Cancer Chemother Pharmacol (1994) 34: 344–350

Cancer Chemotherapy and Pharmacology

Increased intracellular Ca^{2+} signaling caused by the antitumor agent helenalin and its analogues

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Received: 29 November 1993 / Accepted: 11 March 1994

Abstract. The antitumor sesquiterpene lactone helenalin, which is found in species of the plant genus *Helenium*, caused a marked potentiation of the increases in intracellular free Ca^{2+} concentration ($[Ca^{2+}]_i$) produced by mitogens such as vasopressin, bradykinin, and platelet-derived growth factor in Swiss mouse 3T3 fibroblasts. Removing external Ca^{2+} partly attenuated the increased $[Ca^{2+}]_i$ responses caused by helenalin. The increased $[Ca^{2+}]_i$ responses occurred at concentrations of helenalin that inhibited cell proliferation. At higher concentrations, helenalin inhibited the $[Ca^{2+}]_i$ responses. No change in resting $[Ca^{2+}]_i$ was caused by helenalin even at high concentrations. Other helenalin analogues also increased the $[Ca^{2+}]_i$ response. Helenalin did not inhibit protein kinase C (PKC) and PKC appeared to play a minor role in the effects of helenalin on $[Ca^{2+}]_i$ responses in intact cells. Studies with saponin-permeabilized HT-29 human colon carcinosarcoma cells indicated that helenalin caused an increased accumulation of Ca^{2+} into nonmitochondrial stores and that the potentiating effect of helenalin on mitogen-stimulated $[Ca^{2+}]_i$ responses was due in part to an increase in the inositol-(1,4,5)-trisphosphate-mediated release of Ca^{2+} from these stores.

Key words: Intracellular Ca^{2+} – Helenalin

Introduction

The cytoplasmic free Ca^{2+} concentration ($[Ca^{2+}]_i$) is very tightly regulated in eukaryotic cells with resting levels of around 0.1 μM [9]. Transient increases in $[Ca^{2+}]_i$ to micromolar levels are used by cells as important intracellular signaling events that mediate the effects of a variety of external stimuli, including growth factors, hormones, and neurotransmitters [3]. The most extensively studied pathway of $[Ca^{2+}]_i$ signaling involves the phospholipase C-mediated hydrolysis of phosphatidylinositol(4,5)-bisphosphate [PtdIns(4,5)P_2].

There are four major classes of phosphatidylinositol-specific phospholipase Cs (PtdInsPLC-α, -β, -γ, and -δ) [47]. Recent studies have defined at least two distinct mechanisms of PtdInsPLC activation. In the first mechanism a mitogenic peptide, such as vasopressin, acts on a seven-pass membrane spanning receptor coupled to a specific guanine nucleotide-binding (G) protein with α subunits of the $G\alpha_q$ subfamily to activate membrane-bound PtdInsPLC-β [51, 53, 57]. In the second mechanism the binding of a growth factor, such as platelet-derived growth factor (PDGF), to its plasma membrane receptor causes the receptor monomers to dimerize and then to phosphorylate each other on tyrosine residues [6]. This permits a conformational change in the receptor that enhances its protein tyrosine kinase activity [38] toward substrates including PtdInsPlc-γ, which is activated by tyrosine phosphorylation [10]. Receptor autophosphorylation also provides phosphotyrosine binding sites on the receptor for the recruitment of specific cytoplasmic enzymes that contain an *src*-homology-2 (SH2) domain that binds with high affinity to certain phosphotyrosines [32, 37]. One such cytoplasmic enzyme is PtdInsPLC-γ [55].

PtdInsPLCs hydrolyze PtdIns(4,5)P_2 to give the water-soluble inositol(1,4,5)trisphosphate [Ins(1,4,5)P_3] and a lipophilic diacylglycerol (DAG) [4]. Ins(1,4,5)P_3 releases Ca^{2+} from nonmitochondrial stores, producing a transient increase in $[Ca^{2+}]_i$, whereas diacylglycerol is an activator of a Ca^{2+} and phospholipid-dependent protein serine/threonine kinase, protein kinase C (PKC) [22]. Proteins phosphorylated by PKC include growth factor receptors, ion channels, and transcription factors [20, 34]. The increase in $[Ca^{2+}]_i$ together with the increased activity of PKC leads to a sequence of events that culminate in gene expression and cell proliferation [31]. There are other ways $[Ca^{2+}]_i$ can be increased, including the influx of extracellular Ca^{2+} through

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voltage- or receptor-operated membrane Ca^{2+} channels [45] or the release of Ca^{2+} from nonmitochondrial stores by polyunsaturated fatty acids [50].

