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SHORT COMMUNICATIONS

Binding of maytansinoids to tubulin*

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Maytansine and related maytansinoids are novel benzenic ansamycins that were first isolated from plant materials [1–3] and, more recently, from fermentation broth [4, 5]. Some of these compounds have exhibited antitumor activity in experimental animals [5–7]. At very low concentrations (10^{-12} M) these compounds inhibit growth of cultured mammalian cells [5–7]. At 10^{-7}–10^{-8} M concentrations, maytansinoids inhibit cell division and arrest the cells in mitosis [4–10]. These phenomena have been attributed to binding of these compounds to tubulin [6, 8, 11, 12] and, thus, to inhibition of tubulin polymerization [6, 8]. Resistance of a murine P388 tumor to both vincristine as well as maytansine [9] suggests the possibility that the mechanisms of action of these drugs could be similar. In this paper, competition of maytansinoids to the binding of tritium-labeled vincristine and rat brain tubulin are presented.

Maytansine (NSC 153858), maytanvaline (NSC 219970), normaysine (NSC 219973), maysine (NSC 219972), maysenine (NSC 219974) and vincristine sulfate (NSC 67574) were obtained from the Drug Synthesis and Chemistry Branch, Division of Cancer Treatment, National Cancer Institute, Bethesda, MD. Stock solutions of the maytansinoids were made in ethanol. Further dilutions were made in water or the binding buffer. By thin-layer chromatography on silica gel plates (Eastman Kodak, Rochester, NY) in a diethyl ether–*n*-propanol–triethylamine (24:16:2) solvent system, the ansamycins and the alkaloid gave a single spot with R_f values as follows: maytanvaline 0.80, maytansine 0.72, maysenine 0.77, normaysine 0.76, may-

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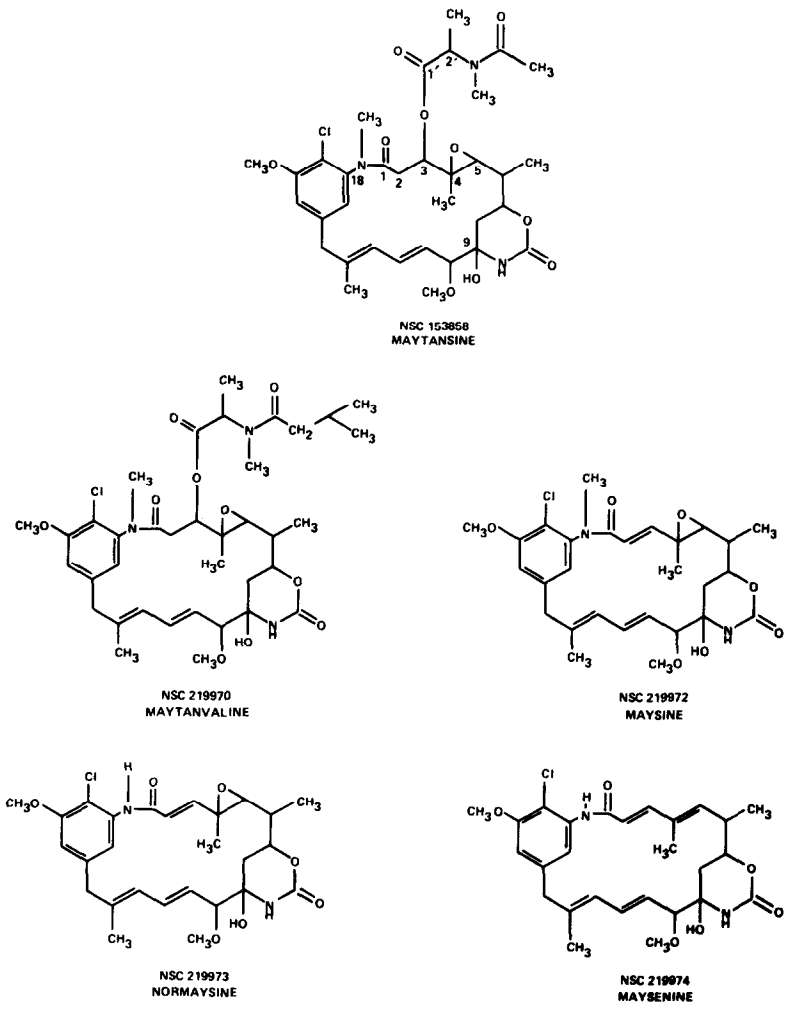

