Vinblastine	$1 \cdot 10^{-5}$ M	0/+	5318
(6)	A23187*** plus colchicine	5 μg/ml $1 \cdot 10^{-5}$ M	+	5387
(7)	Colchicine plus A23187***	$1 \cdot 10^{-5}$ M 5 μg/ml	+	4909
(8)	Valinomycin***	$1 \cdot 10^{-6}$ M	++++	5274

* Agglutination by the indicated concentration of concanavalin A measured and scored from 0 to ++++ as described in Materials and Methods.

** Binding at 0 °C for 30 min as outlined in Materials and Methods.

*** Ionophores A23187 and X537A were dissolved in dimethylsulfoxide (0.1 %, v/v) and valinomycin in ethanol (0.1 %, v/v). Neither solvent when used alone at these concentrations had any effect on cell agglutination or binding of concanavalin A.

and colchicine were acting on a common target site (though not necessarily via the same mechanism). The decrease in SV3T3 cell agglutinability after ionophore treatment may be due to enhanced cap formation of lectin receptors similar to that caused by colchicine treatment [4]. Capping of receptors probably reduces the frequency of contact between surface regions containing aggregated lectin receptors on adjacent cells thereby limiting agglutination [4].

The ability of A23187 and X537A to duplicate the effects of colchicine and *Vinca* alkaloids on cell agglutination by concanavalin A was confirmed in further experiments with untransformed mouse 3T3 cells. We have reported recently that treatment of 3T3 cells with colchicine (or *Vinca* alkaloids) together with cytochalasin B significantly enhanced their susceptibility to agglutination by concanavalin A [22, 48]. We therefore tested whether pretreatment of 3T3 cells with cytochalasin B together with A23187 or X537A in place of colchicine might cause similar enhancement of cell agglutination. The results, shown in Table II, indicate that combined pretreatment with a Ca^{2+} ionophore plus cytochalasin B produces a very marked increase in concanavalin A-mediated agglutination, similar to that caused by pretreatment with cytochalasin B plus colchicine. In the latter situation, enhanced agglutination appears to result from the fact that concanavalin A induces redistribution of its receptors into small patches instead of a single cap (see refs. 22 and 48), a distribution that would increase the opportunities for interaction between receptors on adjacent cells and thus favor agglutination. Thus, if the ionophores dislocate

TABLE II

EFFECT OF IONOPHORES AND DRUGS ACTING ON MICROTUBULES AND MICROFILAMENTS ON CONCANAVALIN A-MEDIATED AGGLUTINATION AND BINDING TO 3T3 CELLS

Results represent mean values derived from five separate experiments.

	Treatment	Concentration	Concentration of concanavalin A (μg/ml) required for maximum cell agglutination*	Specific binding of [^{3}H]concanavalin A (cpm $\times$ 10^7 cells)**
(1)	Untreated	–	1400	5227
(2)	A23187***	5 μg/ml	1200	5024
		2 μg/ml	1200	5369
(3)	X537A***	10 μg/ml	1400	4719
(4)	Cytochalasin B***	15 μg/ml	1100	5193
(5)	Valinomycin***	$1 \cdot 10^{-5}$ M	1400	5266
(6)	A23187 plus cytochalasin B	5 μg/ml 15 μg/ml	250	5092
(7)	X537A plus cytochalasin B	10 μg/ml 15 μg/ml	300	5419
(8)	Colchicine plus cytochalasin B	$1 \cdot 10^{-5}$ M 15 μg/ml	400	5234
(9)	Valinomycin plus cytochalasin B	$1 \cdot 10^{-6}$ M 15 μg/ml	1400	5270

* $>$ 90 % cells agglutinated by the indicated concentration of concanavalin A.

** Measured as described in Table I.

*** A23187, X537A and cytochalasin B were dissolved in Me_2SO (0.1 %, v/v) and valinomycin in ethanol (0.1 %, v/v). Neither Me_2SO or ethanol had any effect at these concentrations on cell agglutination or binding of concanavalin A.

membrane-associated microtubules to produce a colchicine-like effect, a similar mechanism can be advanced to explain the enhanced agglutination response produced by the ionophores and cytochalasin B.

Colchicine-sensitive membrane-associated elements have also been implicated in transmembrane control of cell surface receptor mobility and topography in lymphocytes. Binding of antibodies to surface immunoglobulins on lymphocytes produces patching and subsequent capping of the surface immunoglobulin-antibody complexes [7, 12, 21], but capping can be inhibited by pretreatment of the cells with concanavalin A [11, 12, 21]. This inhibitory effect of concanavalin A can be blocked, however, by colchicine or the *Vinca* alkaloids [11, 12, 21]. To establish whether A23187 or X537A were able to act like colchicine to block concanavalin A-induced inhibition of immunoglobulin capping, concanavalin A-treated BALB/c mouse spleen lymphocytes were incubated with A23187 or X537A and then incubated with fluorescein-conjugated anti-immunoglobulin in the presence or absence of concanavalin A, after which the number of cells with fluorescent caps was measured. Antibody-induced immunoglobulin capping was also determined in replicate cultures incubated with colchicine before addition of anti-immunoglobulin with or without the concanavalin A pretreatment. The results, shown in Table III, indicate that

TABLE III

EFFECT OF IONOPHORES, COLCHICINE AND CONCANAVALIN A ON ANTIBODY-INDUCED CAPPING OF SURFACE IMMUNOGLOBULINS ON SPLEEN LYMPHOCYTES

Results represent mean values from three separate experiments.

