THE JOURNAL OF BIOLOGICAL CHEMISTRY

Vol. 262, No. 14, Issue of May 15, pp. 6531-6538, 1987
Printed in U.S.A.

Manoalide, a Natural Sesterterpenoid That Inhibits Calcium Channels*

(Received for publication, November 18, 1986)

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Manoalide is a marine natural product that has anti-inflammatory and anti-proliferative activities and is an irreversible inhibitor of phospholipase A_2 and phospholipase C. It is now shown that the compound is a potent inhibitor of Ca^{2+} mobilization in several cell types. In A431 cells the increase in epidermal growth factor receptor-mediated Ca^{2+} entry and release from intracellular Ca^{2+} stores were blocked by manoalide in a time-dependent manner with an IC_{50} of 0.4 μM. The effect of manoalide on phosphoinositide metabolism, namely the production of inositol monophosphate, did not coincide with its effect on the epidermal growth factor response. In GH_3 cells, manoalide blocked the thyrotropin-releasing hormone-dependent release of Ca^{2+} from intracellular stores without inhibition of the formation of inositol phosphates from phosphatidylinositol 4,5-bisphosphate. Manoalide also blocked the K^+ depolarization-activated Ca^{2+} channel in these cells as well as the activation of the channel by Bay K8644 with an IC_{50} of 1 μM. In addition, manoalide also inhibited the Ca^{2+} influx induced by concanavalin A in mouse spleen cells in a time- and temperature-sensitive manner with an IC_{50} of 0.07 μM. However, neither forskolin-activated adenylate cyclase in A431 cells nor the distribution of the potential sensitive dye, 3,3′-dipropylthiodicarbocyanide iodide in GH_3 cells was affected by manoalide. Thus, manoalide acts as a Ca^{2+} channel inhibitor in all cells examined. This action may account for its effects on inflammation and proliferation and may be independent of its effect on phospholipases.

Manoalide is a novel marine natural product isolated from the sponge *Luffariella variabilis* that has been shown (1) to be a potent inhibitor of bee venom (IC_{50} = 0.05 μM) and (2) cobra venom (IC_{50} = 2 μM) phospholipase A_2. Glaser and Jacobs (3) recently have shown that manoalide irreversibly binds to bee venom phospholipase A_2 and suggest that it may represent a novel class of anti-inflammatory agent.

The discovery that manoalide is a potent phospholipase A_2 inhibitor provided an opportunity to use it as a pharmacological probe of the role and importance of phospholipases in calcium (Ca^{2+}) mobilization. A wide variety of receptors when activated produce their characteristic effects on cell function through mobilization of Ca^{2+} (for reviews see Refs. 4 and 5). Calcium can be mobilized from two sources—intracellular and extracellular. A key step in the action of Ca^{2+}-mobilizing agonists that release Ca^{2+} from intracellular stores is the activation of phospholipase C and the subsequent hydrolysis (6) of phosphatidylinositol 4,5-bisphosphate to inositol 1,4,5-trisphosphate (IP_3).[1] IP_3, in turn, acts as a second messenger and binds to an intracellular receptor on the rough endoplasmic reticulum, releasing Ca^{2+} from intracellular stores (4, 5). Bennett *et al.* (7) have recently reported that manoalide inhibits a phosphatidylinositol-specific phospholipase C purified from guinea pig uterus (IC_{50} = 1.5 μM). The mechanisms that affect the uptake of Ca^{2+} from extracellular sources are less defined.

Two types of channels have been identified in the plasma membrane—hormone-activated channels and voltage-operated channels (8, 9). Since phospholipases release arachidonic acid from the cell membrane, arachidonic acid or its metabolites have been postulated to play a role in opening the hormone-activated channel (4, 10–12).

We chose representative cells and tissues where the mechanisms of Ca^{2+} mobilization have been established to test manoalide's activity. EGF has been shown to open a hormone-activated plasma Ca^{2+} channel in A431 cells using quin-2 dye techniques (14). In GH_3 cells, Ca^{2+} release from intracellular stores is apparent with TRH stimulation, whereas K^+ depolarization or Bay K8644 opens a voltage-sensitive Ca^{2+} channel that depends on extracellular Ca^{2+} (15–17). Both A431 and GH_3 cells are tumor lines. Therefore, normal mouse spleen cells were used to determine the effects of manoalide in non-transformed cells.

In the study reported here, we show that manoalide is a potent inhibitor of Ca^{2+} mobilization whether it is released from intracellular stores or through opening of plasma membrane voltage-operated or hormone receptor-activated Ca^{2+} channels. Furthermore, Bay K8644 action was inhibited. Results in both A431 and GH_3 cells showed that at a concentration of manoalide that totally blocked Ca^{2+} mobilization (1.5–3.0 μM) there was no effect on phosphatidylinositol hydrolysis, nor on cAMP levels nor on diS-C_3-(5) distribution between cells and medium. These results taken together suggest that a major action of manoalide is inhibition of calcium mobilization.

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[1] The abbreviations used are: IP_3, inositol 1,4,5-trisphosphate $[Ca^{2+}]_i$, cytoplasmic (intracellular) free Ca^{2+} concentration; $[Ca^{2+}]_o$, extracellular Ca^{2+} concentration; ConA, concanavalin A; EGF, epidermal growth factor; EGTA, [ethylenebis(oxyethylenenitrilo)]tetraacetic acid; IP, inositol 1-phosphate; TRH, thyrotropin-releasing hormone; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; diS-C_3-(5), 3,3′-dipropylthiodicarbocyanide iodide, Me_2SO, dimethyl sulfoxide.

EXPERIMENTAL PROCEDURES

Cells—Human A431 (clone 8) epidermoid carcinoma cells were obtained from Dr. Gordon N. Gill, Dept. of Medicine, University of California San Diego School of Medicine, La Jolla, CA. Cells were plated in 75-mm flasks and grown in monolayer culture in RPMI 1640 medium containing 10% fetal calf serum, 10 mM HEPES buffer, penicillin (100 units/ml), streptomycin (100 units/ml), 2 mM L-glutamine, and 40 μM 2-mercaptoethanol. Cells were used 2–3 days after plating when cultures were 80–90% confluent.

GH_3 cells, a clonal strain of rat pituitary tumor cells, were obtained from American Type Tissue Culture collection. Cells were grown either as monolayer cultures or in suspension at 37 °C under 95% air, 5% CO_2 in Ham's F-10 medium supplemented with donor horse serum (15%), fetal calf serum (2.5%), penicillin (100 units/ml), and streptomycin (100 units/ml).

Spleen cells were obtained from BALB/c mice. Single cell suspensions were made and red blood cells lysed with a Tris-buffered ammonium chloride solution. Viable cells were counted with fluorescein diacetate and resuspended at approximately 5×10^6 cells per ml.