Fig. 1. Structural formulae of maytansine and maytansinoids.

sine 0.75, and vincristine 0.58. Concentrations of these drugs were determined spectrophotometrically. The following molar extinction coefficients were used at the λ_{max}: vincristine 15,150 (296 nm); maytansine 5,300 (288 nm); maytanvaline 5,360 (288 nm); maysine 4,280 (280 nm); normaysine 5,770 (280 nm); and maysenine 23,500 (271 nm). Structural formulae of the maytansinoids are given in Fig. 1. Tritium-labeled vincristine (sp. act. 5.50 Ci/mmole) was obtained from the Pharmaceutical Resources Branch, DCT, NCI, and purified by Dr. Manford Castle of the National Cancer Institute by high pressure liquid chromatography. It had a radiochemical purity of 93 per cent as determined by thin-layer chromatography in the above system.

Tubulin was purified from male Sprague–Dawley rat brain extracts by two cycles of polymerization and depolymerization according to Shelanski *et al.* [13] in 0.1 M 2-(*N*-morpholino) ethanesulfonic acid (Mes), pH 6.4, containing 1 mM ethylene glycol-bis (β-aminoethyl ether)-*N*, *N'*-tetraacetic acid (EGTA), 1 mM GTP and 0.5 mM $MgCl_2$. Protein concentration was determined by the method of Lowry *et al.* [14], with crystalline bovine albumin as a standard. The tubulin preparation at 0.7 mg/ml concentration was stored in the above buffer containing 8 M glycerol in small aliquots at −15°. Sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis performed on this preparation gave a single major band [15] that appeared to be composed of two closely spaced components representing α- and β-subunits of a tubulin dimer. A molecular weight of 110,000 daltons was used for tubulin.

The DEAE-cellulose disc filtration assay for colchicine [16] was modified to make it suitable for vincristine binding. The binding buffer consisted of 10 mM sodium phosphate, 10 mM $MgCl_2$, and 0.1 mM GTP adjusted to pH 6.8 at 4°. In a total volume of a 400 μl or 850 μl reaction mixture containing tubulin, various amounts of [^{3}H]-vincristine with or without maytansinoids, and the binding buffer, were incubated for 30 min at 37°. The reactions were stopped by chilling in ice and were diluted with 1.5 ml of ice-cold binding buffer. The reaction solutions were then transferred onto two DE-81 (Whatman) 2.5 cm diameter discs, which were previously soaked in the binding buffer and placed on a Millipore filtration apparatus. The mixtures were allowed to filter by gravity and subsequently were washed three times with 4 ml each of ice-chilled buffer under mild suction. Radioactivity in each reaction was determined by counting the filters in 10 ml of a solution of Hydromix (Yorktown Co., PA). Radioactivity was measured for 10 min and corrected for quench by the filters. All binding assays were run in triplicate. The control samples were without tubulin. Radioactivity in the control samples was less than 3 percent of the total input counts and was subtracted from all test samples.

In preliminary experiments a linear range of [^{3}H]-vincristine binding to tubulin was obtained between 20 and 100 μg of the tubulin preparation. For binding studies, 0.02 μM to 0.7 μM [^{3}H]-vincristine (sp. act. 111 dpm/pmole) was allowed to bind with 35 μg tubulin preparation in a volume of 850 μl reaction mixture, and the data were plotted according to Scatchard [17] (Fig. 2). By linear regression of these data points, an association constant (K_a) of 1.8×10^7 M^{-1} was calculated. These results are in agreement with those obtained for high affinity binding sites of vinblastine to tubulin [18]. The capacity of the alkaloid binding was 1.88 nmoles vincristine per mg tubulin preparation. Analogous to vinblastine–tubulin binding [18], vincristine concentrations chosen in these experiments represented a high affinity tubulin binding with a stoichiometry of 1:1. These results, therefore, suggested that our tubulin preparation had 20.7 per cent activity with respect to its binding to the alkaloid. This factor was taken into account in all calculations.