There exists considerable evidence that $[Ca^{2+}]_i$ signaling is important for cell growth. First, lymphocytes and oocytes can be induced to undergo proliferation using the Ca^{2+} ionophore A23187 to increase $[Ca^{2+}]_i$, together with a nonmetabolizable diacylglycerol analogue to activate PKC [30, 52]. Second, lowering external Ca^{2+} [8] or the use of a Ca^{2+} channel blocker [56] to decrease Ca^{2+} influx during mitogenic stimulation inhibits cell growth. Third, intracellular Ca^{2+} chelators that buffer the increases in $[Ca^{2+}]_i$ caused by growth factors and mitogens prevent cell growth [11, 54]. It has also been observed that cancer cells are less susceptible than normal cells to growth inhibition by a low extracellular Ca^{2+} concentration [8].

Constitutive activation of intracellular signaling pathways by oncogenes can lead to unregulated cell growth in cancer cells [43, 44]. It is possible that if the $[Ca^{2+}]_i$ signaling pathways activated by oncogenes could be regulated with drugs, this might offer a way of controlling cancer cell growth. As part of our studies to identify antiproliferative drugs that modulate growth factor-induced $[Ca^{2+}]_i$ signaling, we found an unusual activity in extracts of *Helenium autumnale* L. (sneezeweed), a plant ommonly found in the southern United States. At concentrations that inhibited cell proliferation, the extract potentiated growth factor-induced $[Ca^{2+}]_i$ responses in Swiss 3T3 fibroblasts, and at higher concentrations it inhibited them. We could show that the active constituent of the extract is most likely helenalin, a cytotoxic pseudoguaianolide sesquiterpene lactone present in many species of the genus *Helenium* [13]. Helenalin has previously been shown to have antitumor activity in a number of animal tumor models [27].

Materials and methods

Cells and chemicals. Swiss mouse 3T3 fibroblasts were obtained from Dr. H.R. Herschmann, University of California (Los Angeles, Calif.) and HT-29 human colon carcinosarcoma cells, from the American Tissue Type Collection (Rockville, Md.). Cells were maintained in Dulbeccos modified Eagles medium (DMEM) containing 10% fetal bovine serum and were harvested with 0.05% trypsin and 0.5 m*M* ethylenedioninetetraacetic acid (EDTA) before becoming confluent. Helenalin and the related compounds helenine, multistatin, mexicanin E, fastigilin C, and autumnolide were obtained from the Drug Synthesis and Chemistry Branch, National Cancer Institute (Bethesda, Md.). The Ca^{2+}-sensitive photoprotein aequorin was purchased from Dr. John Blinks, Friday Harbor Laboratories (Seattle, Wash.), $Ins(1,4,5)P_3$, from Calbiochem (San Diego, Calif.), human PDGF (B-chain homodimer), from Bachem (Torrence, Calif.); [Arg^8]vasopressin, bradykinin, and TPA (phorbol 12-myristate 13-acetate), from Sigma Chemical Co. (St. Louis, Mo.); H-7 [1-(5-isoquinoline sulfonyl)-2-methyl piperazine dihydrochloride], from Seikagaku, Inc. (Rockville, Md.); and $^{45}Ca^{2+}$(25 mCi/mg), from Amersham Corp. (Arlington Heights, Ill.).

Measurement of $[Ca^{2+}]_i$. Measurements of $[Ca^{2+}]_i$ were made in Swiss 3T3 fibroblasts that give well-defined agonist-induced $[Ca^{2+}]_i$ responses [39]. The cells were loaded with aequorin by a low-Ca^{2+} centrifugation technique as previously described [39]. The aequorin-loaded cells were plated at a concentration of 10^6 cells in a 35-mm plastic culture dish containing 2 ml DMEM with 10% fetal calf serum. After 18 h in an incubator containing 5% CO_2 in air, the medium was replaced with 2 ml DMEM for 3 h. $[Ca^{2+}]_i$ was estimated by light emission from the aequorin-loaded cells by placing the cells over a sensitive photomultiplier tube in a light-tight apparatus, with the culture dish being maintained at 37° C and continually flushed with 5% CO_2 in air. The mitogens bradykinin (2×10^{-7} *M*), vasopressin (10^{-7} *M*) and PDGF (3.3×10^{-9} *M*), dissolved in 0.2 ml warmed DMEM, were added sequentially to the dish at 10-min intervals through separate light-tight, temperature-controlled inlet lines. We have previously reported that this order of addition of mitogens does not interfere with the $[Ca^{2+}]_i$ responses produced by the individual mitogens [41].