Reagents—Fura-2 and diS-C_3-(5) were purchased from Molecular Probes Inc., (Junction City, OR) and were stored as 1 mM stock solutions in dimethyl sulfoxide or methanol, respectively, at −70 °C. EGF (culture grade) was purchased from Collaborative Research (Lexington, MA). Bay K8644 was kindly provided by Dr. Fairhurst (Dept. of Pharmacology, University of California, Irvine). Other substances were obtained from the following sources: TRH from Behring Diagnostics; concanavalin A, pyruvate, digitonin from Sigma; Dowex AG 1-X8 (formate form, 100–200 mesh) anion-exchange resin from Bio-Rad; *myo*-[2-^{3}H]inositol (16.3 Ci/mmol) from Amersham Corp. Manoalide was purified from the *L. variabilis* sponge by the Synthetic Chemistry Department, Discovery Research, Allergan Inc./Herbert Labs. Manoalide was stored dry at −70°C in polypropylene tubes and solubilized in Me_2SO or polyethylene glycol 200 for experimental use.

Fura-2 and quin-2 Loading of Cells for Determining $[Ca^{2+}]_i$—A431 cells were detached using a 5–10-min trypsin-EDTA treatment, whereas GH_3 cells were treated 2–5 min with a 1% pancreatin solution. Cells were immediately washed twice in a 20 mM HEPES buffer (pH 7.4) containing 120 mM NaCl, 6 mM KCl, 1 mM $MgSO_4$, 1 mg/ml glucose, 1 mg/ml pyruvate, and 1.4 mM calcium (medium A). Approximately 5×10^6 cells were suspended in medium A and incubated with 4 μM fura-2-AM for 15 min at 37 °C. After washing the fura-2-loaded cells, the uptake of dye was checked using fluorescence microscopy and found to be evenly distributed in the cytosol of all cells. Fluorescence was continuously recorded with a Perkin-Elmer LS-5 spectrofluorometer. The excitation wavelength was set at 340 nm and emission wavelength set at 500 nm. The cell suspension was continually stirred, maintained at 37 °C, and equilibrated for approximately 5 min before addition of various agents. $[Ca^{2+}]_i$ was calculated using the following formula:

$$[Ca^{2+}]_i = 220 \frac{F - F_{min}}{F_{max} - F}$$

All fluorescence values were measured relative to an EGTA-quenched signal determined as follows: F was the relative fluorescence measurement of the sample. F_{max} was determined by lysing the cells with digitonin (100 μl/ml) in Me_2SO. After F_{max} was determined the pH was adjusted to 8, with NaOH and Ca^{2+} chelated with 3 mM EGTA to totally quench the fura-2 signal and obtain F_{min}.

When quin-2 was used, cells were incubated with 10 μM quin-2 at 37 °C for 1 h, washed, and then used. The method of calibration was as described by Moolenaar *et al.* (14).

Uptake of DiS-C_3-(5) by GH_3 Cells—A solution of medium A and 1 μM diS-C_3-(5) was continually stirred, maintained at 37 °C, and equilibrated for at least 5 min before addition of GH_3 cells (1×10^6 cells/ml). Fluorescence was continuously recorded with a Perkin-Elmer LS-5 spectrofluorometer (excitation wavelength = 606 nm; emission wavelength = 664 nm) to follow the uptake and distribution of diS-C_3-(5) between cells and media.

Cyclic AMP in A431 Cells—A431 cells were plated at 4×10^5 cells per well in 24-well cluster dishes. Media from confluent cultures were replaced with Earle's medium containing 1 mM 3-isobutyl-1-methylxanthine and 6 mM ascorbic acid. The cultures were treated with vehicle or manoalide in 0.1% Me_2SO for 10 min. Vehicle or forskolin in ethanol was then added and the incubation continued for an additional 15 min. The reaction was stopped by the addition of trichloroacetic acid. The cells were scraped from the culture dishes and transferred, together with the incubation medium, to 12 × 75-mm tubes. The trichloroacetic acid was removed with water-washed ethyl ether, the samples acetylated according to the method of Harper and Brooker (18), and the cyclic AMP content measured by radioimmunoassay (19).

[^{3}H]Inositol Labeling of A431 and GH_3 Cells, Isolation, and Quantitation of [^{3}H]Inositol Metabolites—Medium M199 was used for labeling cells because it contains a low concentration of cold inositol and enhanced cellular incorporation of [^{3}H]inositol.

A431 cells were plated at 4×10^5 per well (24-well cluster dish) in M199 medium plus 10% fetal bovine serum and incubated overnight with 4 μCi/ml [^{3}H]inositol (Amersham Corp., 16.3 Ci/mmol). Cells were left attached for EGF stimulation. The reaction was stopped by adding chloroform/methanol/4 N HCl (100:200:2, v/v/v). The assay of water-soluble [^{3}H]inositol phosphates was done as described by Beaven *et al.* (13). Suspensions of GH_3 cells were plated in 150-mm Petri dishes at 5×10^5 cell per ml in M199, 15% horse serum, 2.5% fetal calf serum, and [^{3}H]inositol (4 μCi/ml). The cells were incubated another 72 h before the experiment. Isolation and quantitation of inositol phosphates are as described above.

Dose-response Curves for Manoalide—Since this compound shows a time- and temperature-dependent effect, the IC_{50} values stated in the text refer to only the specific conditions of the experiments (5 min preincubation at 37 °C).

RESULTS

Effect of Manoalide on $[Ca^{2+}]_i$ Changes

A431 Cells—The addition of EGF induced a rapid, transient rise in $[Ca^{2+}]_i$ in fura-2-loaded cells (Fig. 1*A*). A maximal response was obtained after about 30–60 s and had not returned to base line by 16 min. The peak values ranged from 246 to 394 nM ($X = 315 \pm 20$, $n = 16$), which represented a 2-fold increase of $[Ca^{2+}]_i$ over basal levels (148 ± 5, $n = 41$). The EGF-dependent rise in $[Ca^{2+}]_i$ was dose-dependent with a maximal response at 100 ng/ml. In A431 cells loaded with quin-2, no change in $[Ca^{2+}]_i$ was seen even with maximal levels of EGF in Ca^{2+}-free medium as described previously (14). Quin-2 is a stronger Ca^{2+} buffer than fura-2, and some inhibitory actions of this dye have been described on Na^+/Ca^{2+} exchange (20). When fura-2-loaded cells in the same Ca^{2+}-free medium were treated with EGF, a transient increase in $[Ca^{2+}]_i$ was observed. Again, there was an approximate doubling of the $[Ca^{2+}]_i$ level, from 118 to 284 nM (Fig. 1*B*). The time course of the response was similar, but $[Ca^{2+}]_i$ levels returned to base line after 2 min. When Ca^{2+} was then added to the medium to raise the concentration to 1.4 mM, $[Ca^{2+}]_i$ increased to 284 nm, after which cells slowly reduced Ca^{2+} toward resting levels (Fig. 1*B*). An alternative means of showing intracellular Ca^{2+} release by an agonist is to block Ca^{2+} entry. As shown in Fig. 1*C*, 100 μM La^{3+} was not able to prevent the EGF-induced rise in $[Ca^{2+}]_i$. However, as in Ca^{2+}-free media, the cells reduced $[Ca^{2+}]_i$ rapidly to resting levels. These data indicated that EGF was able both to mobilize intracellular Ca^{2+} stores, and to increase Ca^{2+} entry into A431 cells.

Preincubation of these cells with manoalide blocked these EGF responses. The addition of manoalide in this cell type produced a small transient change in $[Ca^{2+}]_i$ that was dependent on $[Ca^{2+}]_o$ (Fig. 1, *D* and *E*). 150 nM manoalide required about 30 min incubation at 37 °C to abolish the EGF effect, whereas 1.5 μM required only 5 min preincubation. Similar results were obtained in Ca^{2+}-free medium, showing that manoalide did not discriminate between the Ca^{2+} pathways affected by the stimulus, EGF.

