There is a possibility that the administration itself induces the stress response. However, in a preliminary study, we immunohistochemically checked the expression of HSP in the cochlea of animals that received phosphate-buffered saline in the right middle ear cavity, and found no such upregulation.

After single-dose administration of GGA, mRNA expression of HSP in the brain was observed from 4 h then slowly decreased, and disappeared by 48 h (Nikaido et al., 2004). Western immunoblotting analysis in the rat heart revealed that HSP was present from 6 h and peaked at 24 h after GGA administration (Ooie et al., 2001). As shown in the present study, the effect of protection by HSP in the cochlea against trauma may also peak at 24 h after GGA administration.

Inner ear disorders caused by inflammation in the middle ear that are not treated early could lead to permanent inner ear damage. HSP has been considered to protect cells during inflammation associated with the toxicity of nitric oxide (Jacquier-Sarlin et al., 1994). A study using an HSP-inducer, sodium arsenite, demonstrated that the expression of HSP protected against iNOS expression and attenuated hypotension in rats treated with LPS (Hauser et al., 1996). Other studies have also shown that members of the HSP70 family inhibited iNOS expression (Matsuda et al., 2000; Dobbin et al., 2002). The upregulation of HSP70 in the cochlea following GGA administration, as seen in the present study, prompted us to also investigate whether GGA protects the cochlear lateral wall from damage induced by inflammation.

The rebound effect we observed after the release of AICA clamping is thought to reflect the autoregulation of blood flow in the inner ear (Yamamoto et al., 2003). This finding demonstrates physiologically that GGA can protect the lateral wall from damage induced by LPS-inoculation. The result was confirmed morphologically by electron microscopy, which revealed that the appearance of the stria vascularis was virtually normal in rats pretreated with GGA. GGA itself might influence iNOS expression in the cochlea, but oral administration of GGA was shown not to induce iNOS in rat hearts (Ooie et al., 2001). Antiapoptotic effects following GGA administration have been reported in the liver (Ikeyama et al., 2001), and the cochlear lateral walls in the present study might be partially protected by this mechanism.

GGA administration induced HSP70 in hair cells and supporting cells of the organ of Corti, the spiral ganglion, and the perivascular portion of modiolar vessels, as well as in the lateral wall. These findings suggest that GGA protects the cochlea against other injuries to these target structures through the upregulation of HSP70, such as induced by noise, ototoxic drugs, and ischemia. GGA might induce other HSPs in the cochlea besides HSP70, suggesting the prophylactic use of this drug in clinical applications.

Acknowledgement

This work was supported in part by research grants from the Ministry of Education, Culture, Sports, Science and Technology of the Japanese Government.

References

Altschuler, R.A., Fairfield, D., Cho, Y., Leonova, E., Benjamin, I.J., Miller, J.M., Lomax, M.I., 2002. Stress pathways in the rat cochlea and potential for protection from acquired deafness. Audiol. Neurootol. 7, 152–156.

Becker, J., Craig, E.A., 1994. Heat-shock proteins as molecular chaperones. Eur. J. Biochem. 219, 11–13.

Chopp, M., Chen, H., Ho, K.-L., Dereski, M.O., Brown, E., Hetzel, F.W., Welch, K.M.A., 1989. Transient hyperthermia protects against subsequent forebrain ischemic cell damage in the rat. Neurology 39, 1396–1398.

Dobbin, C.A., Smith, N.C., Johnson, A.M., 2002. Heat shock protein 70 is a potential virulence factor in murine toxoplasma infection via immunomodulation of host NF-κB and nitric oxide. J. Immunol. 169, 958–965.

Fairfield, D.A., Kanicki, A.C., Lomax, M.I., Altschuler, R.A., 2004. Induction of heat shock protein 32 (HSP32) in the rat cochlea following hyperthermia. Hear. Res. 188, 1–11.

Fujiki, M., Kobayashi, H., Abe, T., Ishi, K., 2003. Astroglial activation accompanies heat shock proteins upregulation in rat brain following single oral dose of geranylgeranylacetone. Brain Res. 991, 254–257.

Hauser, G.J., Dayao, E.K., Wasserloos, K., Pitt, B.R., Wong, H.R., 1996. HSP induction inhibits iNOS mRNA expression and attenuates hypotension in endotoxin-challenged rats. Am. J. Physiol. 271, H2529–H2535.

Hayes, S.A., Dice, J.F., 1996. Roles of Molecular chaperones in protein degradation. J. Cell. Biol. 132, 255–258.

Hirakawa, T., Rokutan, K., Nikawa, T., Kishi, K., 1996. Geranylgeranylacetone induces heat shock proteins in cultured guinea pig gastric mucosal cells and rat gastric mucosa. Gastroenterology 111, 354–357.

Ikeyama, S., Kusumoto, K., Miyake, H., Rokutan, K., Tashiro, S., 2001. A non-toxic heat shock protein 70 inducer, geranylgeranylacetone, suppresses apoptosis of cultured rat hepatocytes caused by hydrogen peroxide and ethanol. J. Hepatol. 35, 53–61.

Jacquier-Sarlin, M.R., Fuller, K., Dinh-Xuan, A.T., Richard, M.J., Polla, B.S., 1994. Protective effects of hsp70 in inflammation. Experientia 50, 1031–1038.

Lim, H.H., Jenkins, O.H., Myers, M.W., Miller, J.M., Altschuler, R.A., 1993. Detection of HSP 72 synthesis after acoustic overstimulation in rat cochlea. Hear. Res. 69, 146–150.

Matsuda, H., Kagerura, T., Toguchida, I., Ueda, H., Morikawa, T., Yoshikawa, M., 2000. Inhibitory effects of sesquiterpenes from bay leaf on nitric oxide production in lipopolysaccharide-activated macrophages: structure requirement and role of heat shock protein induction. Life Sci. 66, 2151–2157.

Minowada, G., Welch, W.J., 1995. Clinical implications of the stress response. J. Clin. Invest. 95, 3–12.

Niu, X., Canlon, B., 2002. Protective mechanisms of sound conditioning. Adv. Otorhinolaryngol. 59, 96–105.