At the end of the experiment, the cells were lysed with 1% Triton X-100 solution containing 5 m*M* $CaCl_2$ and the total light signal was integrated. $[Ca^{2+}]_i$ was calculated by the method of Allen and Blinks [1] employing the ratio of the light signal obtained by exposure to the mitogen to the total light signal obtained following cell lysis. In some studies, 0.5 m*M* ethylene glycol tetraacetic acid (EGTA) was added to nominally Ca^{2+}-free DMEM to reduce the Ca^{2+} concentration to which the cells were exposed to below 10 n*M*. Helenalin and its analogues were added to the cells 20 min or, in some cases, 18 h, before addition of the mitogens.

Ca^{2+} uptake and release. Ca^{2+} uptake and release by saponin-permeabilized HT-29 colon carcinoma cells was measured by a modification of a method previously described elsewhere [50]. Briefly, the cells in suspension at 2×10^6 cells/ml were permeabilized with medium containing 0.005% saponin for 25 min at 37° C with gentle stirring. Cells were washed three times to remove saponin and then incubated at 37° C at 4×10^6 cells/ml in medium containing 1 m*M* adenosine triphosphate (ATP), 3% polyethylene glycol, 50 μ*M* $^{45}Ca^{2+}$ (160 μCi/μmol), and sufficient EGTA to buffer the free Ca^{2+} concentration to 10^{-7} M [12]. Mitochondrial function was inhibited by including in the incubation medium 0.5 m*M* dinitrophenol, 16 μ*M* antimycin A, and 2 μg oligomycin/ml. Aliquots (0.1 ml) of the cell suspension were taken at various times and cells were collected on glass microfiber filters (GF/A, Whatman International Ltd, Maidstone, England) and washed with medium containing 1 m*M* $LaCl_3$. The cells were then digested in 0.5 ml 1 *N* KOH at 60° C for 1 h prior to liquid scintillation counting. In some studies, 10 μ*M* $Ins(1,4,5)P_3$, a supramaximal concentration for Ca^{2+} release [50], was added to the cells after 8 min incubation and $^{45}Ca^{2+}$ release was measured as the difference in the cellular $^{45}Ca^{2+}$ content 1 min later as compared with that of nontreated cells.

PKC assay. Studies of the modulation of PKC activity used partially purified rat brain PKC, which consists primarily of PKCα and PKCβ isoforms, with histone serving as the substrate, and PKC modulation was assayed as previously described [23]. Phosphatidylserine and phorbol esters, but not Ca^{2+}, were included for the partial activation of PKC.

Cell colony formation. Inhibition of the growth of Swiss 3T3 fibroblasts growing on plastic culture surfaces in DMEM supplemented with 10% fetal bovine serum was measured with continuous drug exposure over 4 days. Inhibition of HT-29 colon carcinoma colony formation in soft agarose by helenalin was measured with continuous drug exposure over 7 days as previously described [2].

Results

Potentiation of [Ca2+]i signalling

Studies of aqueous methanol extracts of *Helenium autumnale* L. (sneezeweed) revealed an activity that at low concentrations of <50 μg crude extract/ml enhanced the mitogen-induced increases in $[Ca^{2+}]_i$ in intact Swiss 3T3 fibroblasts but at a concentration of >50 μg crude extract/ml caused inhibition of the $[Ca^{2+}]_i$ responses. A biologi-

