JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Influence of Cucumariosides upon Intracellular $[Ca^{2+}]_i$ and Lysosomal Activity of Macrophages

IRINA G. AGAFONOVA,* DMITRY L. AMININ, SERGEY A. AVILOV, AND VALENTIN A. STONIK

Pacific Institute of Bioorganic Chemistry, Far Eastern Branch of RAS, Vladivostok 690022, Russia

Biological effects of the triterpene glycosides, cucumariosides A_2-2 and A_7-1 from the edible sea cucumber *Cucumaria japonica* and their aglycones were investigated using embryos of the sea urchin *Strongylocentrotus nudus* and the BALB/C line mouse peritoneal macrophages. Cucumariosides were highly cytotoxic in a sea urchin embryo development test with EC_{50} values of 0.3 and 1.98 μg/mL, respectively. The aglycone was completely lacking in cytotoxicity. In subtoxic concentrations (0.001−0.1 μg/mL), cucumarioside A_2-2 showed more then 2-fold stimulation of lysosomal activity and induced a rapid short-term increase in cytosolic Ca^{2+} content in mouse macrophages. The maximal stimulatory effect was detected after 1−2 h of cultivation of cells with this glycoside. Cucumarioside A_7-1 demonstrated more weak effects and even slightly inhibited lysosomal activity, while the aglycone was completely ineffective. At the toxic concentration (1 μg/mL), cucumarioside A_2-2 induced the sharp irreversable increase of intracellular Ca^{2+} concentration. We suggested that cucumariosides, especially A_2-2, may act as Ca^{2+} agonists due to their membranolytic properties.

KEYWORDS: Sea cucumber; triterpene glycosides; cucumariosides; Ca^{2+} signaling; cytotoxicity; immunomodulation; lysosomal activity; mechanism of action

INTRODUCTION

The Far Eastern edible sea cucumber (holothurian) *Cucumaria japonica* is at the least a source of 13 different triterpene oligoglycosides cucumariosides (*1*−*4*). Aglycones of cucumariosides are derivatives of so-called holostane (lanostane (18→20) lactone). All of these compounds have carbohydrate chains with five monosaccharide units linked to C-3 of the aglycones. Glycosides of this series contain one, two, or three sulfate groups bounded with sugars.

Some triterpene glycosides from the Far Eastern holothurian *C. japonica* are known as active substances of a veterinary immunostimulatory preparation approved for the treatment of mink, pigs, dogs, and other animals in Russia (*5*, *6*). These cucumariosides in low doses were shown to increase significantly the animal resistance to bacterial infections elicited by various pathogens (*7*−*9*). The effect of cucumariosides is mediated in part by the increase in macrophage phagocytic activity, which was at least doubled 7−14 days after the intraperitoneal injection of the mixture of cucumariosides (0.03 μg/mouse). Cucumariosides were also shown to possess strong adjuvant properties potentiating the effect of some bacterial vaccines (*8*, *9*) and demonstrated some antiviral activity, possibly through the activation of T- and B-cell cooperation (*10*). The substances blocked the mitotic activity of rat liver cells at the concentration of 0.05 μg/kg during the first 28−32 h of treatment but increased the hepatocyte proliferation 12 h later (*11*).

Recently, we found that the intraperitoneal injection of picogram to nanogram doses of some cucumariosides significantly induced the macrophage lysosomal activity in a dose-dependent manner. The stimulatory effect was related to chemical structures of cucumariosides and was influenced by the number and positions of sulfate groups in the carbohydrate moiety of the molecules (*12*).

Despite comprehensive knowledge concerning chemical structures and properties of these glycosides as well as their practical use in veterinary medicine and medicine, the mechanism underlying immunostimulatory properties of the individual glycosides from *C. japonica* remains to be elucidated. To address this question, effects of two predominant cucumariosides, A_2-2 and A_7-1, from *C. japonica* (**Figure 1**) and their chemically obtained aglycone on the course and amplitude of intracellular calcium signals and lysosomal activity in mouse peritoneal macrophages are studied using *in vitro* models.

MATERIAL AND METHODS

Compounds. Cucumarioside A_2-2 (*1*) and cucumarioside A_7-1 (*2*) from the sea cucumber *C. japonica* were obtained as described (**Figure 1**). Aglycones were obtained using acid hydrolysis of glycosides (*13*). Chemical structures and purities of the obtained compounds were confirmed by the determination of their physical constants, by the mobility on silica gel TLC plates, and by ^1H and ^{13}C NMR spectra (*1*, *2*).