Nikaido, H., Tsunoda, H., Nishimura, Y., Kirino, T., Tanaka, T., 2004. Potential role for heat shock protein 72 in antagonizing cerebral vasospasm after rat subarachnoid hemorrhage. Circulation 110, 1839–1846.

Oda, H., Miyake, H., Iwata, T., Kusumoto, K., Rokutan, K., Tashiro, S., 2002. Geranylgeranylacetone suppresses inflammatory

responses and improves survival after massive hepatectomy in rats. J. Gastrointest. Surg. 6, 464–473.

Oh, S.H., Yu, W.S., Song, B.H., Lim, D., Koo, J.W., Chang, S.O., Kim, C.S., 2000. Expression of heat shock protein 72 in rat cochlea with cisplatin-induced acute ototoxicity. Acta Otolaryngol. 120, 146–150.

Ooie, T., Takahashi, N., Saikawa, T., Nawata, T., Arikawa, M., Yamanaka, K., Hara, M., Shimada, T., Sakata, T., 2001. Single oral dose of geranylgeranylacetone induces heat-shock protein 72 and renders protection against ischemia/reperfusion injury in rat heart. Circulation 104, 1837–1843.

Sone, M., Hayashi, H., Yamamoto, H., Tominaga, M., Nakashima, T., 2003. A comparative study of intratympanic steroid and NO synthase inhibitor for treatment of cochlear lateral wall damage due to acute otitis media. Eur. J. Pharmacol. 482, 313–318.

Sone, M., Hayashi, H., Tominaga, M., Nakashima, T., 2004. Changes of cochlear blood flow due to endotoxin-induced otitis media. Ann. Otol. Rhinol. Laryngol. 113, 450–454.

Suzuki, K., Sawa, Y., Kaneda, Y., Ichikawa, H., Shirakura, R., Matsuda, H., 1997. In vivo gene transfection with heat shock protein 70 enhances myocardial tolerance to ischemia-reperfusion injury in rat. J. Clin. Invest. 99, 1645–1650.

Unoshima, M., Iwasaka, H., Eto, J., Takita-Sonoda, Y., Noguchi, T., Nishizono, A., 2003. Antiviral effects of geranylgeranylacetone: enhancement of $M \times A$ expression and phosphorylation of PKR during influenza infection. Antimicrob. Agents Chemother. 47, 2914–2921.

Yamamoto, H., Tominaga, M., Sone, M., Nakashima, T., 2003. Contribution of stapedial artery to blood flow in the cochlea and its surrounding bone. Hear. Res 186.

Yoshida, N., Kristiansen, A., Liberman, M.C., 1999. Heat stress and protection from permanent acoustic injury in mice. J. Neurosci. 19, 10116–10124.

Weiss, Y.G., Maloyan, A., Tazelaar, J., Raj, N., Deutschman, C.S., 2002. Adenoviral transfer of HSP-70 into pulmonary epithelium ameliorates experimental acute respiratory distress syndrome. J. Clin. Invest. 110, 801–806.

THE JOURNAL OF BIOLOGICAL CHEMISTRY
© 2005 by The American Society for Biochemistry and Molecular Biology, Inc.

Vol. 280, No. 35, Issue of September 2, pp. 31059–31067, 2005
Printed in U.S.A.

Involvement of Intracellular Ca^{2+} Levels in Nonsteroidal Anti-inflammatory Drug-induced Apoptosis*

Received for publication, March 17, 2005, and in revised form, May 25, 2005
Published, JBC Papers in Press, June 29, 2005, DOI 10.1074/jbc.M502956200

Ken-ichiro Tanaka‡, Wataru Tomisato‡, Tatsuya Hoshino‡, Tomoaki Ishihara‡, Takushi Namba‡, Mayuko Aburaya‡, Takashi Katsu§, Keitarou Suzuki‡, Shinji Tsutsumi‡, and Tohru Mizushima‡¶

From the ‡Graduate School of Medical and Pharmaceutical Sciences, Kumamoto University, Kumamoto 862-0973, and the §Faculty of Pharmaceutical Sciences, Okayama University, Okayama 700-8530, Japan

We recently reported that nonsteroidal anti-inflammatory drug (NSAID)-induced gastric lesions involve NSAID-induced apoptosis of gastric mucosal cells, which in turn involves the endoplasmic reticulum stress response, in particular the up-regulation of CCAAT/enhancer-binding protein homologous transcription factor (CHOP). In this study, we have examined the molecular mechanism governing this NSAID-induced apoptosis in primary cultures of gastric mucosal cells. Various NSAIDs showed membrane permeabilization activity that correlated with their apoptosis-inducing activity. Various NSAIDs, particularly celecoxib, also increased intracellular Ca^{2+} levels. This increase was accompanied by K^+ efflux from cells and was virtually absent when extracellular Ca^{2+} had been depleted. These data indicate that the increase in intracellular Ca^{2+} levels that is observed in the presence of NSAIDs is due to the stimulation of Ca^{2+} influx across the cytoplasmic membrane, which results from their membrane permeabilization activity. An intracellular Ca^{2+} chelator partially inhibited celecoxib-induced release of cytochrome *c* from mitochondria, reduced the magnitude of the celecoxib-induced decrease in mitochondrial membrane potential and inhibited celecoxib-induced apoptotic cell death. It is therefore likely that an increase in intracellular Ca^{2+} levels is involved in celecoxib-induced mitochondrial dysfunction and the resulting apoptosis. An inhibitor of calpain, a Ca^{2+}-dependent cysteine protease, partially suppressed mitochondrial dysfunction and apoptosis in the presence of celecoxib. Celecoxib-dependent CHOP-induction was partially inhibited by the intracellular Ca^{2+} chelator but not by the calpain inhibitor. These results suggest that Ca^{2+}-stimulated calpain activity and CHOP expression play important roles in celecoxib-induced apoptosis in gastric mucosal cells.

Nonsteroidal anti-inflammatory drugs (NSAIDs)[1] account for nearly 5% of all prescribed medications (1). The anti-inflammatory action of NSAIDs is mediated through their inhibitory effect on cyclooxygenase (COX) activity. COX is an enzyme essential for the synthesis of prostaglandins (PGs), which have a strong capacity to induce inflammation. On the other hand, NSAID use is associated with gastrointestinal complications (2), with about 15–30% of chronic users of NSAIDs suffering from gastrointestinal ulcers and bleeding (3–6).