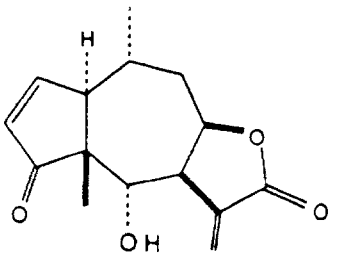

Fig. 1. Structure of helenalin

cally active constituent found in many species of the genus *Helenium* is the pseudoguaianolide sesquiterpine lactone helenalin (Fig. 1) [13]. Studies with helenalin showed that it reproduced the effects of the crude extract of *H. autumnale* on mitogen-induced increases in $[Ca^{2+}]_i$. Helenalin at concentrations of 0.1–0.5 μg/ml potentiated the $[Ca^{2+}]_i$ responses produced by vasopressin and bradykinin, but at 50 μg/ml it inhibited the $[Ca^{2+}]_i$ responses (Fig. 2). The $[Ca^{2+}]_i$ responses produced by PDGF were potentiated by helenalin at 0.1 μg/ml but were inhibited by helenalin at 0.5 μg/ml and higher concentrations. The cells appeared morphologically normal, even with the high concentrations of helenalin. There was no measurable effect of helenalin at any concentration tested on the resting levels of $[Ca^{2+}]_i$, which were (mean values ± SD for at least three determinations); control, 0.12 ± 0.03 μ*M*; helenalin at 0.1 μg/ml, 0.17 ± 0.02 μ*M*; helenalin at 0.5 μg/ml, 0.15 ± 0.05 μ*M*; and helenalin at 50 μg/ml, 0.16 ± 0.03 μ*M*. Since $[Ca^{2+}]_i$ is increased in damaged cells [42], the results confirm the lack of an acute toxic effect of helenalin on the cells. Helenalin inhibited the growth of Swiss 3T3 fibroblasts in serum with a 50% growth-inhibitory concentration (IC_{50}) of 0.2 μg/ml. Incubating cells with helenalin for 18 h before treatment with the mitogens slightly decreased the potentiation of the $[Ca^{2+}]_i$ responses seen at low concentrations of helenalin, and inhibition of the $[Ca^{2+}]$ responses occurred even at the high concentration of helenalin (Fig. 3). The surviving cells were morphologically normal and had normal resting $[Ca^{2+}]_i$. The results suggest that prolonged incubation of cells with helenalin has only a small cumulative effect on $[Ca^{2+}]_i$ responses.