* To whom correspondence should be addressed. Tel: 7(4232)319932. Fax: 7(4232)314050. E-mail: agafonova@piboc.dvo.ru.

10.1021/jf034439x CCC: $25.00
Published on Web 10/17/2003

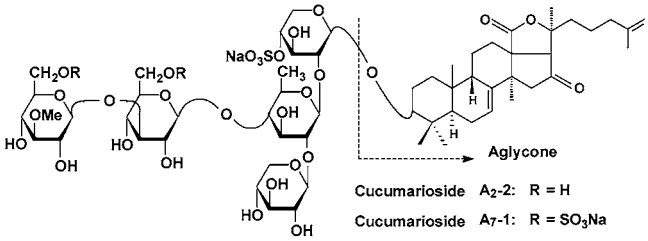

Figure 1. Chemical structures of triterpene glycosides from the sea cucumber *C. japonica*.

Cytotoxicity. The sea urchin *Strongylocentrotus nudus* embryos were used as a test material for cytotoxic bioassays, according to the method of Kobayashi (*14*) with some modifications. To isolate the mature eggs or sperm, 1−2 mL of 0.5 M KCl solution was injected into the cavity of a sea urchin. The eggs and sperm (separately) were collected into glass beakers with seawater. When the female had completly spawned, the eggs were allowed to settle to the bottom of a cultivator box filled with filtered and aspirated seawater and then seawater. This process was repeated three times in order to wash out the egg's jelly coating. After artificial fertilization, 0.9 mL of egg suspension with a density of 1×10^3 cell/mL was put into each well of a 24 well microplate containing the sample solutions (0.1 mL) and the plate was kept at 20−22 °C during 2 h before the stage of eight blastomeres (control). Then, aliquots of formaldehyde solution (2%) were added to each well to fix the embryos. A hundred embryos were examined at each concentration of toxicant, and the number of developed eight blastomeres were determined with the inverted microscope. All experiments were repeated in triplicate. The results were expressed as percent of the controls and plotted. The means and standard errors (SE) for each treatment were calculated, and EC_{50} values were estimated using SigmaPlot 3.02 software (Jandel Scientific, San Rafeal, CA).

Ca^{2+} Signaling in Single Macrophages. Biomembrane permeable Ca^{2+} sensitive fluorescent probes and microcytophotometry techniques were applied to record $[Ca^{2+}]_i$ changes in single macrophages (*15*). BALB/C line mice were used as a source of peritoneal macrophages. The mouse peritoneal macrophage suspension (20 μL) was transferred to glass coverslips and stored for 1 h in an incubator at 37° for cell adhesion. Then, the coverslips were washed (three times) with PBS (pH 7.5) and transferred to 10 μM Calcium Green-1/AM (Molecular Probes) in saline solution to load cells with fluorescent dye for 40 min at 37° C. The components of saline solution were as follows: NaCl, 140 mM; KCl, 5 mM; $CaCl_2$, 1 mM; HEPES, 10 mM, pH 7.4. Then, cells were washed with the same solution but without fluorescent dye and incubated for 15−20 min in serum-containing medium to let cells recover. After that, coverslips were mounted on an epifluorescent microcytometer (LUMAM IUF-1, St.-Petersburg, Russia). The light from a mercury lamp was filtered through a FITC band-pass filter block (488 nm) and focused on a single cell. The emitted fluorescence was directed to a PMT connected with an amplifier and a 14 bit AD/DA converter (Decision Computer International Co., Taipei, Taiwan). The recording and quantitation of fluorescence changes were performed using our own C++ laboratory made software. Changes in single cell fluorescence were expressed as F/F_0, where F_0 is the cell background fluorescence (mV) and F is the resulting maximum fluorescence after glycoside treatment (mV). A micropipet perfusion system with a flow rate of 100 μL/min was used to apply the test solution of glycosides to macrophages plated on the glass coverslip. All experiments were performed at 20−22 °C.