Inhibition of vincristine binding to tubulin by various

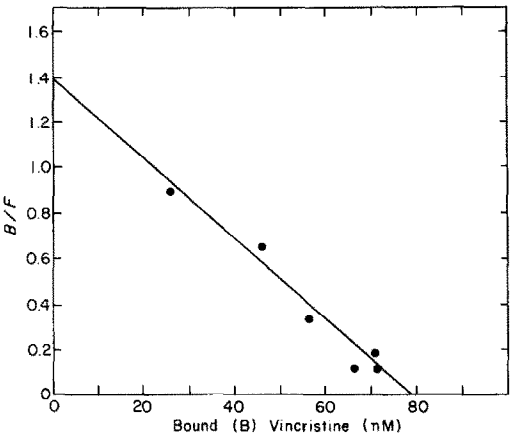

Fig. 2. Scatchard plot of [^{3}H]-vincristine binding to rat brain tubulin. Binding of the alkaloid to tubulin was assayed by the DEAE-filter disc method as described in the text. Increasing [^{3}H]-vincristine concentrations (0.02 to 0.7 μM) were incubated with 35 μg tubulin at 37° for 30 min in triplicate reaction mixtures. After subtracting the background radioactivity, the amounts of the bound (B) and free (F) ligand were calculated for each data point. These results were plotted according to Scatchard [17].

maytansinoids was carried out at various concentrations of the inhibitor. The corrected data were plotted according to Dixon [19]. Linear regression analysis of the data was performed, and the point of intersection determined. The mean ± S.D. value of the intersection point for maytansine, $X = -0.3475 \pm 0.0817$ μM, indicated a competitive type of inhibition with an apparent inhibition constant (K_i) of 0.34 μM (Fig. 3A). The mean ± S.D. value of the point of intersection on the X-axis for maytanvaline was -0.9494 ± 0.277 μM (Fig. 3B). These data suggested that maytanvaline, like maytansine, is a competitive inhibitor of vincristine binding to tubulin with an apparent K_i of 0.95 μM. Inhibition of normaysine was studied at two concentrations (0.758 and 0.374 μM) of vincristine. The regression lines intersected at X = −31.76, indicating that normaysine also was a competitive inhibitor with a K_i of 31.76 μM (Fig. 3C). The inhibitory data with maysenine did not give straight lines, but they fit a parabolic equation (Fig. 3D). These equations at 1.01 μM, 0.758 μM and 0.505 μM vincristine were, respectively, $y = 1.573 + 0.2802\,x - 0.008605\,x^2$, $r = 0.97$; $y = 1.752 + 0.3216\,x - 0.01009\,x^2$, $r = 0.95$; and $y = 2.085 + 0.3700\,x - 0.01174\,x^2$, $r = 0.95$, where x was the maysenine concentration, and r was the correlation coefficient. These data suggested that the nature of inhibition of maysenine was different from that of maytansine. The significance of these data is not clear. Maysine at concentrations up to 20 μM did not inhibit binding of vincristine to tubulin (data not shown).

Maytansine, maytanvaline and normaysine exhibited a competitive inhibition of [^{3}H]-vincristine binding to tubulin with apparent K_i values of 0.35 μM, 0.95 μM and 31.76 μM respectively. These data are in agreement with the K_i of 0.5 μM for maytansine, which was obtained by competition of [^{3}H]-vinblastine with tubulin [12]. Among these inhibitors, maytansine and maytanvaline possess C-3 ester side chains, and these are potent inhibitors. Normaysine, which lacks the C-3 ester side chain and the *N*-methyl group, is a 90-fold weaker inhibitor as compared to maytansine. Maysenine in which the 4,5-epoxide ring is substituted by a double bond and lacks the *N*-methyl and C-3 ester side chain gives inhibition curves of a parabolic type. Interpretation of these data is not clear. Maysine which is devoid of the C-3 hydroxyl function and thus of the ester group