The inhibition of COX by NSAIDs at gastrointestinal mucosa was previously thought to be the sole explanation for the gastrointestinal side effects of NSAIDs because PGs have a strong protective effect on gastrointestinal mucosa (7, 8). However, now it is believed that the induction of gastrointestinal lesions by NSAIDs involves additional mechanisms, since the increased incidence of gastrointestinal lesions and the decrease in PG levels induced by NSAIDs do not always occur in parallel (9, 10). We have previously demonstrated that NSAIDs induce cell death (apoptosis and necrosis) in primary cultures of gastric mucosal cells in a manner independent of COX inhibition (11, 12). Furthermore, we recently suggested that both COX inhibition and NSAID-induced cell death at gastric mucosa are required for NSAID-induced gastric lesions *in vivo* (12). Therefore, elucidation of the mechanisms governing NSAID-induced cell death is important for understanding the mechanisms by which NSAIDs cause gastric lesions and may allow the development of new safer NSAIDs. We recently showed that all of the NSAIDs tested have membrane permeabilization activity, which is implicated in NSAID-induced apoptosis and necrosis (13). However, the mechanism by which the membrane permeabilization activity of NSAIDs causes cell death and in particular apoptosis in gastric mucosal cells remains unknown.

Accumulation of unfolded protein in the endoplasmic reticulum (ER) induces the ER stress response, otherwise known as the unfolded protein response. Cells initially adapt to the accumulation of unfolded proteins by inducing the expression of ER-resident molecular chaperones such as glucose-regulated protein 78 (14–17). However, if this adaptation does not prove sufficient, the apoptotic response is initiated, which is primarily the induction of the CCAAT/enhancer-binding protein homologous transcription factor (CHOP) (18). We recently reported that various NSAIDs induce not only glucose-regulated protein 78 but also CHOP expression. In addition to this, we showed, using CHOP-

* This work was supported by grants-in-aid for scientific research from the Ministry of Health, Labour, and Welfare of Japan as well as by the Suzuken Memorial Foundation, the Tokyo Biochemical Research Foundation, the Kumamoto Technology and Industry Foundation, and the Japan Research Foundation for Clinical Pharmacology. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

¶ To whom correspondence should be addressed: Graduate School of Medical and Pharmaceutical Sciences, Kumamoto University, Kumamoto 862-0973, Japan. Tel./Fax: 81-96-371-4323; E-mail: mizu@gpo.kumamoto-u.ac.jp.

[1] The abbreviations used are: NSAIDs, nonsteroidal anti-inflammatory drugs; COX, cyclooxygenase; PG, prostaglandin; PGE_2, prostaglandin E_2; ER, endoplasmic reticulum; CHOP, CCAAT/enhancer-binding protein homologous transcription factor; SERCA, sarcoendoplasmic reticulum Ca^{2+}-ATPase; BAPTA-AM, 1,2-bis(2-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid; Ho 342, Hoechst 33342; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; DePsipher, 5,5′,6,6′-tetrachloro-1, 1′,3,3′-tetraethylbenzimidaozolylcarbocyanine iodide; Z-Leu-Leu-H, carbobenzoxy-L-leucyl-L-leucinal; AMC, aminomethylcoumarin; PIPES, 1,4-piperazinediethanesulfonic acid; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonic acid.

deficient mice and a dominant negative form of CHOP, that this CHOP induction is essential for NSAID-induced apoptosis (19). Therefore, NSAIDs seem to induce apoptosis by acting as ER stressors. However, the mechanism by which NSAIDs induce the ER stress response has remained unknown. Other groups have pointed out the involvement of mitochondrial dysfunction in NSAID-induced apoptosis; some NSAIDs (such as celecoxib) stimulate the release of cytochrome *c* from mitochondria and decrease the mitochondrial membrane potential (20, 21). Various mechanisms have been proposed for NSAID-induced mitochondrial dysfunction, such as inactivation of phosphatidylinositol 3-kinase/3-phosphoinositide-dependent kinase-1/Akt or mitogen-activated protein kinase/extracellular signal-regulated kinase (20–22). However, data in these studies have some contradictions (may be due to differences in cell species), and the mechanisms by which NSAIDs cause mitochondrial dysfunction have also not been fully characterized.

Increases in intracellular Ca^{2+} levels, due to cellular Ca^{2+} overload or perturbation of intracellular Ca^{2+} compartmentalization, trigger apoptosis. For example, many apoptotic stimuli, such as activation of surface antigen receptors, increase the intracellular Ca^{2+} level, and compounds that directly increase the intracellular Ca^{2+} level (Ca^{2+} ionophores and inhibitors for sarcoendoplasmic reticulum Ca^{2+} ATPase (SERCA)) have been shown to induce apoptosis (23–25). Various mechanisms have been proposed for the Ca^{2+}-induced apoptosis pathway, such as activation of protein kinase C, opening of permeability transition pores in mitochondria, and stimulation of reactive oxygen species synthesis (26–29). In addition, calpain, a Ca^{2+}-dependent cysteine protease, plays an important role in the Ca^{2+}-induced apoptosis pathway. Calpain activates or inhibits Bax and Bid or Bcl-2 and Bcl-X_L, respectively, by their cleavage, resulting in stimulation of the release of cytochrome *c* from mitochondria and a decrease in the mitochondrial membrane potential. Then cytochrome *c* activates caspase-9, which in turn activates caspase-3 and induces apoptosis. It is also suggested that calpain directly activates some caspases (26, 29–32). On the other hand, recent reports show that Ca^{2+} ionophores induce the ER stress response and CHOP expression, suggesting that increases in the intracellular Ca^{2+} level can induce the ER stress response and CHOP expression (26, 33).