Lysosomal Activity. An estimation of intracellular lysosome activity was conducted by staining and the localization of lysosomes in live macrophages with a fluorescent dye Acridine orange (*16*−*18*) followed by fluorescence image analysis. The image analysis of lysosomal fluorescence was done according to the Lowe method (*19*) with modifications. For this purpose, 250 μL of a BALB/C mice peritoneal fluid was applied on a microscope cover glass and left at 37 °C in an incubator for 1 h. After adhesion of macrophages, the cover glasses were washed (three times) with PBS (pH 7.5). A 250 μL amount of Acridine orange solution (Calbiochem, 100 μg/mL in PBS) was added dropwise to the cell monolayer, and glasses were incubated at 37 °C for 30 min. The cell monolayers were then washed (three times) in PBS. Cover glasses were mounted on a microscopic glass of epifluorescent microcytometer (LUMAM IUF-1). The FITC filter block was set for visualization of Acridine fluorescence in lysosomes. The images of red-orange fluorescent cells were acquired using digital CCD color videocamera CCS-212 (Samsung, Seoul, Korea), captured, and transferred to an IBM compatible computer with WinFast 3D S680 frame grabber (Leadtek, Taipei, Taiwan). The true color acquired images were transformed to the 256 grey scale using PhotoShop 3.0 software (Adobe Systems Inc., San Jose, CA), and gray level intensity of cells was determined with Axon Image Workbench 2.1.1 software (Axon Instruments Inc., Foster City, CA). The fluorescence of randomly selected 100 cell images was measured for each concentration of tested compounds as an average pixel intensity of gray level for each cell. The intensity of lysosome fluorescence in cells from treated animals was expressed as a percentage of the average fluorescence of 100 cells in comparison with the fluorescence of control cells.

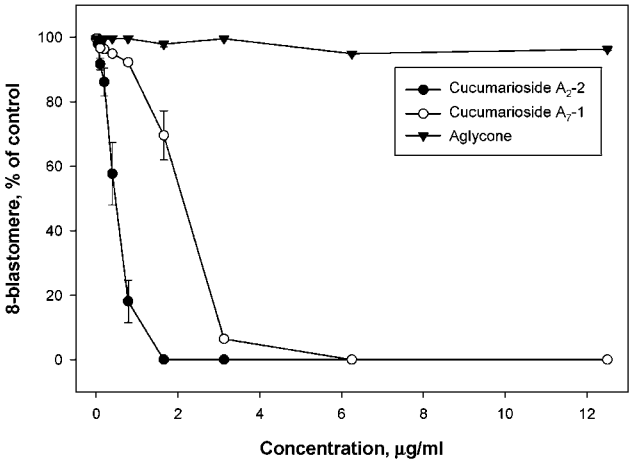

Figure 2. Cytotoxic effects of cucumariosides on the sea urchin *S. nudus* embryo.

RESULTS

Cytotoxicity. The cytotoxic activities of cucumariosides A_2-2, A_7-1, and aglycone were measured in a sensitive sea urchin embryo development test instead of the conventional cytotoxicity methods based on the Trypan blue cell stain to avoid the errors related with dye penetration into the cells through the pores generated by glycosides. It was found that cucumariosides had high antimitotic activities and exhibited many undivided (blocked) and lysed blastomeres when applied at a concentration over 5 μg/mL. Many morphological abnormalities, such as abnormal cleavages (three blastomeres, shaped eggs, and unequal cleavages) and delay of development at lower concentrations of cucumariosides, were observed. EC_{50} values of cucumariosides A_2-2 and A_7-1 were determined as 0.30 and 1.98 μg/mL, correspondingly; the aglycone did not show any activity to 100 μg/mL. In concentration ranges of 0.1−0.001 μg/mL (subtoxic doses), all tested compounds did not influence embryo development of the sea urchins. Representative dose−response curves are illustrated in **Figure 2**.

Ca^{2+} Signaling in Single Macrophages. The increased Ca^{2+} intracellular content in the presence of cucumariosides in the concentration range of 0.001−0.1 μg/mL was detected with a Calcium Green-1/AM preloaded single mouse macrophage. **Figure 3** depicts the typical responses of macrophages to various doses of cucumarioside A_2-2. Changes in basal $[Ca^{2+}]_i$ were observed after several seconds following glycoside addition to the cell monolayer; $[Ca^{2+}]_i$ increased sharply after glycoside treatment and then slowly decreased to the basal level (**Figure 3**). The rise in $[Ca^{2+}]_i$ was transient at the mentioned above