The time dependence of inhibition by manoalide is shown in Fig. 2. Thus, manoalide was preincubated with A431 cells at concentrations of 150 nM, 300 nM, and 1.5 μM. Cells were sampled at different times and the EGF-mediated response

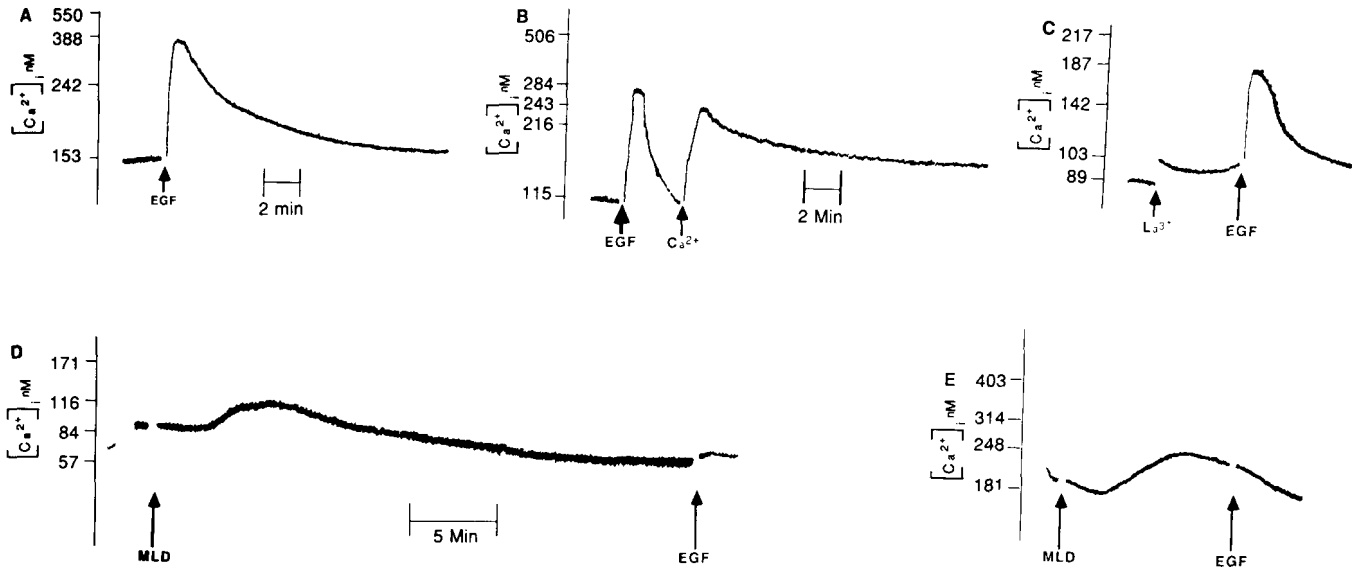

FIG. 1. **Effect of manoalide on the EGF-induced increase in $[Ca^{2+}]_i$.** A431 cells were loaded with fura-2, and $[Ca^{2+}]_i$ was measured as described under "Experimental Procedures." *A*, A431 cells (7×10^4 cells/ml) resuspended in medium A containing 1.4 mM $CaCl_2$. Where indicated, the cells were stimulated with 100 ng/ml EGF. This experiment is representative of 16 others. *B*, A431 cells added to Ca^{2+}-free medium A and stimulated with 100 ng/ml EGF. Where indicated, 1.4 mM $CaCl_2$ were added to the incubation medium ($n = 3$). *C*, A431 cells resuspended in medium A containing 1.4 mM $CaCl_2$. Where indicated, 100 μM $LaCl_3$ and then 100 ng/ml EGF were added to the incubation medium ($n = 3$). *D*, A431 cells suspended in medium A containing 1.4 mM $CaCl_2$. 0.15 μM manoalide from a stock solution of 1 mM in Me_2SO was then added. After 30 min of incubation at 37 °C, the cells were stimulated with 100 ng/ml EGF ($n = 3$). *E*, experimental procedure as in *D*, except that the cells were treated with 1.5 μM manoalide for 5 min before stimulation with EGF ($n = 5$). *MLD*, manoalide.

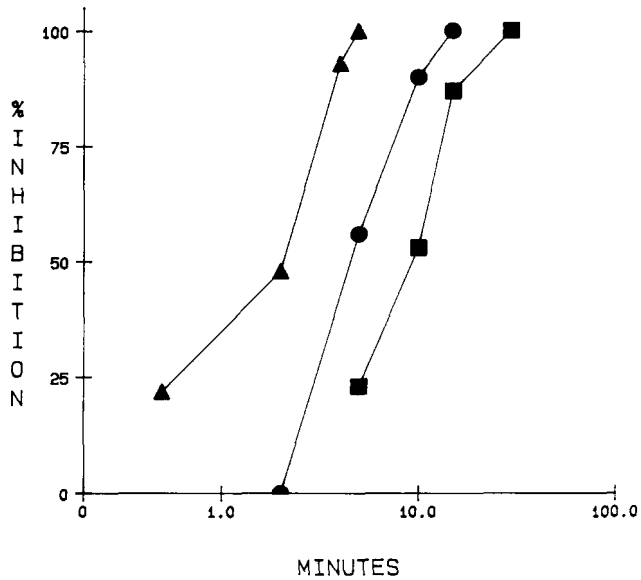

FIG. 2. **Time course of manoalide inhibition of EGF effect in A431 cells at three manoalide concentrations.** A431 cells were loaded with fura-2, and $[Ca^{2+}]_i$ was measured as described under "Experimental Procedures." The cells were then washed and resuspended at a concentration of 4×10^6 cells/ml in medium A containing 1.4 mM $CaCl_2$. 50 μl of the cells were added to 3 ml of medium A containing 1.4 mM $CaCl_2$ and either 0.15 (■), 0.3 (●), or 1.5 μM (▲) manoalide. After the indicated incubation times at 37 °C, the cells were stimulated with 100 ng/ml EGF and $[Ca^{2+}]_i$ was measured. The EGF-mediated increase in $[Ca^{2+}]_i$ in the absence of manoalide (control) was taken as 100% response and was measured before each incubation time with manoalide.

of $[Ca^{2+}]_i$ was monitored. As seen from Fig. 2, 100% inhibition of the response was obtained after 5 min with 1.5 μM drug, 15 min at 300 nM, and 30 min at 150 nM. Therefore, the dose-response curve for manoalide was obtained with a standardized preincubation time of 5 min at 37 °C. Manoalide had an IC_{50} of 0.4 μM for the total response of $[Ca^{2+}]_i$ to EGF in A431 cells. The inhibition of EGF-induced intracellular Ca^{2+} release by manoalide in Ca^{2+}-free media was identical to the combined response seen in medium A with 1.4 mM $CaCl^{2+}$ ($IC_{50} = 0.4$ μM).

GH_3 Cells—This cell line has been used to study two types of Ca^{2+} responses—the release of Ca^{2+} from intracellular stores and depolarization-dependent Ca^{2+} entry induced by elevation of medium K^+ (15). This cell model therefore allows assessment of the effect of manoalide on a proven IP_3-mediated response and on a voltage-operated channel.