Permeabilization of cytoplasmic membranes causes an increase in intracellular Ca^{2+} levels by stimulating Ca^{2+} influx across the cytoplasmic membrane. Some NSAIDs were shown to increase intracellular Ca^{2+} levels (13, 34). Therefore, it is reasonable to speculate that increases in intracellular Ca^{2+} levels are involved in NSAID-induced apoptosis (*i.e.* that the increased intracellular Ca^{2+} level connects the membrane permeabilization and CHOP induction activities of NSAIDs). Furthermore, it is also possible that activation of calpain connects the increases in intracellular Ca^{2+} levels to mitochondrial dysfunction in the presence of NSAIDs. In this study, we showed, in primary cultures of guinea pig gastric mucosal cells, that all NSAIDs tested increased intracellular Ca^{2+} levels, which accompanied the induction of apoptosis. An intracellular Ca^{2+} chelator partially inhibited CHOP induction, release of cytochrome *c* from mitochondria, the decrease in mitochondrial membrane potential, and apoptotic cell death in the presence of celecoxib (the most potent NSAID for apoptosis induction). Furthermore, an inhibitor of calpain partially suppressed the mitochondrial dysfunction and apoptotic cell death but did not affect CHOP induction in the presence of celecoxib. These results suggest that the celecoxib-dependent increase in intracellular Ca^{2+} levels and the resulting calpain activation and CHOP induction are involved in celecoxib-induced apoptosis in gastric mucosal cells.

EXPERIMENTAL PROCEDURES

Chemicals, Media, and Animals—Fetal bovine serum was from Invitrogen. RPMI 1640 and Hanks' solution were obtained from Nissui Pharmaceutical Co. Pronase E and type I collagenase were purchased from Kaken Pharmaceutical Co. and Nitta Gelatin Co., respectively. Pluronic F127, fluo-3/AM, 1,2-bis(2-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid (BAPTA-AM), and BAPTA were from Dojindo Co. Nimesulide and flurbiprofen were from Cayman Chemical Co. Indomethacin was from Wako Co. Hoechst 33342 (Ho 342), ibuprofen, diclofenac, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), mefenamic acid, ketoprofen, and flufenamic acid were from Sigma. 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidaozolylcarbocyanine iodide (DePsipher) was from Trevigen. Celecoxib was from LKT Laboratories Inc. Etodolac was a gift kindly provided by Nippon Shinyaku Co. Peptides for the assay of caspase-3 or calpain and carbobenzoxy-L-leucyl-L-leucinal (Z-Leu-Leu-H) were from Peptide Institute, Inc. Egg phosphatidylcholine was from Kanto Chemicals Co. Antibodies against CHOP and actin were from Santa Cruz Biotechnology Inc. Antibody against cytochrome *c* was from PharMingen. Male guinea pigs weighing 200–300 g were purchased from Kyudo Co. The experiments and procedures described here were carried out in accordance with the Guide for the Care and Use of Laboratory Animals as adopted and promulgated by the National Institutes of Health and were approved by the Animal Care Committee of Kumamoto University.

In Vitro Assay of Cytotoxicity—Gastric mucosal cells were isolated from guinea pig fundic glands as described previously (35). Isolated gastric mucosal cells were cultured for 12 h in RPMI 1640 containing 0.3% (v/v) fetal bovine serum, 100 units/ml penicillin, and 100 μg/ml streptomycin in type I collagen-coated plastic culture plates under conditions of 5% CO_2, 95% air and 37 °C. Nonadherent cells were removed, and the cells attached to the plate were used in the experiments. Guinea pig gastric mucosal cells prepared under these conditions have previously been characterized, with the majority (about 90%) of cells identified as pit cells (35).

NSAIDs were dissolved in Me_2SO or Na_2CO_3 (for indomethacin only) and control experiments (without NSAIDs) were performed in the presence of the same concentrations of Me_2SO or Na_2CO_3. Cells were exposed to NSAIDs by changing the medium. Cell viability was determined by the MTT method.

Apoptotic chromatin condensation was monitored as described previously (36). Cells were washed with PBS, stained with 10 μg/ml Ho 342, and observed under a fluorescence microscope.

Immunoblotting Analysis—Whole cell extracts were prepared as described previously (36). The protein concentration of the samples was determined by the Bradford method. The samples were electrophoresed on polyacrylamide gels containing SDS, and the proteins were then transferred to membranes and detected using antibodies.

Mitochondrial Membrane Potential Assay—Mitochondrial membrane potential was assayed using a fluorometric mitochondrial permeability assay kit (Trevigen) (36). Briefly, after treatment with NSAIDs, cells were treated with DePsipher (5 μg/ml) for 20 min at 37 °C and observed under a fluorescence microscope using 590 nm for red emission and 530 nm for green emission.

Measurement of the Intracellular Ca^{2+} Level—Intracellular Ca^{2+} levels were monitored as described (37). The cells were washed with assay buffer (115 mM NaCl, 5.4 mM KCl, 1.8 mM $CaCl_2$, 0.8 mM $MgCl_2$, 20 mM HEPES, and 13.8 mM glucose). For Ca^{2+}-free conditions, the modified assay buffer (115 mM NaCl, 5.4 mM KCl, 5 mM EGTA, 20 mM HEPES, and 13.8 mM glucose) was used instead of the normal assay buffer. The cells were then incubated with 4 μM fluo-3/AM in assay buffer supplemented with 0.1% bovine serum albumin, 0.04% pluronic F127, and 2 mM probenecid, for 40 min at 37 °C. After washing twice with assay buffer, cells were suspended in assay buffer supplemented with 2 mM probenecid. Cells were transferred to a water-jacketed cuvette (1.6×10^6 cells/cuvette), and the fluo-3 fluorescence was then measured with a Hitachi F-4500 spectrofluorophotometer by recording excitation signals at 490 nm and the emission signal at 530 nm at 1-s intervals. Maximum and minimum fluorescence values (F_{max} and F_{min}) were obtained by adding 10 μM ionomycin and 10 μM ionomycin under Ca^{2+}-free conditions, respectively. The intracellular Ca^{2+} level was calculated according to the equation, $[Ca^{2+}]_i = K_d(F - F_{min})/(F_{max} - F)$, where K_d is the apparent dissociation constant (400 nM) of the fluorescent dye-Ca^{2+} complex.