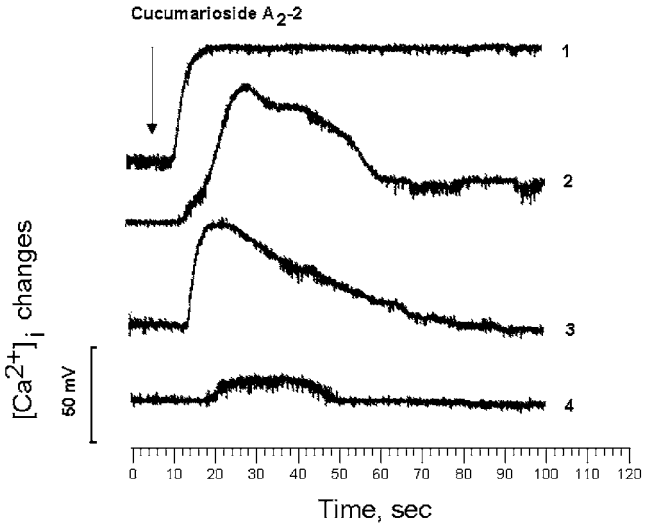

Figure 3. Modulation of $[Ca^{2+}]_i$ in mouse single macrophages preloaded with Calcium Green-1 by cucumarioside A_2-2. Cucumarioside concentrations: 1, 1 μg/mL; 2, 0.1 μg/mL; 3, 0.01 μg/mL; and 4, 0.001 μg/mL.

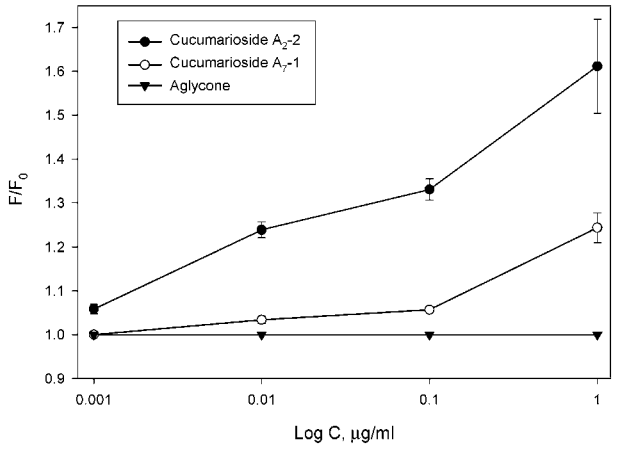

Figure 4. Dose–response relationship between concentration of cucumariosides and $[Ca^{2+}]_i$ in Calcium Green-1 preloaded single mouse macrophages determined by fluorescence microcytophotometry. Values are means ± SE (n = 10).

glycoside concentrations, peaking within 10−60 s and declining slowly to the control value of about 50−150 s.

The intensity of the short-term Ca^{2+} spikes depended on the glycoside concentration in the extracellular incubation medium. When the $[Ca^{2+}]_i$ responses in individual experiments were compared, a dose−response relationship with various doses (0.001−0.1 μg/mL) of cucumariosides was observed (**Figure 4**). The dose−response curve was linear at all nontoxic concentrations examined. Among the studied glycosides monosulfated cucumarioside, A_2-2 was the most active compound in inducing a rapid increase in cytosolic Ca^{2+} content when compared with the polysulfated cucumarioside A_7-1 producing a weak effect. The aglycone was absolutely ineffective (**Figure 4**). Simultaneously, toxic concentration (1 μg/mL) of A_2-2 or A_7-1 gave the sharp increase of intracellular calcium level, but the subsequent recession was not detected.

Lysosomal Activity. Using the Acridine orange technique to stain lysosomes in viable cells, the significant stimulation of mouse BALB/C macrophage activity *in vitro* by cucumarioside A_2-2 was detected (**Figures 5** and **6**). Monosulfated cucumarioside A_2-2 (**Figure 1**) action was estimated to be accompanied by about 2-fold stimulation of macrophage lysosomal activity,

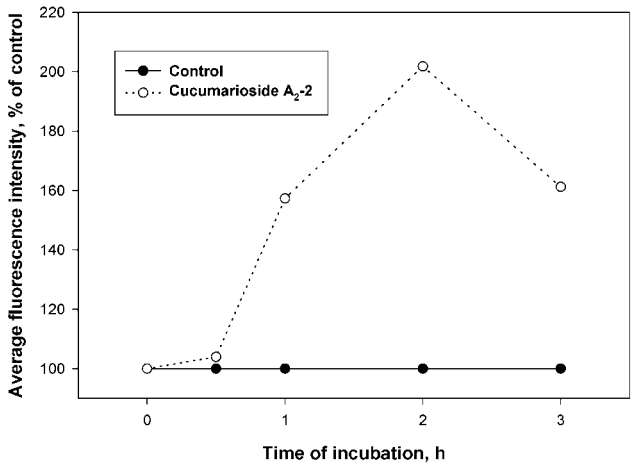

Figure 5. Dependence of macrophage lysosome activity on time of cell incubation with cucumarioside A_2-2 (0.02 μg/mL).