The addition of TRH induced a transient rise in $[Ca^{2+}]_i$ from a basal level of 207 ± 5 nM ($n = 43$) to 511 ± 87 nM ($n = 7$) that decayed to almost base-line values in about 5 min (Fig. 3*A*). 0.1 μM TRH produced a maximal rise in $[Ca^{2+}]_i$ that was independent of medium Ca^{2+} (Fig. 3*B*). However, in the absence of medium Ca^{2+}, the effect of TRH was short-lived compared to the effect in the presence of medium Ca^{2+}. An increase of medium K^+ induced an increase in $[Ca^{2+}]_i$ (Fig. 3*A*) that was dependent on the presence of medium Ca^{2+} (Fig. 3*B*). 0.5 μM manoalide for 20 min or 3.0 μM for 5 min completely blocked both the hormone and voltage effects on $[Ca^{2+}]_i$ (Fig. 3, *C* and *D*).

The Ca^{2+} agonist Bay K8644, when added to GH_3 cells incubated in medium A containing 12 mM K^+, induced a rise in $[Ca^{2+}]_i$ from 208 to 520 nM that was dependent on the presence of medium Ca^{2+} (Fig. 4, *A* and *B*). The effect of Bay K8644 was dose-dependent with an ED_{50} of 0.3 μM (Fig. 4*D*). This compound is known to affect the voltage-operated Ca^{2+} channel (15–17). Manoalide inhibited the effect of Bay K8644,

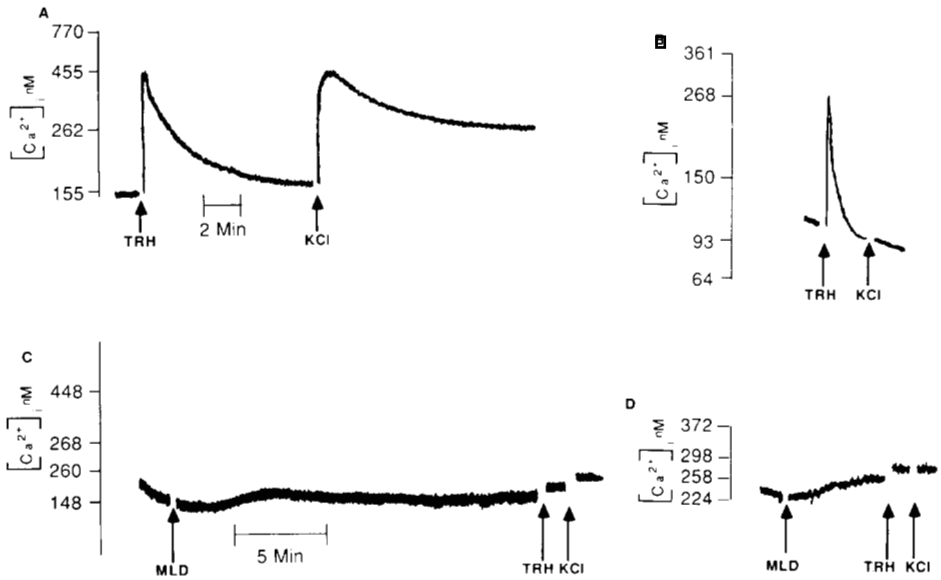

FIG. 3. **Effect of manoalide on TRH and KCl induced increase in $[Ca^{2+}]_i$ in GH_3 cells.** GH_3 cells were detached from culture plates and loaded with fura-2 as described under "Experimental Procedures." *A*, about 10^5 cells/ml suspended in medium A containing 1.4 mM $CaCl_2$ and incubated at 37 °C with continuous stirring in a fluorometer cuvette. Where indicated, 0.1 μM TRH and then 50 mM KCl were added. This experiment is one of six other determinations. *B*, fura-2-loaded GH_3 cells washed once (by centrifugation for 5 min at 300 × *g*) and resuspended in Ca^{2+}-free medium A containing 0.2 mM EGTA. Where indicated, 0.1 μM TRH and 50 mM KCl were added to the incubation medium ($n = 3$). *C*, 0.5 μM manoalide from a 1 mM stock solution in Me_2SO added to GH_3 cells suspended in medium A containing 1.4 mM $CaCl_2$. After 20 min of incubation at 37 °C, 0.1 μM TRH and 50 mM KCl were added as indicated in the figure ($n = 3$). *D*, the experimental protocol was as for *C*, except that the cells were incubated with 3 μM manoalide for 5 min before stimulation with 0.1 μM TRH and 50 mM KCl ($n = 5$). *MLD*, manoalide.

as shown in Fig. 4*C*. With 5 min preincubation at 37 °C, the IC_{50} values for the four responses (TRH, ±1.4 mM medium Ca^{2+}, K^+ depolarization, and Bay K8644) were similar and were found to be 1.0 μM.

Mouse Splenic Lymphocytes—These cells were chosen to provide a model of a non-tumor cell line. In fura-2-loaded cells, the addition of ConA produced a 1.5-fold rise in $[Ca^{2+}]_i$ to a level of 272 ± 11 nM ($n = 8$) followed by a slow reduction toward base line as illustrated in Fig. 5*A*. The ConA-mediated increase in $[Ca^{2+}]_i$ was largely dependent on $[Ca^{2+}]_o$ (Fig. 5*B*). Addition of 1.4 mM Ca^{2+} after ConA increased the $[Ca^{2+}]_i$ to 403 nM (Fig. 5*B*). The addition of Ca^{2+} to unstimulated cells in Ca^{2+}-free medium resulted in a 60 nM increase in $[Ca^{2+}]_i$. As shown in Fig. 5*C*, preincubation of cells with 0.5 μM manoalide for 5 min inhibited the ConA response. The IC_{50} for the compound was found to be 70 nM. As for the tumor cell lines, the effect of manoalide was time-dependent and irreversible.

The effect of the incubation temperature on manoalide inhibition of the ConA response is shown in Fig. 6. In preliminary experiments it was found that the manoalide effect was irreversible, although any excess manoalide can be washed from the cells (not shown). Therefore, to test the effect of the incubation temperature on manoalide effect, lymphocytes were incubated with 0.1 μM manoalide for 5 min at the indicated temperature. Then the cells were washed with manoalide-free medium, resuspended in 3 ml of incubation medium at 37 °C, and stimulated with ConA. The manoalide effect was sensitive to the preincubation temperature; when above 20 °C, the potency of manoalide increased exponentially. An irreversible, temperature-dependent effect was also reported for manoalide inhibition of phospholipase A_2 (2, 3).