Membrane Permeability Assay—Permeabilization of calcein-loaded liposomes was assayed as described previously (13). Liposomes were prepared using the reversed-phase evaporation method. Egg phosphatidylcholine (10 μmol, 7.7 mg) was dissolved in chloroform/methanol (1:2,

v/v), dried, dissolved in 1.5 ml of diethyl ether, and added to 1 ml of 100 mM calcein-NaOH (pH 7.4). The mixture was sonicated to obtain a homogenous emulsion. The diethyl ether solvent was removed, and the resulting suspension of liposomes was centrifuged and washed twice with fresh buffer A (10 mM potassium buffer, containing 150 mM NaCl) to remove untrapped calcein. The final liposome precipitate was resuspended in 5 ml of buffer A. A 0.3-ml aliquot of this suspension was diluted with 19.7 ml of buffer A, and 400 μl of this diluted suspension was then incubated at 30 °C for 10 min in the presence of the NSAID under investigation. The release of calcein from liposomes was determined by measuring fluorescence intensity at 520 nm (excitation at 490 nm).

Assay for K^+ Efflux from Cells—K^+ efflux from cells was monitored as described previously (38) with some modifications. Cells were washed twice with buffer A and then suspended in fresh buffer A (6×10^6 cells/ml). After incubation with NSAIDs for 30 min at 37 °C, K^+ efflux from cells was measured with a K^+ ion-selective electrode.

Assay for Caspase Activity—The activity of caspase-3 was determined as described previously (36). Briefly, cells were collected by centrifugation and suspended in extraction buffer (50 mM PIPES (pH 7.0), 50 mM KCl, 5 mM EGTA, 2 mM $MgCl_2$, and 1 mM dithiothreitol). Suspensions were sonicated and centrifuged, after which the supernatants were incubated with fluorogenic peptide substrates (acetyl-DEVD-methylcoumarin amide) in reaction buffer (100 mM HEPES-KOH (pH 7.5), 10% sucrose, 0.1% CHAPS, and 1 mg/ml bovine serum albumin) for 15 min at 37 °C. The release of aminomethylcoumarin (AMC) was determined using a fluorescence spectrophotometer. One unit of protease activity was defined as the amount of enzyme required to release 1 pmol of AMC/min.

Assay for Calpain Activity—The activity of calpain was determined as described previously (30). Briefly, cells were collected by centrifugation, washed by Hanks' solution, and suspended with the same solution at 2.5×10^5 cells/ml. Suspensions were incubated with fluorogenic peptide substrates succinyl-L-leucyl-L-leucyl-L-valyl-L-tyrosine 4-methylcoumaryl-7-amide for 3 min at 37 °C. The release of AMC was determined using a fluorescence spectrophotometer.

Statistical Analyses—All values are expressed as the means ± S.E. One-way analysis of variance, followed by Scheffe's multiple comparison, was used for evaluation of differences between the groups. Student's *t* test for unpaired results was performed to evaluate differences between two groups. Differences were considered to be significant for values of $p < 0.05$.

RESULTS

Close Relationship between NSAID-induced Apoptosis and Membrane Permeabilization—We recently reported that some NSAIDs (celecoxib, mefenamic acid, flufenamic acid, nimesulide, and flurbiprofen) cause not only apoptosis in primary cultures of guinea pig gastric mucosal cells but also membrane permeabilization in calcein-loaded liposomes (13). To examine the relationship between the apoptosis-inducing and membrane permeabilization activities of NSAIDs, in this study, we have examined these activities of other NSAIDs. As shown in Fig. 1*A*, treatment of primary cultures of guinea pig gastric mucosal cells with celecoxib for 16 h decreased cell viability in a dose-dependent manner, and this is consistent with our previous results (13). Each of the NSAIDs tested here (indomethacin, diclofenac, etodolac, ibuprofen, and ketoprofen) also decreased cell viability in a dose-dependent manner. Because cell death under these conditions was accompanied by apoptotic DNA fragmentation and chromatin condensation (data not shown), it is most likely mediated by apoptosis.

Two subtypes of COX, COX-1 and COX-2, are responsible for the majority of COX activity in gastric mucosal and inflammatory tissues, respectively, and recently a number of COX-2-selective NSAIDs have been developed (39). Among the NSAIDs tested in Fig. 1*A*, etodolac and celecoxib have selectivity for COX-2. No relationship was evident between NSAID-induced apoptosis and selectivity for COX-2 (Fig. 1*A*). We also confirmed that exogenously added PGE_2 (either native PGE_2 or 16,16-dimethyl-PGE_2) did not affect NSAID-induced apoptosis even at a higher concentration of PGE_2 in the culture medium than is present endogenously (10^{-9} M) (data not shown). These

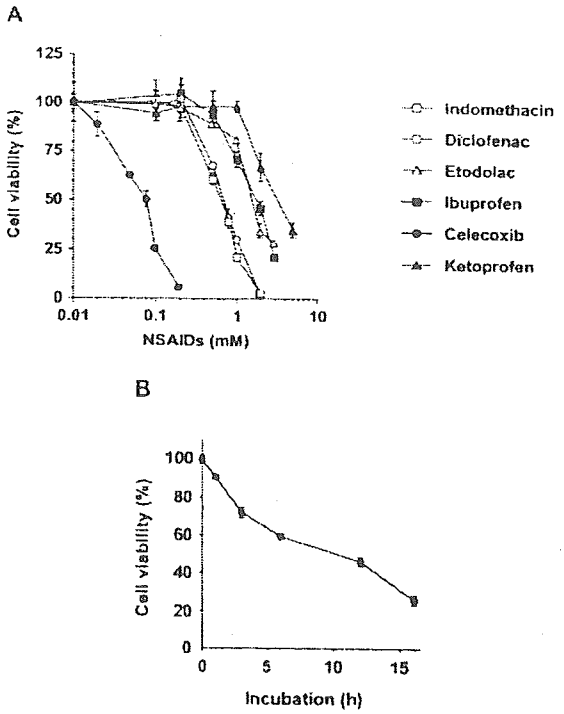

FIG. 1. **Apoptosis induced by NSAIDs.** Cultured guinea pig gastric mucosal cells were incubated with the indicated concentrations of each NSAID for 16 h (*A*) or with 100 μM celecoxib for the indicated periods (*B*). Cell viability was determined by the MTT method. Values are mean ± S.E. ($n = 3$).

data show that NSAID-induced apoptosis is independent of COX-inhibition by NSAIDs and are consistent with our previous conclusion (12, 13).