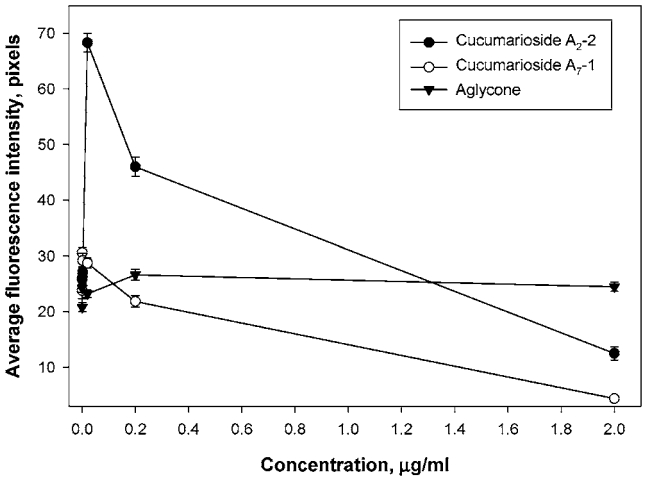

Figure 6. Effects of cucumariosides on BALB/C mouse peritoneal macrophage lysosomal activity *in vitro*. Time of cell incubation with glycosides is 2 h. Values are means ± SE (n = 100).

which was expressed in the increase in number and volume of lysosomes as well as their acidity in mouse peritoneal macrophages. The stimulation of cell activity depended on the cucumarioside A_2-2 concentration in the incubation medium (from 0.002 to 2.0 μg/mL). It was established that the most effective concentration was of 0.02 μg/mL; additional augmentation of cucumarioside concentration in the incubation medium resulted in reduction of effect down to inhibition (**Figure 6**). The peak of the glycoside-mediated stimulatory effect was registered after 2 h of cell cultivation at a glycoside concentration of 0.02 μg/mL. The subsequent increase of incubation time up to 3 h lead to some decrease of the stimulatory effect (**Figure 5**).

However, the trisulfated cucumarioside A_7-1 (**Figure 1**) induced a weak peak of lysosome stimulation at low concentrations (less than 0.1 μg/mL). Moreover, this compound even slightly inhibited lysosomal activity at higher concentrations as 0.2−2 μg/mL. The aglycone alone was completely inactive (**Figure 6**).

DISCUSSION

Triterpene glycosides from holothuriods have a wide spectrum of biological effects including cytotoxic, hemolytic, antifungal, ichthiotoxic, and other activities. A majority of the activities are based on the interaction of these compounds with membrane

sterols. This interaction results in the formation of pores, changes in membrane ion permeability and viscosity, and profound inhibition of some membrane enzymes, especially ATPases, that finally lead to cell death (*4*, *20*).

At the same time, subtoxic concentrations of these triterpene glycosides may result in cellular activation and potentiation of cellular functions. Applied in low concentrations, some glycosides induced an increase of phagocytosis by human polymorphonuclear leucocytes (*21*) and showed mitogenic activities and modulated the immune response in mice T- and B-lymphocytes, while high concentrations were found to suppress cellular function (*22*).

Nevertheless, these studies do not deal with the mechanism by which holothurian triterpene glycosides stimulate cellular activity. For some higher plant glycosides, the activation of Ca^{2+} uptake from extracellular environment was shown. The triterpene glycoside cauloside C was found to increase $[Ca^{2+}]_i$ in human fibroblasts, which was accompanied with more than 2-fold stimulation of cell proliferation in serum-free culture medium. The stimulation was caused by activation of membrane-associated Ca^{2+} channels at the excess of calcium incorporated into cells with glycoside (*23*). Digitonin at subskinning concentrations caused the formation of pores in plasma membranes as a result of the interaction with cholesterol and produced an increase in cultivated cardiac cell motion and an augmentation in $[Ca^{2+}]_i$ while membrane fluidity was decreased (*24*). Ginseng glycoside Rg1 stimulated the proliferation of lymphocytes, influenced the fluidity of lymphocyte cytoplasmatic membrane (*25*), and protected cardiomyocytes in the case of ischemia disease. These findings were associated with the glycoside modulation of K^+-dependent Ca^{2+} channel current and K^+, Na^+, and Ca^{2+} transport across the cellular membrane (*26*−*28*).