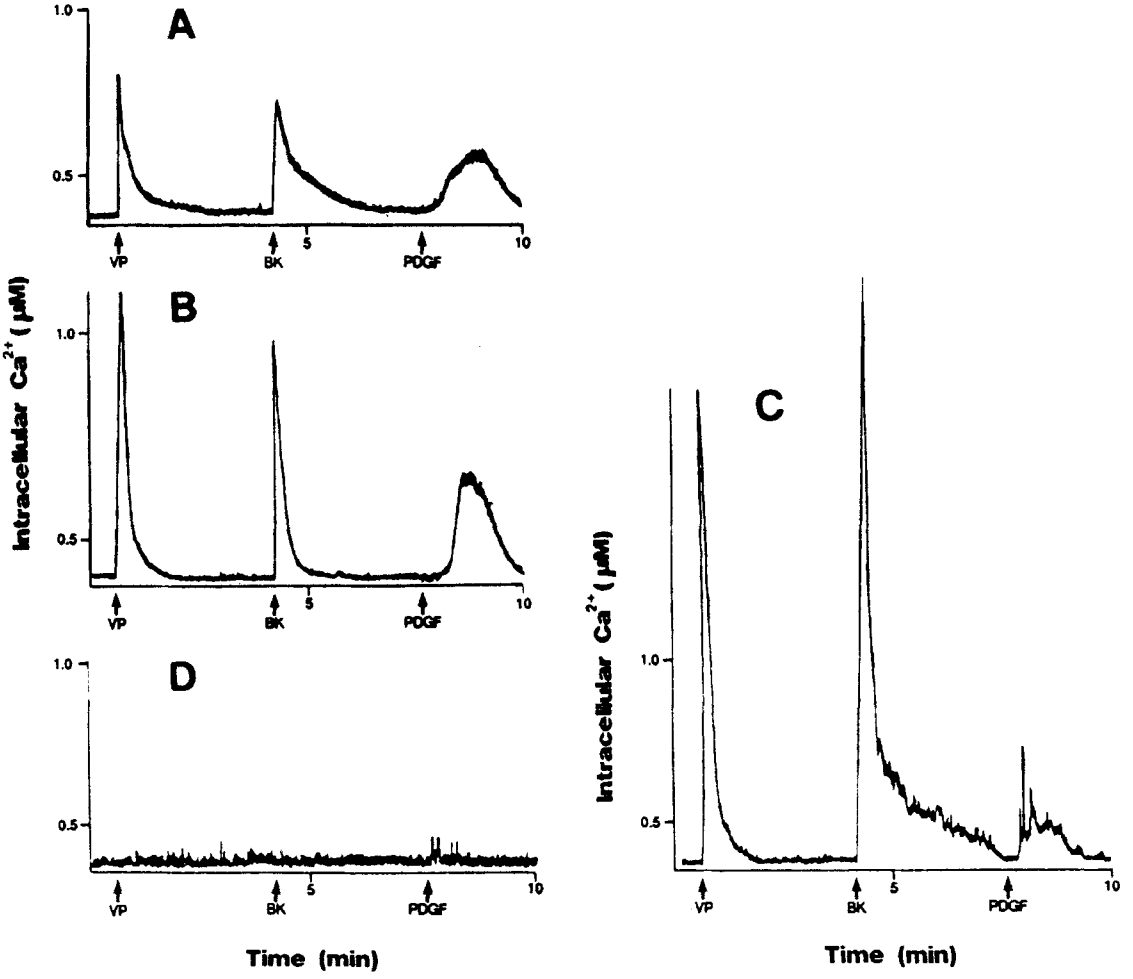

Fig. 2A–D. Changes in mitogen-induced $[Ca^{2+}]_i$ responses caused by helenalin. Swiss 3T3 cells loaded with the Ca^{2+}-sensitive photoprotein aequorin 21 h previously were allowed to attach in 10% fetal calf serum containing DMEM for 18 h and were serum-deprived for 3 h before addition of the mitogens bradykinin (*BK*, 2×10^{-7} *M*), vasopressin (*VP*, 10^{-7} *M*), and PDGF (3.3×10^{-9} *M*) at the *arrows*. **A** Control. **B** Helenalin, 0.1 μg/ml. **C** Helenalin, 0.5 μg/ml. **D** Helenalin, 50 μg/ml. Helenalin was added 20 min before the mitogens

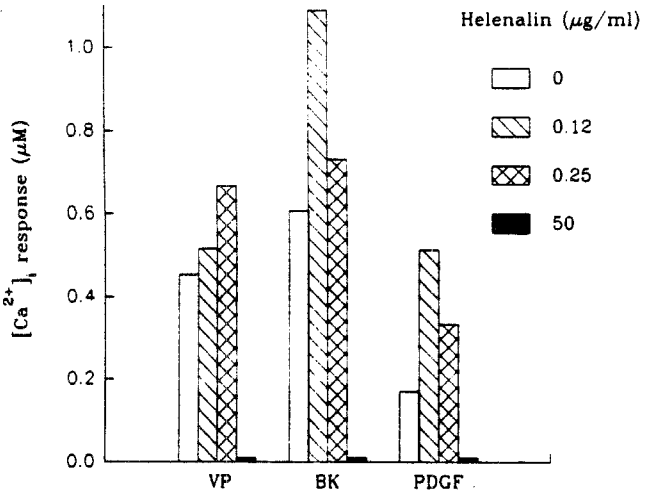

Fig. 3. Effect of 18 h incubation with helenalin on mitogen-induced $[Ca^{2+}]_i$ responses. The mitogens used were bradykinin (*BK*, 2×10^{-7} *M*), vasopressin (*VP*, 10^{-7} *M*), and PDGF (3.3×10^{-9} *M*). *Open boxes*, controls; *shaded boxes*, helenalin added 18 h before the mitogens at the concentrations shown. Resting $[Ca^{2+}]_i$ was not altered by helenalin exposure of these cells. The results are the mean values for 2 separate determinations

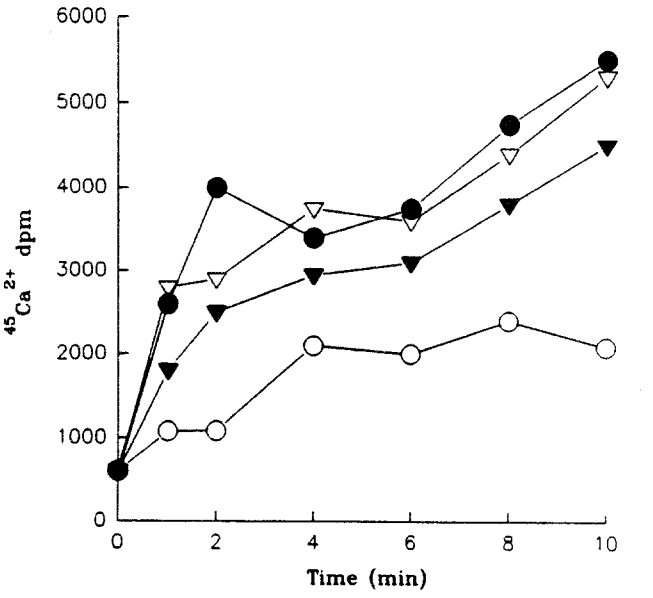

Fig. 4. Effect of helenalin on $^{45}Ca^{2+}$ uptake by nonmitochondrial stores of saponin-permeabilized HT-29 colon carcinoma cells. $^{45}Ca^{2+}$ uptake was measured in the presence of an ATP-generating system at 37° C as described in Materials and methods. Control cells (○); cells with helenalin at 0.1 μg/ml (▼), 1.0 μg/ml (▽), and 10 μg/ml (●). Each point is the mean of 3 determinations; SD bars are omitted for clarity

Table 1. Effects of modulators of PKC on $[Ca^{2+}]$ responses in the presence of helenalin