Effect of Manoalide on Inositol Phosphate Production

A431 Cells—The stimulation of phosphatidylinositol turnover in A431 cells by EGF has been shown to depend on extracellular Ca^{2+} (21). Due to the newly described ability of EGF to release Ca^{2+} from intracellular stores (Fig. 1), we reexamined the Ca^{2+} requirement for EGF-induced phosphatidylinositol turnover (Fig. 7). In the absence of Ca^{2+} in the medium after 15 min there was no elevation of inositol phosphate production, but at longer time intervals there was a significant increase even in the absence of medium Ca^{2+}. An increase of the effective medium Ca^{2+} concentration from 50 nM to 5 μM restored 70% of the EGF-induced increment of IP levels. The IP levels increased linearly for 60 min. The maximal increase of IP was produced at 100 μM medium Ca^{2+} and was the same as that found at 1.4 mM. Separation of the inositol phosphates showed that the major product was inositol monophosphate, and no increase in IP_3 or inositol 1,4-bisphosphate was detected (Table I). The apparent EGF-induced release of Ca^{2+} (Fig. 1) suggested that IP_3 would be formed in the first 2 min. However, in spite of lengthy incubation of the cells in *myo*-[^{3}H]inositol, no effect of EGF was found on IP_3. As will be seen later, this was not true of TRH stimulation of GH_3 cells.

Fig. 8 shows the effect of 1.5 μM manoalide for 10 min on the EGF-stimulated formation of inositol phosphates over a 60 min observation period. As before, in the absence of EGF there was no increase of IP formation over the basal level. The effect of EGF was statistically significant at 5 min and rose thereafter. Manoalide at 1.5 μM did not inhibit EGF-stimulated IP production. This experiment provides further evidence that a change in $[Ca^{2+}]_i$ is not required for EGF-mediated stimulation of IP production. However, higher manoalide concentrations (10 μM) produced a 40% inhibition of EGF action on IP formation. When the EGF-dose response of IP formation at 60 min was compared in the absence and presence of manoalide, no effect of 1.5 μM manoalide was observed at doses of EGF up to 150 ng/ml (Fig. 9). Thus, the

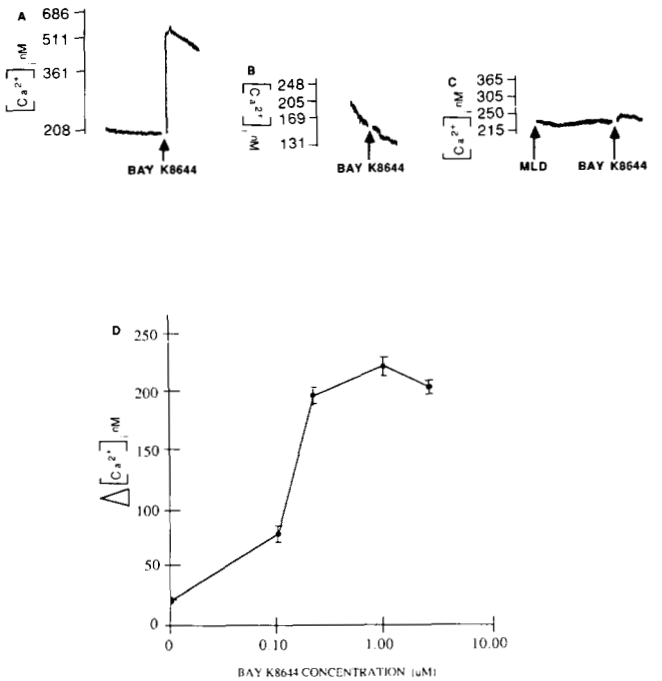

FIG. 4. **Effect of Bay K8644 and manoalide on $[Ca^{2+}]_i$ in GH_3 cells.** Fura-2-loaded GH_3 cells (10^5 cells/ml) were suspended in medium A containing 1.4 mM $CaCl_2$ and 12 mM KCl. *A*, where indicated, 1 μM Bay K8644 from a stock solution of 1 mM in ethanol:water (50:50, v/v) was added to the incubation medium. *B*, cells washed and suspended in Ca^{2+}-free medium A containing 12 mM KCl and 0.2 mM EGTA as described in the legend to Fig. 3. Where indicated, the cells were stimulated with 1 μM Bay K8644. *C*, GH_3 cells suspended in incubation medium similar to that in *A* incubated with 1 μM manoalide for 5 min before stimulation with 1 μM Bay K8644 ($n = 3$). *D*, to obtain the dose-response curve for Bay K8644-mediated increase in $[Ca^{2+}]_i$ in GH_3 cells, the protocol of experiment A was used except that the indicated concentrations of Bay K8644 were added and the peak increase in $[Ca^{2+}]_i$ was measured. *MLD*, manoalide.

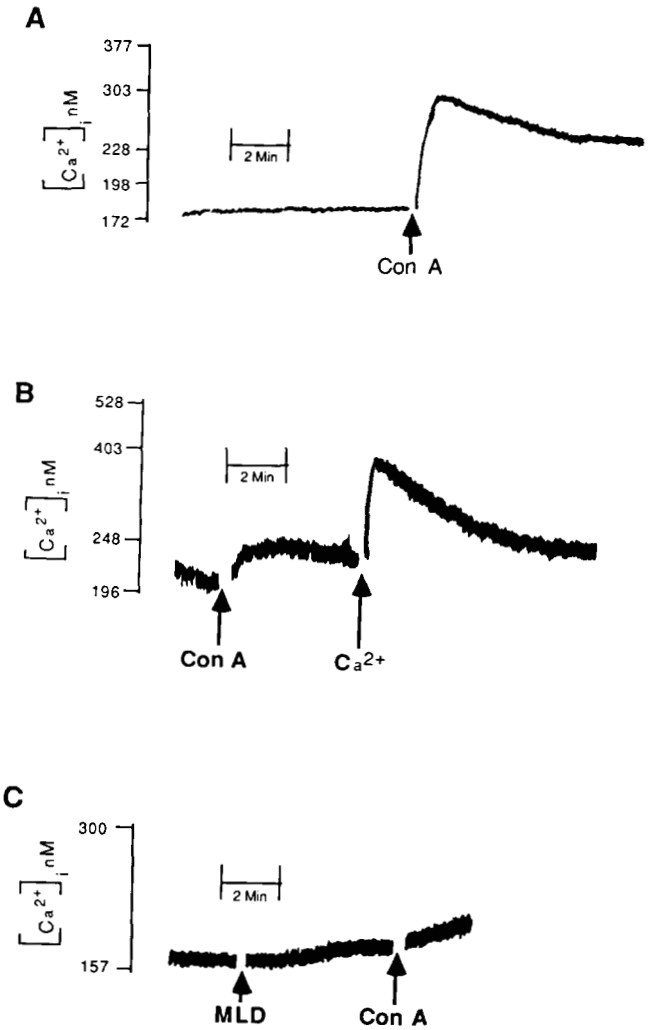

FIG. 5. **Effect of manoalide on ConA-induced increase in $[Ca^{2+}]_i$ in mouse spleen lymphocytes.** Mouse spleen lymphocytes were isolated and loaded with fura-2 as described under "Experimental Procedures." *A*, about 7×10^5 cells/ml resuspended in medium A containing 1.4 mM $CaCl_2$ and then stimulated with 25 μg/ml of ConA. The experiment shown is representative of eight others. *B*, cells washed and resuspended in Ca^{2+}-free medium A before stimulation with 25 μg/ml ConA. When $[Ca^{2+}]_i$ stabilized, 1.4 mM $CaCl_2$ was added to the medium. Addition of 1.4 mM $CaCl_2$ to cells suspended in Ca^{2+}-free medium and without ConA increased $[Ca^{2+}]_i$ only by about 60 nM. *C*, cells in medium A containing 1.4 mM $CaCl_2$ incubated with 0.5 μM manoalide for 5 min before stimulation with 25 μg/ml ConA. *MLD*, manoalide.