It is well-known that a change in intracellular free Ca^{2+} concentration is a crucial signal for diverse cellular functions (*29*, *30*). A $[Ca^{2+}]_i$ increase is usually contributed by Ca^{2+} mobilization from intracellular stores and/or Ca^{2+} entry from extracellular space through specific natural membrane Ca^{2+} channels, artificial pores, ion exchangers, and due to Ca^{2+}-ionophore activity. In the present study, the involvement of intracellular Ca^{2+} as a possible messenger in the cucumarioside effects was investigated. Because the macrophage lysosomal activity stimulation occurred at the same subtoxic concentration range (picograms to nanograms per milliliter) of cucumarioside A_2-2 that reversibly increased $[Ca^{2+}]_i$, it is quite possible that this stimulatory effect was Ca^{2+}-dependent and the lysosomal activity stimulation was implemented via the regulation of cellular membrane permeability for calcium ions. The toxic concentrations of cucumarioside A_2-2 (as well as cucumarioside A_7-1) triggered a rapid irreversable prolonged lifting of the basal Ca^{2+} level into macrophages corresponding to reducing in lysosomal activity. Apparently, the previously known membranolytic and acute cytotoxic activities of sea cucumber glycosides may be caused by this effect. Therefore, some cucumariosides may probably act as Ca^{2+} agonists due to their membranolytic properties.

The difference between mono- and trisulfated glycosides and aglycone in potentiation of $[Ca^{2+}]_i$ increasing and the stimulation of macrophage lysosomal activity *in vitro* shows the importance of the carbohydrate chain and especially the presence and number of sulfate groups in carbohydrate moieties to exert the maximal stimulation of the immune response. This findings support the data on cytotoxicity obtained with sea urchin embryo in the present study and with differences in K^+ release from erythrocytes followed by hemolysis at the action of cucumariosides with different chemical structures (*31*).

On the other hand, a significant stimulatory effect of some cucumariosides on macrophage lysosomal activity was not observed during 0.5 h after glycoside addition. It may indicate that a complex pathway of signal transduction is responsible for the lysosome stimulation. Perhaps, the observed biological effects of the studied compounds may be generated by both the biomembrane barrier damage and a signal transduction mechanism involving an interaction with a membrane steroid receptor, modulating the rapid opening of Ca^{2+} channels.

In fact, some authors assume that some ginsenosides bind with steroid receptors or their analogues because steroid hormones and ginsenosides are similar in their gross molecular shapes and have certain structural conformity. Moreover, steroids with glucocorticoid activities are reported to induce a similar response to glycosides that affects macrophages and granulocytes (*32*, *33*). It may be suggested that the cucumarioside interaction with biomembrane steroid receptors may also contribute to macrophage $[Ca^{2+}]_i$ mobilization and lysosome alteration.

Finally, our results are in a good agreement with the previously obtained data on immunostimulatory activities of some cucumariosides in *in vivo* tests. It was demonstrated that the intraperitoneal injection of picogram to nanogram doses of several cucumariosides induced macrophage lysosomal activities of mice at day 4 after the treatment. However, the mechanism of this immunopotentiating remained unclear (*12*). The present study reveals the direct *in vitro* interaction of cucumariosides with target peritoneal macrophages evoking $[Ca^{2+}]_i$ response followed by macrophage activation.

ABBREVIATIONS USED

FITC, fluorescein isothiocyanate; EC_{50}, effective concentration causing 50% effect; $[Ca^{2+}]_i$, intracellular Ca^{2+} concentration; PBS, phosphate-buffered saline; HEPES, N-[2-hydroxyethyl]-piperazine-N′-[2-ethanesulfonic acid]; TLC, thin-layer chromatography; PMT, photomultiplier tube; NMR, nuclear magnetic resonance.

ACKNOWLEDGMENT

We are grateful to Dr. George Waller (Department of Biochemistry and Molecular Biology, Oklahoma State University, U.S.A.) for a helpful review and discussion of the manuscript.

LITERATURE CITED

(1) Avilov, S. A.; Stonik, V. A.; Kalinovsky, A. I. Structures of four new triterpene glycosides from the sea cucumber *Cucumaria japonica*. *Khim. Prirodn. Soedin.* **1990**, *6*, 787−792.

(2) Drozdova, O. A.; Avilov, S. A.; Kalinovsky, A. I.; Stonik, V. A.; Milgrom, Yu. M.; Rashkes, Ya. W. Trisulfated glycosides from the sea cucumber *Cucumaria japonica*. *Khim. Prirodn. Soedin*. **1993**, *3*, 369−374.