	Helenalin (μg/ml)	Peak $[Ca^{2+}]_i$ response (μM)		
		VP	BK	PGDF
Control	0	0.68 ± 0.08	0.60 ± 0.07	0.31 ± 0.03
	0.25	1.00 ± 0.06	1.05 ± 0.09*	0.40 ± 0.04
	50	0.0*	0.0*	0.0*
H-7, 5×10^{-5} M	0	0.79 ± 0.09	0.93 ± 0.02	0.37 ± 0.07
	0.25	0.89 ± 0.12	0.97 ± 0.07	0.30 ± 0.07
	50	0.0*	0.0*	0.0*
TPA (18 h)	0	0.66 ± 0.06	0.98 ± 0.06	0.36 ± 0.04
	0.25	0.81 ± 0.20	1.19 ± 0.02	0.44 ± 0.09
	50	0.0*	0.0*	0.0*
Without external-Ca^{2+}	0	0.67 ± 0.10	0.75 ± 0.17	0.03 ± 0.03
	0.25	0.78 ± 0.12	0.80 ± 0.20	0.12 ± 0.06
	50	0.04 ± 0.04*	0.26 ± 0.17*	0.08 ± 0.04*

* $P < 0.05$ compared to value without helenalin

Changes in $[Ca^{2+}]_i$ in Swiss 3T3 cells loaded with the Ca^{2+}-sensitive photoprotein aequorin were measured as described in Materials and methods. The mitogens used were vasopressin (VP, 10^{-7} *M*), bradykinin, (BK, 2×10^{-7} *M*), and PDGF (3.3×10^{-9} *M*). Helenalin was added at 0.25 and 50 μg/ml 20 min before the addition of mitogens. Treatment with the PKC inhibitor H-7 (5×10^{-5} *M*) was carned out for 20 min before and treatment with TPA (10 ng/ml) for 18 h before the addition of mitogens. Ca^{2+} was omitted from the external medium and 0.5 m*M* EGTA was added 20 min before addition of the mitogens. Values are means ± SE. of at least four separate measurements

Role of PKC

Activation of PKC has been reported to exert a negative inhibitory control on $[Ca^{2+}]_i$ responses [33]. We therefore investigated whether inhibition of PKC with H-7 [18] or down-regulation of PKC by prolonged exposure to TPA [20] would affect the ability of helenalin to modulate $[Ca^{2+}]_i$ responses. As expected, the treatments themselves caused an increase in the $[Ca^{2+}]_i$ responses to vasopressin and bradykinin (Table I). Helenalin caused no additional increase in $[Ca^{2+}]_i$ responses in H-7 treated cells, but there was a further increase in $[Ca^{2+}]_i$ responses in TPA-treated cells. The inhibition of $[Ca^{2+}]_i$ responses by high concentrations of helenalin was not affected by treatments that modulated PKC activity. Helenalin was found not to be a direct inhibitor of purified PKC at concentrations of up to 1.6 μg/ml. Taken together, the results suggest that PKC plays only a minor role in the modulation of $[Ca^{2+}]_i$ responses by helenalin.

Role of extracellular Ca2+

The origin of the Ca^{2+} responsible for the increases in $[Ca^{2+}]_i$ responses caused by helenalin was investigated by omitting Ca^{2+} from the extracellular medium (Table 1). Without extracellular Ca^{2+}, the $[Ca^{2+}]_i$ response to PDGF was decreased, whereas the $[Ca^{2+}]_i$ responses to bradykinin and vasopressin were unaffected, thus confirming earlier observations that PDGF, but not bradykinin or vasopressin, causes the early influx of external Ca^{2+} in Swiss 3T3 cells [40]. The increased $[Ca^{2+}]_i$ responses caused by helenalin were attenuated when there was no Ca^{2+} in the external medium (Table 1). Surprisingly, there remained small but significant mitogen-induced $[Ca^{2+}]_i$ responses at high helenalin concentrations in the absence of external Ca^{2+}.