Calcein fluoresces very weakly at high concentrations due to self-quenching, so the addition of membrane-permeabilizing drugs to a medium containing calcein-loaded liposomes should cause an increase in fluorescence by diluting out the calcein (13). As shown in Fig. 2*A*, not only celecoxib but also other NSAIDs increased the calcein fluorescence, suggesting that they have membrane permeabilization activity.

Combining the results from the previous (13) and present studies, we obtained dose-response curves of 10 different NSAIDs for both induction of apoptosis and membrane permeabilization. To examine the relationship between NSAID-induced apoptosis and membrane permeabilization, we determined ED_{50} values of the 10 NSAIDs for apoptosis (concentrations of NSAIDs required for 50% inhibition of cell viability by apoptosis) and ED_{20} values for membrane permeabilization (concentration of NSAIDs required for 20% release of calcein) (Table I). We used ED_{20} values instead of ED_{50} values for estimating the activity of each NSAID for membrane permeabilization, because ED_{50} values of etodolac for calcein release could not to be determined (Fig. 2*A*). Plotting ED_{50} values for apoptosis *versus* ED_{20} values for membrane permeabilization (calcein release) yielded an r^2 value of 0.929 (Fig. 3*A*), which suggests that NSAID-induced apoptosis is mediated by their ability to permeabilize membranes.

Mechanism whereby the Intracellular Ca^{2+} Level Is Increased by NSAIDs—Fig. 4*A* shows the effect of various NSAIDs on the intracellular Ca^{2+} level. Not only celecoxib but also all of the other NSAIDs tested increased the intracellular Ca^{2+} level in a dose-dependent manner. Since indomethacin absorbs fluo-3 fluorescence (530 nm), we could not measure the intracellular Ca^{2+} level in the presence of indomethacin by this assay system. In order to estimate the activity of each NSAID for Ca^{2+} increase, we determined the concentration of each

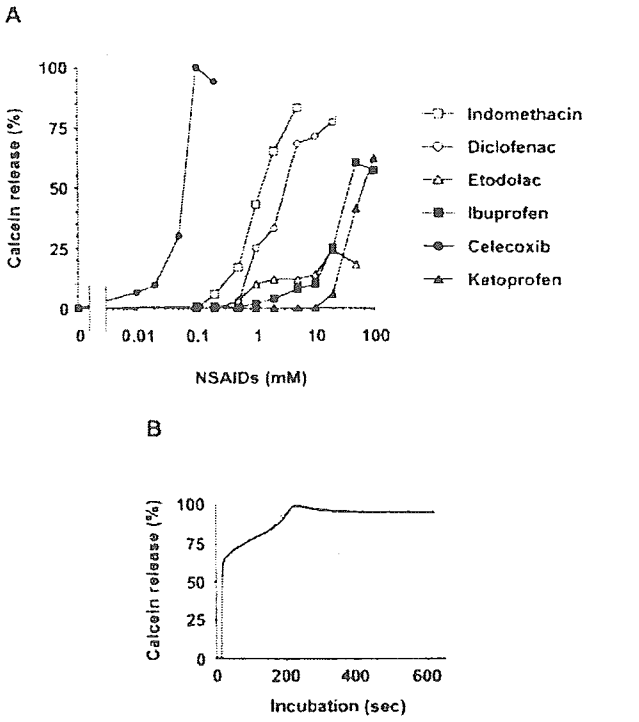

FIG. 2. **Membrane permeabilization by NSAIDs.** Calcein-loaded liposomes were incubated for 10 min with the indicated concentrations of each NSAID (*A*) or with 100 μM celecoxib for the indicated periods (*B*). The release of calcein from liposomes was determined by measuring fluorescence intensity. Melittin (10 μM) was used to determine the 100% level of membrane permeabilization (38).

TABLE I
NSAID concentrations required for apoptosis, membrane permeabilization, and Ca^{2+} increase

ED_{50} values of NSAIDs for apoptosis (concentrations of NSAIDs required for 50% inhibition of cell viability by apoptosis) were calculated based on results provided in Fig. 1 in the present study and Fig. 2*B* in our previous study (13). ED_{20} values of NSAIDs for calcein release (concentrations of NSAIDs required for 20% release of calcein) were calculated based on results provided in Fig. 2 in the present study and Fig. 4 in our previous study (13). ED_{double} values of NSAIDs for Ca^{2+} increase (concentrations of NSAIDs required for 2-fold increase in the intracellular Ca^{2+} level) were from Fig. 4 in the present study. ND, not determined.

NSAID	ED_{50} of apoptosis	ED_{20} of calcein release	ED_{double} of Ca^{2+} increase
		mM	
Indomethacin	0.6	0.5	ND
Diclofenac	0.8	0.9	0.15
Ibuprofen	1.8	17	0.3
Flurbiprofen	1.2	13	0.6
Ketoprofen	3.2	28	1.2
Mefenamic acid	0.7	3.0	0.5
Flufenamic acid	0.2	1.3	0.08
Celecoxib	0.08	0.02	0.03
Nimesulide	1.6	14	0.55
Etodolac	1.6	15	5.6

NSAID required for 2-fold increase in the intracellular Ca^{2+} level based on results in Fig. 4*A* (ED_{double} value for Ca^{2+} increase in Table I). Compared with ED_{20} values for membrane permeabilization (calcein release), etodolac showed a very high ED_{double} value for Ca^{2+} increase (Table I). Plotting the ED_{double} value for Ca^{2+} increase *versus* ED_{20} values for membrane permeabilization (calcein release) of NSAIDs other than etodolac and indomethacin yielded an r^2 value of 0.738 (Fig. 3*B*), which suggests that increase in the intracellular Ca^{2+} level by most of NSAIDs is mediated through their ability to permeabilize membranes. The membrane permeabilizing activity of etodolac may cause apoptosis independently of the Ca^{2+} increase; however, the mechanism is unknown at present. As well as showing the greatest potency for induction of apoptosis and membrane permeabilization (Figs. 1 and 2) (13), celecoxib increased intracellular Ca^{2+} levels more potently than the other NSAIDs (Fig. 4*A*). We therefore selected celecoxib for use in subsequent experiments.