(3) Drozdova, O. A.; Avilov, S. A.; Kalinin, V. I.; Kalinovsky, A. I.; Stonik, V. A.; Rugiera, R.; Jimenez, C. Cytotoxic triterpene glycosides from Far-Eastern sea cucumber belonging to the genus *Cucumaria*. *Liebigs Ann*. **1997**, *11*, 2351−2356.

(4) Stonik, V. A.; Kalinin, V. I.; Avilov, S. A. Toxins from sea cucumbers (Holothuroids): chemical structures, properties, taxonomic distribution, biosynthesis and evolution. *J. Nat. Toxins* **1999**, *8* (2), 235−248.

(5) Grishin, Y. I.; Kovalevskaya, A. M.; Stonik, V. A.; Avilov, S. A.; Vlaznev, V. P.; Slugin, V. S. Immunostimulators KM and KM-2 for farming. *News Farming* **1991**, *2*, 19−23.

(6) Grishin, Y. I.; Kovalevskaya, A. M.; Stonik, V. A.; Avilov, S. A.; Vlaznev, V. P.; Slugin, V. S. Agent for prophylaxis and treatment of mink Aleutian illness. Patent of Russian Federation, No. 2036654, 1995.

(7) Sedov, A. M.; Elkina, S. I.; Sergeev, V. V. Ability of triterpene glycosides from holothurians to stimulate antibacterial resistance in model of experimental salmonellosis of mice. *J. Microbiol. Epidemiol. Immunobiol*. **1984**, *5*, 55−58.

(8) Sedov, A. M.; Shepeleva, I. B.; Zakharova, N. S.; Sakandelidze, O. G.; Sergeev, V. V.; Moshiashvili, I. Influence of cucumarioside (triterpene glycoside from the holothurian *Cucumaria japonica*) upon mice immune response evaluation on corpuscular vaccine. *J. Microbiol. Epidemiol. Immunobiol*. **1984**, *9*, 100−104.

(9) Sedov, A. M.; Apollonin, A. V.; Sevastyanova, E. K. Stimulation of mice nonspecific antibacterial resistance to conditionally pathogenic Gram-negative microorganisms. *Antibiot. Khimioter*. **1990**, *35* (1), 23−26.

(10) Grishin, Y. I.; Besednova, N. N.; Stonik, V. A.; Kovalevskaya, A. M.; Avilov, S. A. The regulation of hemopoiesis and immunegenesis by triterpene glycosides from holothurians. *Radiobiology* **1990**, *30* (4), 556.

(11) Turischev, S. N.; Bolshakova, G. B.; Sakandelidze, O. G. Influence of complexes of triterpene glycosides from holothurians on liver regeneration. *Izvestija Akad. Nauk SSSR, Ser. Biol.* **1991**, *2*, 306−310.

(12) Aminin, D. L.; Agafonova, I. G.; Berdyshev, E. V.; Isachenko, E. G.; Avilov, S. A.; Stonik, V. A. Immunomodulatory properties of cucumariosides from the edible Far-Eastern holothurian *Cucumaria japonica*. *J. Med. Food* **2001**, *4* (3), 127−135.

(13) Avilov, S. A.; Kalinin, V. I.; Makarieva, T. N.; Stonik, V. A.; Kalinovsky, A. I. Structure of cucumarioside G_2, a novel nonholostane glycoside from the sea cucumber Eupentacta fraudatrix. *J. Nat. Prod.* **1994**, *57* (8), 1166−1171.

(14) Kobayashi, N. Marine ecotoxicological testing with echinoderms. In *Ecotoxicological Testing for the Marine Environment*; Persoone, G., Jaspers, E., Claus, C., Eds.; State University Ghent and Inst. Mar. Scient. Res.: Bredene, Belgium, 1984; Vol. 1, p 798.

(15) Haugland, R. P. In *Handbook of Fluorescence Probes and Research Chemicals*, 6th ed.; Spence, M. T. Z., Ed.; Molecular Probes, Inc.: Eugene, OR, 1996.

(16) Allison, A. C.; Young, M. R. Vital staining and fluorescence microscopy of lysosomes. In *Lysosomes in Biology and Pathology*; Dingle, J. T., Fell, H. B., Eds.; North Holland: Amsterdam, 1969; Vol. 2, pp 600−628.

(17) Millot, C. Characterization of acidic vesicles in multidrug-resistant and sensitive cancer cells by Acridine orange staining and confocal microspectrofluorometry. *J. Histochem. Cytochem.* **1997**, *45*, 1255−1259.