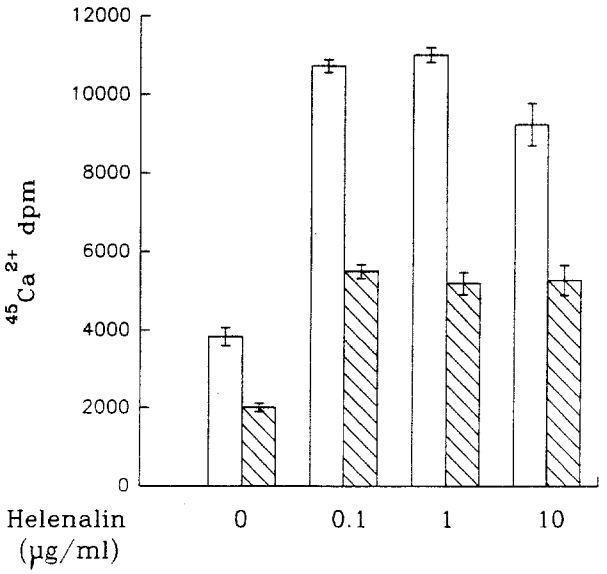

Fig. 5. Effect of helenalin on $^{45}Ca^{2+}$ uptake and Ins(1,4,5)P_3-mediated $^{45}Ca^{2+}$ release by nonmitochondrial stores of saponin-permeabilized HT-29 colon carcinosarcoma cells. $^{45}Ca^{2+}$ uptake was measured in the presence of an ATP-generating system at 37° C over 8 min as described in Materials and methods. Ins(1,4,5)P_3 (10 μ*M*) was added at 8 min, and the $^{45}Ca^{2+}$ released was measured at 9 min as the difference between the amount of $^{45}Ca^{2+}$ that remained in the cells and the amount determined in cells without Ins(1,4,5)P_3. Helenalin was added at the concentrations shown. Each point is the mean of 5 determinations and each bars is the SE

Uptake and release of 45Ca2+ by permeabilized cells

The effect of helenalin on the rate of uptake of $^{45}Ca^{2+}$ into the nonmitochondrial stores of saponin-permeabilized HT-29 colon carcinoma cells was examined (Fig. 4). Helenalin at concentrations of 0.1–10 μg/ml, which encompassed the concentration range that stimulated $[Ca^{2+}]_i$ responses, caused a marked increase in the rate of uptake of $^{45}Ca^{2+}$. In a second study, the effect of helenalin on $^{45}Ca^{2+}$ uptake into non-mitochondrial stores and on the Ins(1,4,5)P_3-mediated release of $^{45}Ca^{2+}$ from the same stores was investigated (Fig. 5). Helenalin at between 0.1 and 10 μg/ml caused a 175% increase in $^{45}Ca^{2+}$ uptake. The same concentrations of helenalin caused a 260% increase in the Ins(1,4,5)P_3-dependent release of $^{45}Ca^{2+}$. However, in the helenalin-treated cells the ratio of Ins(1,4,5)P_3-dependent $^{45}Ca^{2+}$ release to $^{45}Ca^{2+}$ uptake was 0.52, the same as that in the non-helenalin-treated cells. Thus, the major effect of helenalin is to increase the uptake of $^{45}Ca^{2+}$ and, presumably, the size of the non-mitochondrial $^{45}Ca^{2+}$ store without changing the fraction of the $^{45}Ca^{2+}$ store that is released by Ins(1,4,5)P_3.

Helenalin analogues

The ability of some helenalin analogues to modulate $[Ca^{2+}]_i$ responses was studied. An increase in the bradykinin-mediated $[Ca^{2+}]_i$ response (the percentage increase seen in cells exposed to the analogues as compared with non-exposed cells is given in parentheses) was seen at 0.5 μg/ml with fastigilin C (93%), autumnolide (45%), and multistatin (103%), whereas $[Ca^{2+}]_i$ responses were completely inhibited by the analogues at 50 μg/ml. Mexicanin E had no effect on the $[Ca^{2+}]_i$ response at 0.5 μg/ml but stimulated the response (103%) at 50 μg/ml. The analogues were all growth-inhibitory to HT-29 cells with IC_{50}s of < 0.1 μg/ml. Helenalin inhibited the growth of HT-29 cells with an IC_{50} of 0.1 μg/ml. Helenine, which inhibited HT-29 cell growth with an IC_{50} of 7 μg/ml, did not stimulate $[Ca^{2+}]_i$ responses at concentrations of up to 50 μg/ml.

Discussion

Sesquiterpene lactones such as helenalin exhibit antiinflammatory [16], hypolipidemic [17], and antibacterial activity [28]. It has been suggested that the biological activity of the sesquiterpene lactones is due to the irreversible alkylation of thiol groups by Michael addition at the carbonyl moiety [24, 26]. Helenalin is cytotoxic to tumor cells in culture [15, 25, 29] and has shown in vivo antitumor activity against Walker 256 carcinosarcoma in rats, Ehrlich ascites carcinoma in mice, and P-388 lymphocytic leukemia in mice [27].

The cytotoxic activity of the sesquiterpene lactones is dependent upon the presence of an α,β-unsaturated carbonyl moiety, O = C−C = CH [25], although the cytotoxicity does not correlate directly with thiol reactivity, suggesting that other mechanisms might also be operative [29]. Helenalin at high concentrations decreases cellular glutathione levels in L1210 leukemia cells and inhibits several thiol-containing enzymes involved in DNA, RNA, and protein synthesis [15].