ED_{50} for EGF was unchanged by manoalide. Apparently, in A431 cells, the action of manoalide on $[Ca^{2+}]_i$ responses could be dissociated from its effect on IP formation and hence from its effect on phospholipase C.

GH_3 Cells—The action of TRH on the level of IP_3 is shown in Fig. 10. The change in IP_3 levels was found to be independent of medium Ca^{2+} and similar to previously described experiments (22–24). Preincubation with manoalide at 1 and 3 μM had no effect on the TRH-induced increment of IP_3, inositol 1,4-bisphosphate (not shown), and IP (not shown). However, at 10 μM, there was significant inhibition of IP_3 release. Thus, manoalide at high concentrations inhibited IP_3 formation, presumably due to its effect on phospholipase C. However, at concentrations where the $[Ca^{2+}]_i$ increase was blocked, no effect of manoalide on IP_3 levels was found. Thus, as for the A431 cells, the effect of the compound on Ca^{2+} signals was dissociated from its effect on inositide metabolism.

Effect of Manoalide on Other Membrane Functions—In A431 cells forskolin stimulated dose-responsive increases in cAMP that were not inhibited by 1 and 10 μM manoalide. The action of the compound did not extend, therefore, to the membrane-bound adenylate cyclase (Table II). GH_3 cells respond to depolarization by activation of a voltage-dependent Ca^{2+} channel. Thus, if manoalide altered cell membrane potential, this voltage effect might be blocked. Accordingly, the uptake of the potential sensitive dye, diS-C_3-(5) was monitored in control and manoalide-treated cells. No difference in the redistribution of the dye was noted (Fig. 11) in GH_3 cells even in the presence of Ca^{2+} inhibitory concentrations of manoalide.

DISCUSSION

The data from these studies show that manoalide can block a variety of Ca^{2+} responses, namely hormone-operated plasma membrane Ca^{2+} channels, voltage-operated plasma membrane Ca^{2+} channels, and intracellular Ca^{2+} release. Both tumor cell lines and normal cells were chosen, to ensure that the actions described had general applicability. In addition, the relationship between the Ca^{2+} changes and alterations in phosphoinositide metabolism was investigated as a function of the production of water-soluble inositol phosphates.

A431 cells were selected initially because of the previous finding (14) that EGF raised intracellular Ca^{2+} due to Ca^{2+} entry. In these studies quin-2, which has a greater buffering capacity than fura-2, was used but this dye or its metabolites

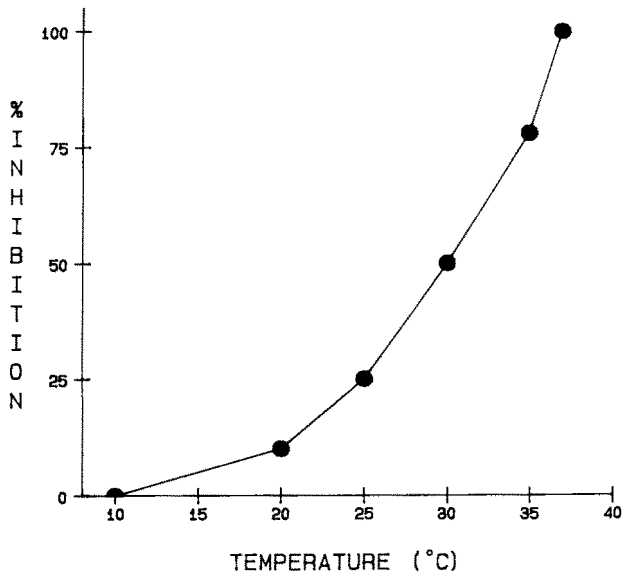

FIG. 6. **Temperature dependence of manoalide inhibition of ConA-mediated increase in $[Ca^{2+}]_i$ in mouse spleen lymphocytes.** Fura-2-loaded mouse spleen lymphocytes (7×10^5 cells/ml) were incubated with 0.1 μM manoalide for 5 min at the indicated temperature. Cells were then centrifuged for 10 s at 12,000 × *g* in an Eppendorf centrifuge. The cells were resuspended in 3 ml of prewarmed, manoalide-free medium A. The cells were then stimulated with 25 μg/ml ConA, and $[Ca^{2+}]_i$ was measured.

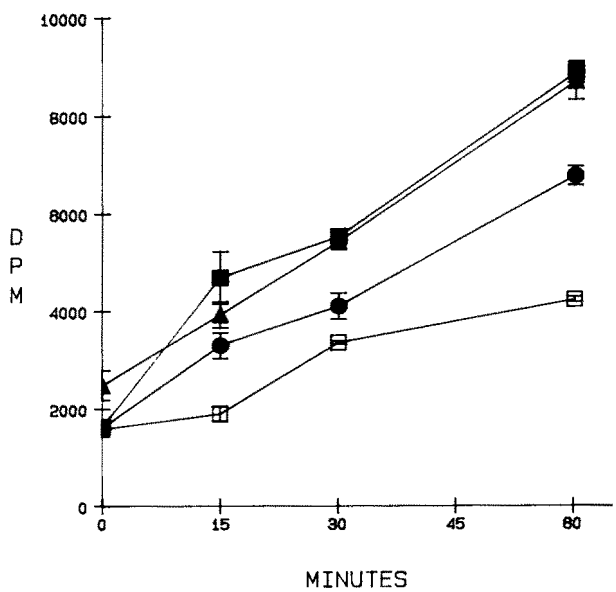

FIG. 7. **Effect of extracellular Ca^{2+} on the EGF-induced release of [^{3}H]inositol phosphates in A431 cells.** Monolayers of A431 cells were incubated with [^{3}H]inositol (4 μCi/ml) overnight in M199 medium as described under "Experimental Procedures." The cells were washed with medium A containing 1.4 mM $CaCl_2$ and 5 mM unlabeled *myo*-inositol and then 200 μl of new medium A containing 5 mM LiCl and the following $CaCl_2$ concentrations placed in each well: Ca^{2+}-free and 0.2 M EGTA (□), no Ca^{2+} or EGTA were added and the Ca^{2+} concentration was not determined (●), 0.1 mM $CaCl_2$ (▲), 1.4 mM $CaCl_2$ (■). After 10 min of incubation at 37 °C the cells were stimulated with 200 ng/ml EGF. At the indicated times, the reactions were terminated with 750 μl of chloroform/methanol/4 N HCl (100:200:2, v/v/v), and [^{3}H]inositol phosphates were separated as described by Beaven *et al.* (13). Control cells contained 31,556 ± 2,964 dpm in the lipid fraction. Results are mean ± S.E. of triplicate determinations.