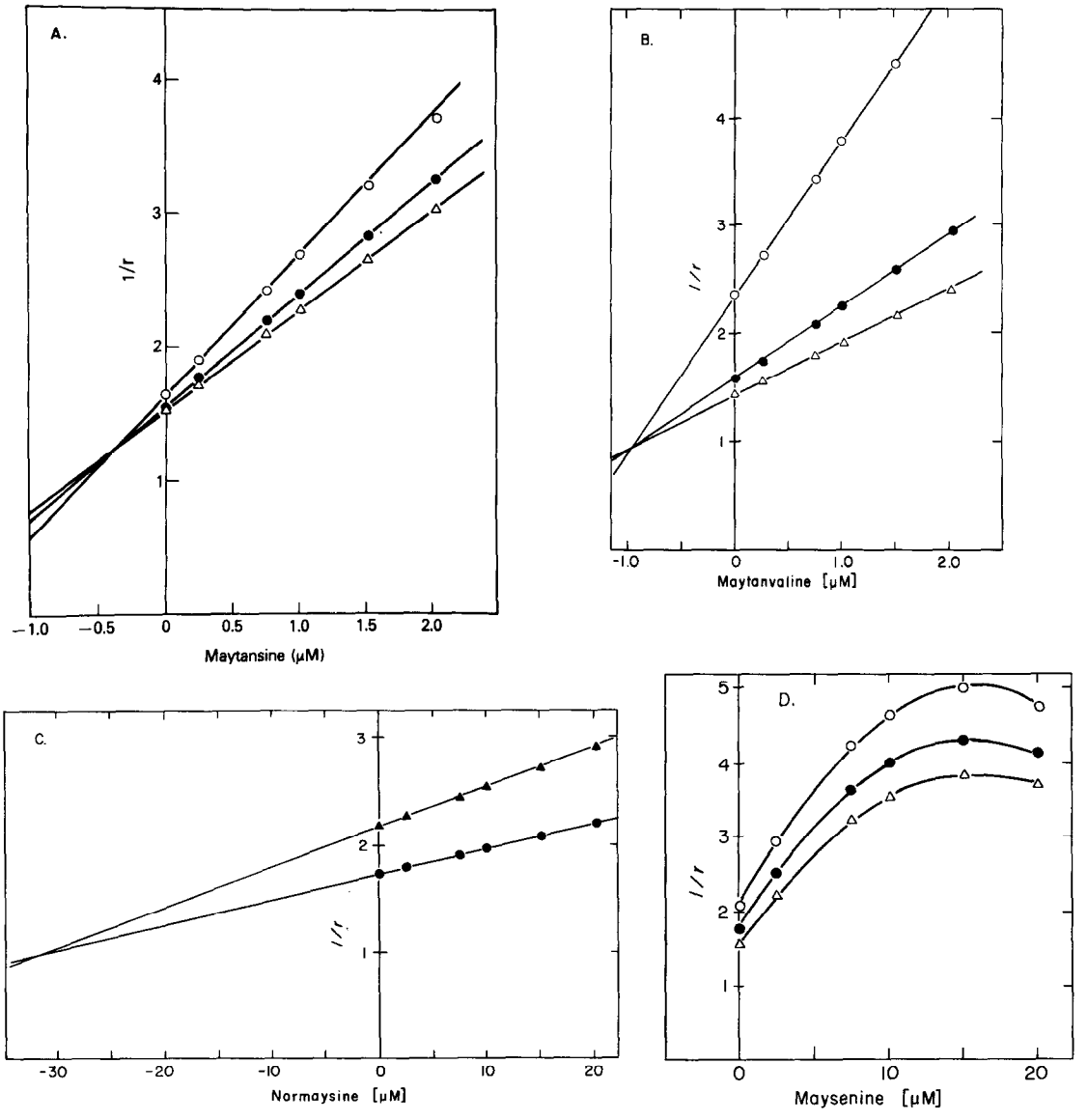

Fig. 3. Dixon plots of maytansine (A), maytanvaline (B), normaysine (C), and maysenine (D) for the binding of [^{3}H]-vincristine to rat brain tubulin. Reaction mixtures with 400 μl volume containing various concentrations of maytansinoids, different concentrations [1.01 μM (△—△) 0.758 μM (●—●), 0.505 μM, (○—○) and 0.374 μM (▲—▲)] of [^{3}H]-vincristine, 0.098 to 1.39 μM tubulin, and binding buffer were incubated for 30 min at 37° and processed as described. Some of these experiments contained 0.5 to 2% ethanol, which had no influence on the binding of [^{3}H]-vincristine to tubulin. After subtracting background the corrected data were calculated as a ratio (r) of pmoles bound vincristine over pmoles tubulin. These data were plotted according to Dixon [19], i.e. $1/r$ versus inhibitor concentration.

does not inhibit the binding of vincristine to tubulin. The apparent K_i values of these compounds correlate well with the cytoxicity of KB cells in culture [6] and with the antileukemic activity against a murine P388 leukemia system [6] (Table 1). Other biological properties, such as inhibition of tubulin polymerization and the arrest of sea urchin egg cells in mitosis [6], do not seem to be related to their binding to tubulin (Table 1). Moreover, in L1210 cultured cells, about 100-fold higher concentrations of maysine as compared to maytansine are needed to achieve 70 per cent mitotic arrest and cell kill as determined by colony formation assay [15]. These data clearly lend further support to the conclusion that the C-3 ester chain is necessary for antitumor activity [6].