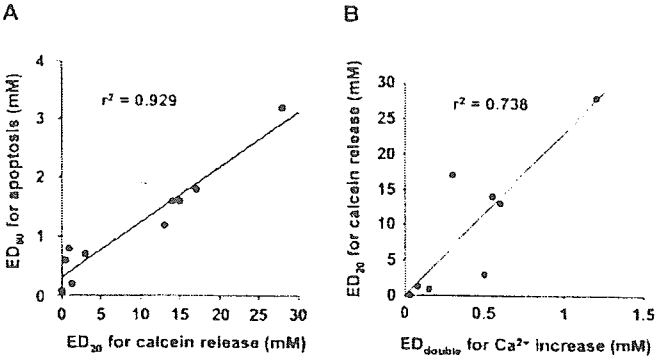

FIG. 3. **Relationship between apoptosis-inducing, membrane permeabilization, and Ca^{2+}-increasing activities of NSAIDs.** ED_{20} values for membrane permeabilization (calcein release), ED_{50} values for apoptosis and ED_{double} values for Ca^{2+} increase are from Table I. All 10 NSAIDs and NSAIDs except etodolac and indomethacin are plotted in *A* and *B*, respectively.

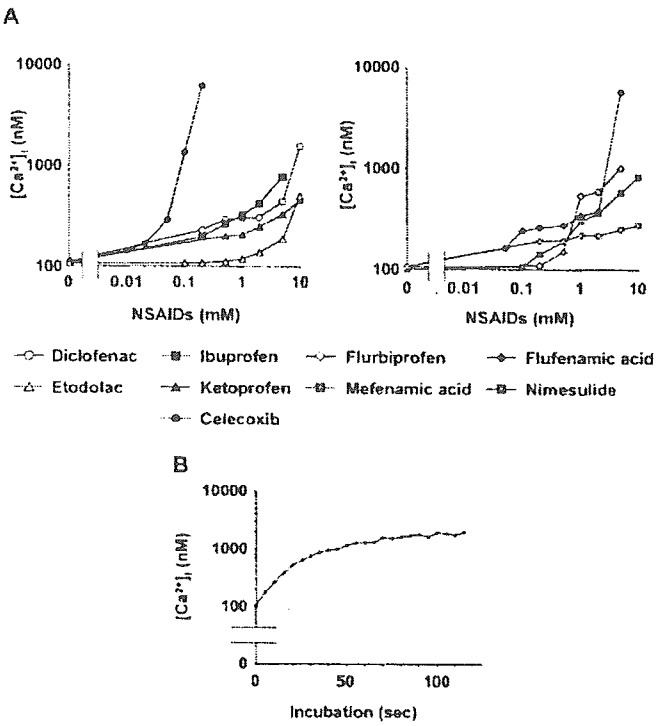

FIG. 4. **Changes in the intracellular Ca^{2+} level induced by NSAIDs.** The intracellular Ca^{2+} level was monitored using a fluo-3/AM assay system. Indicated concentrations of each NSAID were added to fluo-3/AM-loaded cells, and the time course of fluo-3 fluorescence change was monitored. The maximum values for the intracellular Ca^{2+} level ($[Ca^{2+}]_i$) are shown (*A*). The time course of fluo-3 fluorescence change in the presence of 100 μM celecoxib is shown (*B*).

In order to reveal the mechanism by which intracellular Ca^{2+} levels are increased by celecoxib, we monitored intracellular Ca^{2+} levels under Ca^{2+}-free conditions (see "Experimental Procedures"). As shown in Fig. 5*A*, the increase in the intracellular Ca^{2+} level induced by 50–100 μM celecoxib (apoptosis-inducing conditions) was drastically inhibited under Ca^{2+}-free conditions, suggesting that most of the increase is derived from extracellular Ca^{2+} and not from intracellular compartments (such as the ER