TABLE I

Effect of Ca^{2+} on production of [^{3}H]inositol phosphates in EGF-stimulated A431 cells

Monolayers of A431 cells were incubated with 4 μCi/ml [^{3}H]inositol as described under "Experimental Procedures." The cells were then washed three times with medium A containing 5 mM unlabeled *myo*-inositol to remove extracellular label and were incubated for 10 min with 200 μl of medium A containing 5 mM Li^+. After the indicated incubation time with 200 ng/ml EGF, the reaction was terminated by the addition of 750 μl of choroform/methanol/4 N HCl (100:200:2, v/v/v). The [^{3}H]inositol phosphates were isolated and quantitated as described by Beaven *et al.* (13). A shows the result obtained with cells treated with EGF in the presence of 1.4 mM $[Ca^{2+}]_o$. B shows the results obtained with cells treated with EGF in Ca^{2+}-free media plus 0.2 mM EGTA. For the experiment in C, A431 cells were incubated for 2.5 days with 4 μCi/ml *myo*-[^{3}H]inositol before the stimulation with EGF in medium containing 1.4 mM Ca^{2+}. Numbers are means ± S.E. ($N = 3$). IP_2 = inositol 1,4-bisphosphate.

Incubation time	Inositol phosphates		
	IP	IP_2	IP_3
		dpm	
A. 0 s	1,192	117	174
15 s	1,087	93	131
30 s	755	64	110
1 min	734	88	124
2 min	1,163	119	198
15 min	4,761	124	183
30 min	6,968	150	155
60 min	11,723	100	136
B. 0 s	937	124	160
15 s	817	164	148
30 s	1,118	153	126
1 min	882	68	188
2 min	1,099	124	160
15 min	1,873	98	243
30 min	2,428	52	191
60 min	4,373	114	205
C. 0 s	2,818 ± 224	234 ± 17	263 ± 26
15 s	2,902 ± 438	305 ± 68	281 ± 29
30 s	2,506 ± 199	184 ± 32	231 ± 11
1 min	2,891 ± 229	176 ± 27	219 ± 33
15 min	3,675 ± 29	163 ± 39	213 ± 83

may have additional toxic effects (20). When fura-2 was used to monitor the EGF-mediated changes in $[Ca^{2+}]_i$ in A431 cells, the change in $[Ca^{2+}]_i$ was only partially dependent on $[Ca^{2+}]_o$. Thus, in this cell type, EGF mobilized Ca^{2+} from intracellular stores, allowed Ca^{2+} entry across the plasma membrane, and increased turnover of phosphoinositides. We could not obtain evidence for a relationship between intracellular Ca^{2+} release and the formation of IP_3. This may be due to a small pool of IP_3 being formed, beyond the limits of our measurement techniques and therefore, if manoalide blocked this pool, we would not be able to detect its inhibition. Release of Ca^{2+} from intracellular stores could also be due to an alternative signaling system. Removal of medium Ca^{2+} abolished the EGF-dependent IP formation for the first 15 min of incubation, without affecting Ca^{2+} release from intracellular stores. Furthermore, in the presence of medium Ca^{2+}, EGF did not affect the production of inositol phosphates for the first 2 min of incubation, by which time $[Ca^{2+}]_i$ has passed its peak. At concentrations of manoalide that obliterated either type of $[Ca^{2+}]_i$ response, no effect of manoalide on the production of inositol phosphates was seen. At higher concentration (10 × IC_{50}), manoalide blocked production of inositol phosphates as expected from studies on inhibition of phospholipases. It would appear that manoalide is able to dissociate in A431 cells, inositide turnover, and changes in

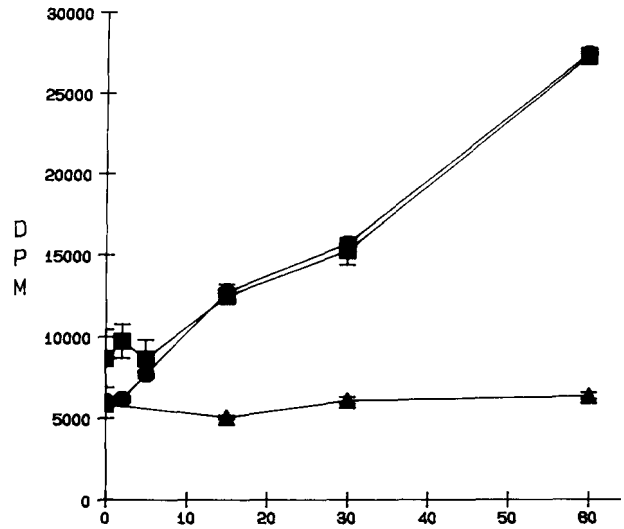

FIG. 8. **Effect of manoalide on EGF-stimulated [^{3}H]IP production in A431 cells.** Cells were labeled with [^{3}H]inositol as described under "Experimental Procedures." The cells were prepared as described in the legend to Fig. 8. Manoalide at 1.5 μM (■) or vehicle (●) was then added and incubated at 37 °C for 10 min. The cells were then stimulated with 200 ng/ml EGF. Addition of manoalide (1.5 μM) with no EGF stimulation is represented by the *solid triangle*. At the indicated times, the reaction was terminated, and total water-soluble [^{3}H]inositol phosphates determined. Control cells contained 96,269 ± 3,835 dpm of ^{3}H label in the lipid fraction. The data shown are mean ± S.E. of triplicate determinations.

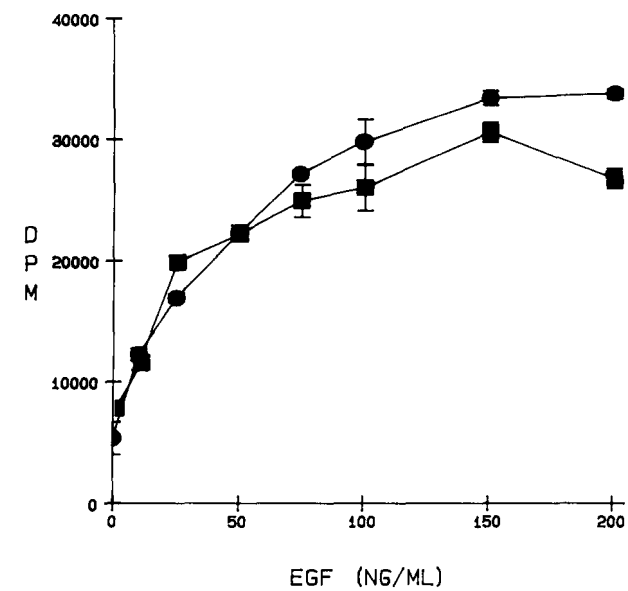

FIG. 9. **Dose-dependent curve for EGF stimulation of IP formation in control and manoalide-treated A431 cells.** Labeling of cells with [^{3}H]inositol and the experimental protocol was the same as in the legend to Fig. 8. The cells in medium A containing 1.4 mM $CaCl_2$ and 5 mM LiCl were incubated with 1.5 μM manoalide (■) or vehicle (●) for 10 min at 37 °C before stimulation with the indicated concentration of EGF. After 60 min of incubation at 37 °C, the reaction was terminated, and total water-soluble [^{3}H]inositol phosphates quantitated. The results shown are the mean ± S.E. of triplicate determinations.

$[Ca^{2+}]_i$. This would be anticipated if manoalide was acting as an inhibitor of Ca^{2+} channels.