Among the known ansamycins maytansins and geldanamycins have been shown to be highly cytotoxic [7], but only maytansine possesses significant antitumor properties [7]. It is not clear whether other ansamycins, rifamycins, streptovaricins and geldanamycins, have any tubulin binding properties. It has been noted, however, that geldanamycin which lacks the carbinolamide function is 1000 times less active in inhibition of sea urchin egg cleavage [8]. Rifamycins and streptovaricins are known to be potent inhibitors of bacterial and mammalian nucleic acid polymerases [7], but maytansinoids do not inhibit mammalian DNA polymerase α and DNA polymerase β, RNA polymerase I and RNA polymerase II, poly (A) polymerase or RNA tumor virus reverse transcriptase activities (Ref. 7,

Table 1. Relationship of K_i for competitive inhibition of [^{3}H]-vincristine binding to purified tubulin, tubulin polymerization as compared to maytansine, antitumor activity, cytotoxicity (I_{50}) towards KB cells, and inhibition of sea urchin mitosis of maytansinoids

Compound	K_i (μM)	Tubulin polymerization*†	Antitumor activity*, % T/C‡ (μg/kg)	I_{50}* (M)	Mitosis inhibition* (M)
Maytansine	0.35	1.0	220 (25)	8.8×10^{-12}	10^{-7}
Maytanvaline	0.95	0.9	187 (12.5)	3.1×10^{-13}	10^{-8}
Maysenine	4.83§	0.9	120 (100)	3.9×10^{-9}	10^{-8}
Maysine	‖	0.3	80 (50)	4.5×10^{-8}	10^{-5}–10^{-6}
Normaysine	31.76	0.3	115 (3.1)	3.5×10^{-8}	10^{-5}–10^{-6}

* These values are obtained from Ref. 6 with the kind permission of the publisher and authors.

† This is the ratio of drug to maytansine turbidity formation due to tubulin polymerization as measured by absorbance at 310 nm.

‡ T/C is the ratio (expressed as a percent) of the median survival time of the treated group of mice divided by the median survival time of the control group at the indicated doses of μg drug/kg given in parentheses. Compounds are thought to be active by the NIH protocol if T/C $\geq$ 125%.

§ Noncompetitive inhibition.

‖ No inhibition up to 20 μM.

V. S. Sethi, unpublished observations). It has been suggested that maytansine due to the carbinolamide group may be involved in the alkylation of sulfhydryl groups of tubulin [8, 20, 21]. These observations along with conformational changes and the chemical nature of the binding sites on tubulin need further exploration so as to better understand the mechanism of action of structurally diverse antitumor agents, dimeric *Catharanthus* alkaloids (vinblastine and vincristine) and maytansinoids.

In summary, the binding of five maytansinoids to purified rat brain tubulin was investigated in a competition binding assay with tritium-labeled vincristine. By Scatchard analysis, an association constant of $1.8 \times 10^7\ M^{-1}$ was obtained for the binding site of vincristine with tubulin. Using Dixon plots, maytansine and maytanvaline showed competitive inhibition with an apparent inhibition constant (K_i) of 0.35 μM and 0.95 μM respectively. Normaysine, which lacked the C-3 ester group and the *N*-methyl group, also showed competitive inhibition in the binding assay with a K_i of 31.76 μM. Maysenine without the C-3 ester, the *N*-methyl and the 4,5-epoxide groups exhibited a partial non-competitive inhibition of binding to tubulin. Maysine which was devoid of the C-3 ester group did not show any competition of binding up to 20 μM drug concentrations. The K_i values of these maytansinoids correlate well with the antitumor activity in the murine P388 leukemia system, and the cytotoxicity towards KB cells. These data emphasize the importance of the ester group at the C-3 ansa chain in maytansinoids for their abilities to compete with vincristine for binding to tubulin and their biological activity.

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Changes in brain polyphosphoinositide metabolism induced by cationic amphiphilic drugs *in vitro*

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Propranolol, which contains both a hydrophobic moiety and a basic amino group, is a cationic amphiphilic drug. A number of such drugs in chronic clinical use are known to induce phospholipidosis in mammals, characterized by occurrence in tissues of cytoplasmic inclusion bodies with properties of lysosomes and containing polar lipids [1–4]. Propranolol, an agent with β-adrenergic receptor blocking and other pharmacological properties, which is widely used in the treatment of hypertension and is of possible utility in schizophrenia [5, 6], may have the potential to be a lipidosis-inducing drug, since it can profoundly alter the pattern of phospholipid metabolism and induce acidic lipid accumulation in tissues *in vitro* [7–14].