The effect of helenalin at cell growth-inhibitory concentrations in increasing mitogen-induced $[Ca^{2+}]_i$ signaling is an unusual $[Ca^{2+}]_i$ response. Depletion of intracellular glutathione by thiol-reactive agents is most frequently associated with a sustained increase in resting $[Ca^{2+}]_i$ leading to cell cytotoxicity [42]. For this to occur, intracellular glutathione must be depleted by over 80% [21]. We did not see an increase in resting $[Ca^{2+}]_i$, even at helenalin concentrations of 50 μg/ml, which is well above cell growth-inhibitory concentrations. Thus, it is unlikely that the cytotoxic activity of helenalin in Swiss 3T3 cells is dependent on a mechanism that involves a major depletion of intracellular glutathione and an increase in resting $[Ca^{2+}]_i$.

The organic mercurial thiol-active agent thimerosal has been reported to evoke $[Ca^{2+}]_i$ spikes in HeLa cells and to sensitize the Ins(1,4,4)P_3 receptor to basal Ins(1,4,5)P_3 levels, thus increasing Ca^{2+} release from intracellular stores [7, 35, 49]. Thimerosal causes a sustained increase in $[Ca^{2+}]_i$, empties intracellular Ca^{2+} stores, and causes the influx of external Ca^{2+} [14]. Other thiol-oxidizing agents, including oxidized glutathione and *t*-butylhydroperoxide, also sensitize the Ins(1,4,5)P_3 receptor and increase basal Ca^{2+} release [5, 19, 35, 46]. Helenalin did not increase resting $[Ca^{2+}]_i$ but potentiated mitogen-induced $[Ca^{2+}]_i$ responses, and it appears to be acting by a mechanism different from that of other thiol-active agents.

Inhibition of PKC can remove a feedback inhibition on mitogen-induced $[Ca^{2+}]_i$ responses [33]. Helenalin was

found not to be a direct inhibitor of PKC, and our studies in intact cells indicated that PKC plays only a minor role in the effects of helenalin on $[Ca^{2+}]_i$ responses. The most likely explanation for the increase in mitogen-induced $[Ca^{2+}]_i$ responses caused by helenalin is an increase in the uptake of Ca^{2+} into nonmitochondrial stores associated with an increased release of Ca^{2+} by $Ins(1,4,5)P_3$, although the fraction of the total Ca^{2+} released from the store remains unchanged. These results suggest that the release of Ca^{2+} from the non-mitochondrial stores is controlled by the Ca^{2+} content of the stores, as has previously been suggested [36]. There may also be an increased influx of extracellular Ca^{2+} following mitogen stimulation caused by helenalin. The mechanism by which helenalin increases the uptake of Ca^{2+} by the nonmitochondrial intracellular stores and the influx of extracellular Ca^{2+} is not known but may involve the alkylation of thiol groups on proteins involved in the Ca^{2+} uptake process.

The potentiation of mitogen-induced $[Ca^{2+}]_i$ signaling occurred at concentrations of helenalin that inhibited cell proliferation. It is not known if the increase in mitogen-induced $[Ca^{2+}]_i$ responses is responsible for the growth-inhibitory effects of helenalin and its analogues. Higher concentrations of helenalin inhibited $[Ca^{2+}]_i$ signaling. We do not know the mechanism of inhibition of $[Ca^{2+}]_i$ signaling by helenalin, but it is significant that it occurred without resulting in an increase in resting $[Ca^{2+}]_i$ or a change in cell morphology, indicating that the cells were not grossly damaged. A helenalin analogue, helenine, that was less active than other helenalin analogues in inhibiting cell proliferation did not increase $[Ca^{2+}]_i$ responses. A priori, an increase in $[Ca^{2+}]_i$ signaling might be expected to promote mitogenesis, although there is evidence that inappropriate increases in $[Ca^{2+}]_i$ might be toxic to cells, for example, driving them into a pathway of programmed cell death [48].

In summary, the present study shows that helenalin and some of its analogues at concentrations that are cytotoxic to cells cause an increase in mitogen-induced $[Ca^{2+}]_i$ responses. Resting levels of $[Ca^{2+}]_i$ are not affected by helenalin. The effect appears to be due to an increase in the uptake of Ca^{2+} into nonmitochondrial stores by helenalin and an increase in the $Ins(1,4,5)P_3$-mediated release of Ca^{2+} together with an increased influx of extracellular Ca^{2+}. At high concentrations, helenalin inhibited the mitogen-induced $[Ca^{2+}]_i$ responses.

Acknowledgements. This work was supported by NCI grants CA52995 and CA42286.

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