GH_3 cells possess two systems of interest in this connection. TRH mobilized intracellular Ca^{2+} stores by means of hydrol-

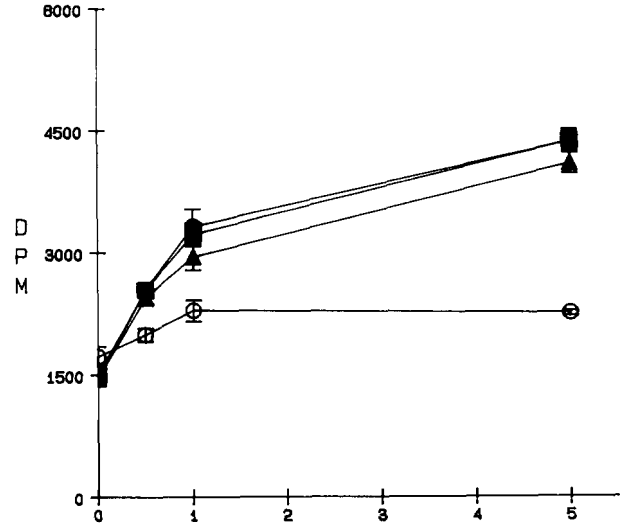

FIG. 10. **Effect of manoalide on TRH-mediated IP_3 production in GH_3 cells.** Suspensions of GH_3 cells were labeled with 4 μCi/ml [^{3}H]inositol as described under "Experimental Procedures." The cells were then washed twice in medium A containing 5 mM *myo*-inositol and 1.4 mM $CaCl_2$, and then resuspended in medium A containing 1.4 mM $CaCl_2$ and 5 mM LiCl and incubated for 10 min at 37 °C. Cells were incubated for 5 min at 37 °C with either vehicle (●), 1 μM (■), 3 μM (▲), or 10 μM (○) manoalide, before stimulation with 0.1 μM TRH. At the indicated times, samples were removed, the reaction was terminated, and IP_3 was separated on Dowex columns as described (13). The results shown are the mean ± S.E. of three determinations.

TABLE II

Effect of manoalide on forskolin-stimulated cyclic AMP production in A431 cells

A431 cells were incubated with 0, 1, or 10 μM manoalide for 10 min at 37 °C. Then the indicated concentrations of forskolin were added and the incubation at 37 °C allowed to proceed for a further 15 min. At the end of the incubation with forskolin, the reaction was terminated by the addition of 0.5 ml of 30% trichloroacetic acid. The supernatant was collected for measurements of cAMP as described under "Experimental Procedures." Values for control and 1 μM manoalide represent the mean ± S.E. of three experiments in which the effect of each drug concentration was determined in triplicate. Values for 10 μM manolide represent the mean ± S.E. of triplicate determinations in one experiment.

Additions	Cyclic AMP		
	Control	Manoalide, 1 μM	Manoalide, 10 μM
	nmol/mg protein		
Vehicle	0.08 ± 0.04	0.04 ± 0.003	0.10 ± 0.05
Forskolin			
1 μM	0.20 ± 0.07	0.22 ± 0.07	ND[a]
10 μM	1.23 ± 0.33	1.34 ± 0.34	ND
100 μM	6.94 ± 1.90	7.55 ± 2.00	8.49 ± 0.36

[a] ND, not determined.

ysis of phosphatidylinositol 1,4,5-bisphosphate and release of IP_3 as well as increasing Ca^{2+} entry. TRH more than doubled $[Ca^{2+}]_i$, and the initial increase was independent of $[Ca^{2+}]_o$. Manoalide inhibition of the TRH-dependent $[Ca^{2+}]_i$ signal had an apparent IC_{50} of 1 μM; a 10-fold greater manoalide concentration was required to inhibit partially the TRH induced increase of IP_3 and other inositol phosphates (data not shown). In contrast to A431 cells; we were able to show that, in GH_3 cells, manoalide's effect on $[Ca^{2+}]_i$ is dissociated from an effect of the drug on IP_3 production. Manoalide also blocked the K^+ depolarization-activated Ca^{2+} channel as well

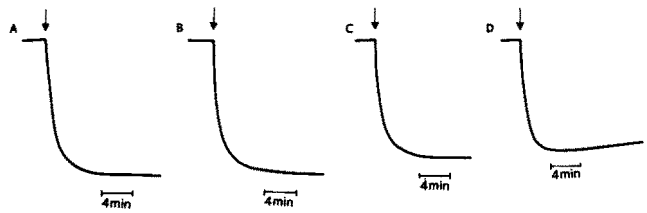

FIG. 11. **Effect of manoalide on diS-C_3-(5) distribution in GH_3 cells.** GH_3 cells were treated with vehicle (*A*), 1 μM (*B*), 3 μM (*C*), or 10 μM (*D*) manoalide for 5 min at 37 °C before addition to medium A containing 1.4 mM $CaCl_2$ and 1 μM diS-C_3-(5). The fluorescence of the dye was measured as described under "Experimental Procedures."

as the activation of the channel by Bay K8644. Evidently, $[Ca^{2+}]_i$ changes with this protocol are due to Ca^{2+} entry. Manoalide readily blocked these responses with an IC_{50} of 1.0 μM with similar characteristics as shown for the other Ca^{2+} pathways, in terms of IC_{50} and time dependence. Thus, in these two cultured cell lines, manoalide was able to block hormone-operated plasma membrane Ca^{2+} pathways, pathways of intracellular Ca^{2+} release, and voltage-operated plasma membrane pathways. The action of manoalide appears to inhibit IP_3-linked agonist-induced release of intracellular Ca^{2+} distal to the generation of inositol phosphates.

Manoalide was found not to inhibit forskolin-stimulated adenylate cyclase in A431 cells or the uptake of the membrane potential sensitive dye diS-C_3-(5). These two membrane-associated activities were quantitated to determine whether the hydrophobicity (calculated log p = 4.5; method described in Ref. 25) of manoalide might produce nonspecific effects on cell membranes. DiS-C_3-(5) is a lipid-permeable cation that accumulates in membrane-enclosed spaces as a function of a negative interior potential, and thus serves as a monitor of plasma membrane potential as well as mitochondrial potential. Because manoalide did not alter the distribution of the dye, we conclude that manoalide affects Ca^{2+} mobilization across plasma and endoplasmic reticular membranes without interference with processes that determine potential differences.

In the case of both bee venom (26, 27) and cobra venom (2) phospholipase A_2, manoalide has been suggested to bind irreversibly to a lysine group at or near the active site. The time and temperature dependence of the action of manoalide on Ca^{2+} mobilization, as well as the difficulty of reversal by washing, suggests that derivatization of cellular sites other than phospholipase C or phospholipase A_2 might be responsible for the effects observed in these experiments. The activity of manoalide allows some dissection of the Ca^{2+} signals from phosphoinositide metabolism and thus provides a probe for studying Ca^{2+} signaling in a variety of normal and transformed cell types and cellular compartments. The anti-inflammatory and anti-proliferative activities of manoalide may derive more from its Ca^{2+} effects rather than from its effects on lipid metabolism.

Acknowledgements—We thank S. Docheff and M. A. Pitale for their secretarial assistance and typing the manuscript. We also thank Drs. M. Garst and E. Tallman for isolating, purifying, and supplying the manoalide used in these studies and Dr. S. Dietrich for doing the log p calculation for manoalide.

Note Added in Proof—EGF-induced IP_3 turnover has been recently reported by Pike and Eakes (Pike, L. J., and Eakes, A. T. (1987) *J. Biol. Chem.* **262,** 1644–1651) which is consistent with the release of Ca^{2+} from intracellular stores reported in this study.

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