(18) Zoccarato, F.; Cavallini, L.; Alexandre, A. The pH-sensitive dye Acridine orange as a tool to monitor exocytosis/endocytosis in synaptosomes. *J. Neurochem.* **1999**, *72*, 625.

(19) Lowe, D. M.; Moore, M. N.; Evance, B. M. Contaminant impact on interaction of molecular probes with lysosomes in living hepatocytes from dab Limanda limanda. *Mar. Ecol.: Prog. Ser.* **1992**, *2*, 1−6.

(20) Anisimov, M. M. Triterpene glycosides and structure−functional properties of biomembranes. *Biol. Nauki* **1987**, *10* (286), 49−63.

(21) Lasley, B. J.; Nigrelli, R. F. The effect of crude holothurin on leucocyte phagocytosis. *Toxicon* **1970**, *8*, 301−306.

(22) Popov, A. M.; Atopkina, L. N.; Samoshina, N. F.; Uvarova, N. I. Immunomodulating activity of tetracyclic triterpene glycoides of the dammaran and holostan series. *Antibiot. Khimioter*. **1994**, *39* (9−10), 19−25.

(23) Aminin, D. L.; Agafonova, I. G.; Gnedoi, S. N.; Strigina, L. I.; Anisimov, M. M. The effect of pH on biological activity of plant cytotoxin cauloside C. *Compar. Biochem. Physiol.* **1999**, *A 122*, 45−51.

(24) Ishida, H.; Hirota, Y.; Nakazava, H. Effect of sub-skinning concentration of saponin on intracellular Ca^{2+} and plasma membrane fluidity in cultured cardiac cells. *Biochim. Biophys. Acta* **1993**, *1145*, 58−62.

(25) Awang, D. V. C. Immune stimulants and antiviral botanicals: Echinacea and ginseng. In *Perspectives on New Crops and New Uses*; Janick, J., Ed.; ASHS Press: Alexandria, VA, 1999; pp 450−456.

(26) Yamasaki, Y.; Ito, K.; Enomoto, Y.; Sutko, J. Alterations by saponins of passive Ca^{2+} permeability and Na^+/Ca^{2+} exchange activity of canine cardiac sarcolemmal vesicles. *Biochim. Biophys. Acta* **1987**, *897*, 481−487.

(27) Li, Y.-L.; Deng, H.-W.; Chen, X. The protective effective effect of ginsenosides and its components on myocytes anoxia/re oxygenation and myocardial reperfusion injury. *Acta Pharm. Sin.* **1987**, *22* (1), 1−5.

(28) Kim, I. A.; Abdrasilov, B. S.; Nurieva, R. I.; Park, K. D. Action of triterpene glycosides of the dammarane series and their aglycones on the K^+ and H^+ ion currents in erythrocytes, induced by the ionophore A23187 and divalent ions. *Antibiot. Khimioter*. **1995**, *40*, 20−24.

(29) Bootman, M. D.; Berridge, M. J.; Lipp, P. Cooking with calcium: the recipes for composing global signals from elementary events. *Cell* **1993**, *91*, 367−373.

(30) Berridge, M. J. Elementary and global aspects of calcium signaling. *J. Physiol*. **1997**, *499*, 291−306.

(31) Kalinin, V. I.; Prokofieva, N. G.; Likhatskaya, G. N.; Schentsova, E. B.; Agafonova, I. G.; Avilov, S. A.; Drozdova, O. A.; Stonik, V. A. Hemolytic activity of triterpene glycosides from the holothurian order Dendrochirotida: Some trends in the evolution of this group of toxins. *Toxicon* **1996**, *34* (4), 475−483.

(32) Lee, H. Y.; Kim, S. I.; Lee, S. K.; Chung, H. Y.; Kim, K. W. Differentiation mechanism of ginsenosides in cultured murine F9 teratocarcinoma stem cells. *Proceedings of the International Ginseng Symposium*, Seoul, Korea, 1993; pp 127−131.

(33) Odashima, S.; Ohta, T.; Arich, S.; Abe, H.; Kitagawa, I.; Kohno, H.; Matsuda, T. Control of phenotypic expression of cultured B-16 Melanoma cells by plant glycosides. *Cancer Res*. **1985**, *45* (6), 2781−2784.

Received for review April 29, 2003. Revised manuscript received September 11, 2003. Accepted September 16, 2003. This study was partially supported by the Russian Foundation for Basic Research (Grant No. 00-15-97806).

JF034439X