Treatment		Concentration	Stained cells (%)	Immunoglobulin caps in stained cells (%)
(1)	Untreated	–	44	81
(2)	A23187*	10 μg/ml	47	90
		2 μg/ml	44	87
(3)	Colchicine	$1 \cdot 10^{-5}$ M	41	84
(4)	Valinomycin*	$1 \cdot 10^{-6}$ M	42	85
(5)	Concanavalin A	100 μg/ml	47	17
(6)	Concanavalin A plus A23187	100 μg/ml 10 μg/ml	39	65
(7)	Concanavalin A plus A23187	100 μg/ml 2 μg/ml	42	60
(8)	Concanavalin A plus colchicine	100 μg/ml $1 \cdot 10^{-5}$ M	43	69
(9)	Concanavalin A plus valinomycin	100 μg/ml $1 \cdot 10^{-6}$ M	40	14

* A23187 was dissolved in Me_2SO (0.1 %, v/v) and valinomycin in ethanol (0.1 %, v/v). Neither solvent alone at these concentrations had any significant effect on cell staining or capping.

A23187, X537A or colchicine each blocked concanavalin A-induced inhibition of immunoglobulin capping, though none of these drugs alone had any significant effect on immunoglobulin capping in the absence of concanavalin A.

The various effects of A23187 and X537A in modifying ligand-induced redistribution of surface receptors shown in Tables I–III were all dependent upon the presence of Ca^{2+} in the culture medium ($1.8 \cdot 10^{-3}$–$4 \cdot 10^{-4}$ M). Incubation of cells with ionophores and ligands in Ca^{2+}-free media completely inhibited the ionophore effects. However, removal of Mg^{2+} from the incubation media did not affect ionophore activity.

Data presented in Tables I–III also show that the K^+-selective ionophore, valinomycin, had no detectable effect on the response of cells to lectins or antibodies. These results are consistent with the conclusion that the ability of A23187 and X537A to modify cellular responses to ligands is related to their capacity to transport divalent cations, notably Ca^{2+}, across the plasma membrane.

DISCUSSION

The present results indicate that A23187 and X537A cause changes in cell surface organization similar to those produced by the microtubule-disruptive drugs, colchicine and vinblastine, indicating that the ionophores, among a variety of effects, can impair the function of membrane-associated microtubules.

In contrast to the inhibitory action of colchicine and the *Vinca* alkaloids on microtubules which results from direct binding of these drugs to tubulin [42], we consider that ionophores indirectly affect microtubules by increasing the level of

Ca^{2+} in the cytoplasm, though it is acknowledged that the present data do not exclude direct binding to microtubules. Ionophore-mediated increases in cytoplasmic Ca^{2+}, resulting from influx of Ca^{2+} from the external medium [32, 33], or from release of Ca^{2+} from internal membranes [43, 44], or both, would create conditions that would not only favor dissociation of tubulin subunits and the breakdown of existing microtubules but would also reduce polymerization of tubulin subunits to form new tubules.

The ability of Ca^{2+} ionophores to produce colchicine-like effects, presumably as a result of functional alterations in cellular microtubule systems, has not been reported previously. However, in the light of the present findings, a number of previous observed effects of Ca^{2+} ionophores on cultured cells might be reasonably interpreted as resulting from the effect of these drugs on cellular microtubules. For example, the action of A23187 in preventing cyclic AMP-induced changes in cell morphology [45] could be due to an effect on membrane-associated microtubules. This interpretation gains support from the earlier observation that the changes in cell shape induced by cyclic AMP can similarly be inhibited by colchicine [46, 47].

The effect of A23187 on cellular microtubules suggested by the present results may also be pertinent to the reported mitogenic activity of this agent [18, 37]. Edelman and his colleagues [13, 18, 21] have suggested that cell surface topography is controlled by a modulating assembly composed of both microtubules and microfilaments which is linked to membrane receptors. They suggest that this assembly is not only responsible for regulating receptor mobility within the membrane but may also be important in regulating mitogenesis. Specifically, these investigators have proposed that the colchicine-sensitive component (microtubules?) of the modulating assembly functions as a system of "anchors" to restrict receptor mobility and that mitogen-induced changes in the state of association of these anchoring elements to membrane receptors may be an important initial event in the cellular commitment to mitosis. Consequently, the mitogenic effect of A23187 might well reflect a functional alteration in the organization of the colchicine-sensitive assembly produced by ionophore-mediated influx of Ca^{2+} into the cell.

The present demonstration that Ca^{2+} ionophores can impair the function of cellular microtubules raises the possibility that this class of drug could serve as a new pharmacological probe to complement the use of colchicine and the *Vinca* alkaloids in studies on the role of membrane-associated microtubules in controlling the mobility and topography of cell surface receptors.

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