The principal effects of propranolol in pineal gland are to stimulate *de novo* synthesis of the liponucleotide phosphatidyl-CMP (CDP-diacylglycerol) and its acidic metabolic products, phosphatidylinositol and phosphatidylglycerol, and to reduce labeling of phosphatidylcholine. In brain cortex mince, on the other hand, the main phospholipid showing increased labeling is phosphatidic acid [11, 12], although decreased incorporation of $^{32}P_i$ into phosphatidylcholine is also characteristic of the action of propranolol in this preparation.

In the course of the study on propranolol-induced alterations of the labeling pattern of phospholipids in rat tissues *in vitro*, we obtained evidence that in addition to the changes described earlier [11, 12] the labeling of polyphosphoinositides from $^{32}P_i$ in preparations of brain is markedly enhanced. This effect of propranolol on brain was compared with those on other tissues and of other cationic amphiphilic drugs on brain cortex. The influence on polyphosphoinositide labeling of different treatments of the tissue preparation and of additions to the incubations has also been investigated.

Materials and methods

Materials. Materials were obtained from the following suppliers: [^{32}P]orthophosphate and [2-^{3}H]myoinositol from New England Nuclear, Boston, MA; (±)-propranolol from Ayerst Laboratories, Inc., New York, NY; haloperidol from McNeil Laboratories, Inc., Fort Washington, PA; pimozide from Janssen Pharmaceutical N.V., Beerse, Belgium; *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES) and chloroquine from the Sigma Chemical Co., St. Louis, MO; and diacylglycerol from pig liver lecithin from Serdary Research Laboratories, London, Ontario, Canada. 4,4'-Bis(diethylaminoethoxy)α,β-diethyldiphenylethane was a gift from Dr. J. Eichberg, Houston, TX. Silica gel H thin-layer plates were purchased from Analtech, Inc., Newark, DE. Silica gel G-60 EM precoated plates were from Brinkmann Instruments, Inc., Westbury, NY. Kodak X-omat RP film was used for radioautography. Adult male rats were provided by Charles River Breeding Laboratories, Wilmington, MA.

Methods. Rats were decapitated and their brains removed immediately. Cerebral cortices were carefully freed of underlying white matter and a mince was prepared with a McIlwain tissue chopper. The mince was suspended at a concentration of approximately 40 mg wet tissue/ml in an incubation medium containing 144 mM NaCl, 6 mM KCl, 5 mM glucose, 2.5 mM $CaCl_2$ and 1.2 mM $MgCl_2$, buffered to pH 7.4 with 50 mM HEPES. Whenever the mince was washed, homogenized, or dialyzed, the same incubation medium was used. The incubations were carried out with 10 mg of mince together with 5–10 μCi $^{32}P_i$, plus drugs and lipid precursors when indicated in a final volume of 0.5 ml for 1 hr at 37° in air. Propranolol was used at a concentration of 0.1 or 0.2 mM and other drugs at 0.2 mM. Myoinositol and choline, when added, were 1 mM; diacylglycerol (as sonicate) and cytidine were 0.1 mM. The reactions were terminated by the addition of 7.5 ml chloroform–methanol (2:1, v/v). The extraction, washing and separation of lipids were essentially as described by Smith and Hauser [15].

The method of Hauser and Eichberg [16] was used when extraction of polyphosphoinositides from the tissue residue was desired. Polyphosphoinositides obtained in this way were separated by one-dimensional chromatography using two successive solvent systems [16]. Lipids were visualized by radioautography, scraped from the plates, and counted in toluene scintillation fluid. Radioactivity data were normalized to 1×10^7 cpm added to the incubation mixture.

Results and discussion

Incubation in air of cerebral cortex mince with 0.1 mM propranolol caused a large increase in the incorporation of $^{32}P_i$ into phospholipids as compared to controls (Table 1). When the medium was equilibrated and the incubation performed with 95% O_2–5% CO_2, total incorporation was increased, but the changes induced by propranolol remained the same. Washing the mince with incubation medium to reduce endogenous P_i enhanced its ability to incorporate $^{32}P_i$ into phospholipids more than 2-fold and retained its susceptibility to the action of propranolol. Dialysis of the mince reduced its ability to incorporate $^{32}P_i$ as compared to unwashed mince but did not lower the incorporation in the presence of the drug. Homogenization of the washed preparation reduced incorporation consider-