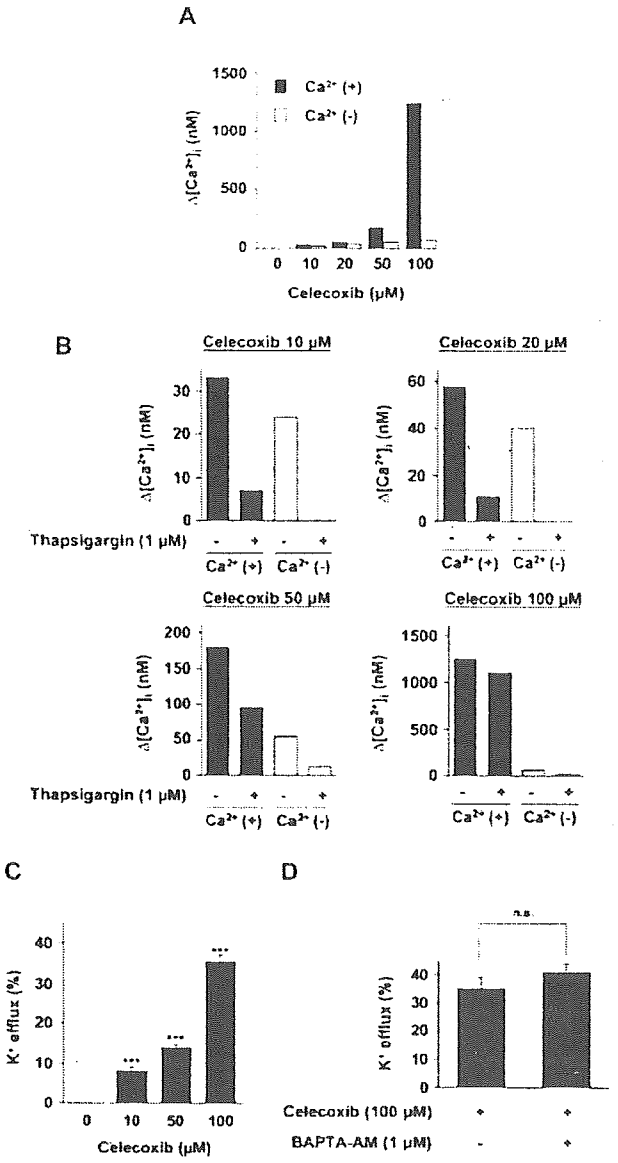

FIG. 5. **Mechanism for celecoxib-induced increases in the intracellular Ca^{2+} level.** Indicated concentrations of celecoxib were added to fluo-3/AM-loaded cells under Ca^{2+}-containing (+) or Ca^{2+}-free (−) conditions (*A* and *B*). Fluo-3/AM-loaded cells were pretreated with 1 μM thapsigargin for 10 min before the celecoxib treatment (*B*). The maximum value for the intracellular Ca^{2+} level ($[Ca^{2+}]_i$) was determined as described in the legend to Fig. 4 (*A* and *B*). Cultured guinea pig gastric mucosal cells were incubated with the indicated concentrations of celecoxib for 30 min (*C* and *D*). Cells were pretreated with BAPTA-AM for 10 min before the celecoxib treatment (*D*). The level of K^+ efflux was measured using a K^+ ion-selective electrode. Melittin (10 μM) was used to determine the 100% level of K^+ efflux (38). Values are mean ± S.E. ($n = 3$). ***, $p < 0.001$; *n.s.*, not significant (*C* and *D*).

and mitochondria). In other words, stimulation of Ca^{2+} influx across the cytoplasmic membrane due to membrane permeabilization by celecoxib seems to be responsible for the increase in the intracellular Ca^{2+} level in the presence of 50–100 μM celecoxib. Since permeabilization of cytoplasmic membranes should stimulate K^+ efflux from cells, we examined the effect of celecoxib on K^+ concentrations in the medium. As shown in Fig. 5*C*, K^+ concentration in the medium was increased, depending on the dose of celecoxib, suggesting that celecoxib stimulated K^+ efflux across the cytoplasmic membranes. These data suggest that celecoxib (50–100 μM) makes the cytoplasmic membranes permeable and stimulates Ca^{2+} influx, resulting in the observed increase in the intracellular Ca^{2+} level.

Since the increase in intracellular Ca^{2+} levels by 10–20 μM celecoxib was not clearly inhibited under Ca^{2+}-free conditions (Fig. 5, *A* and *B*), a mechanism other than stimulation of Ca^{2+} influx across the cytoplasmic membranes seems to be involved. We examined the contribution of the ER Ca^{2+} pool to the celecoxib-induced increase in the intracellular Ca^{2+} levels. Cells were pretreated with thapsigargin, an inhibitor of SERCA, which pumps Ca^{2+} from the cytoplasm to the ER, and then treated with celecoxib under either Ca^{2+}-containing or Ca^{2+}-free conditions (Fig. 5*B*). This pretreatment with thapsigargin depleted the ER Ca^{2+} pool (data not shown). The increase in the intracellular Ca^{2+} level induced by 10–20 μM celecoxib was inhibited more drastically by this thapsigargin pretreatment than by the depletion of extracellular Ca^{2+} (Fig. 5*B*). This contrasts with the results for 50–100 μM celecoxib (Fig. 5*B*). These results suggest that the ER Ca^{2+} pool (and perhaps permeabilization of ER membrane) plays an important role in the increase in the intracellular Ca^{2+} level induced by 10–20 μM celecoxib. In other words, the results indicate that most of the increase in intracellular Ca^{2+} levels that is induced by 10–20 μM celecoxib is derived from the ER and not from extracellular sources. A previous report showed that celecoxib inhibits SERCA (34). Therefore, the inhibitory action of celecoxib on SERCA may also contribute to the increase in the intracellular Ca^{2+} level induced by 10–20 μM celecoxib. In the presence of various concentrations of celecoxib, pretreatment with thapsigargin under Ca^{2+}-free conditions caused almost complete inhibition of the celecoxib-induced increase in intracellular Ca^{2+} levels (Fig. 5*B*). Therefore, both stimulation of Ca^{2+} influx across cytoplasmic membranes and depletion of the ER Ca^{2+} pool are responsible for the celecoxib-dependent increase in intracellular Ca^{2+} levels, which is consistent with previous results (34, 40).

Contribution of the Increase in the Intracellular Ca^{2+} Level to Celecoxib-induced Apoptosis—In order to examine the contribution of the membrane permeabilization and the resulting increase in intracellular Ca^{2+} level to celecoxib-induced apoptosis, at first, we examined the kinetics of celecoxib-induced apoptosis, membrane permeabilization, and increase in intracellular Ca^{2+} level (Figs. 1*B*, 2*B*, and 4*B*). Comparing to induction of apoptosis by celecoxib, the membrane permeabilization (calcein release) and increase in intracellular Ca^{2+} level by celecoxib occurred very rapidly, suggesting that the membrane permeabilization and the resulting increase in intracellular Ca^{2+} level are located upstream of the induction of apoptosis.

We then examined induction of apoptosis under Ca^{2+}-free conditions that is achieved by the addition of BAPTA, Ca^{2+} chelator, to the culture medium. As shown in Fig. 6*A*, the activation of caspase-3-like activity in cells treated with celecoxib for 6 h was partially suppressed by the addition of BAPTA, suggesting that the increase in intracellular Ca^{2+} level by celecoxib is important for celecoxib-induced apoptosis. However, depletion of Ca^{2+} in medium was toxic to cells; treatment of cells with 1 mM BAPTA for 16 h or incubation of cells with Ca^{2+}-free medium decreased the cell viability even in the absence of celecoxib (data not shown). Furthermore, since BAPTA is not permeable for cytoplasmic membranes, BAPTA cannot chelate intracellular Ca^{2+}. In order to address these issues, we used BAPTA-AM, an intracellular Ca^{2+} chelator that is permeable for cytoplasmic membranes. As shown in Fig. 6*B*, BAPTA-AM partially inhibited celecoxib-induced cell death. At the concentrations used, BAPTA-AM did not affect cell viability in the absence of celecoxib (data not shown). Furthermore, treatment of cells with celecoxib caused apoptotic chromatin condensation, which was also partially inhibited by the addition of BAPTA-AM in culture medium (Fig. 6, *D* and *E*).

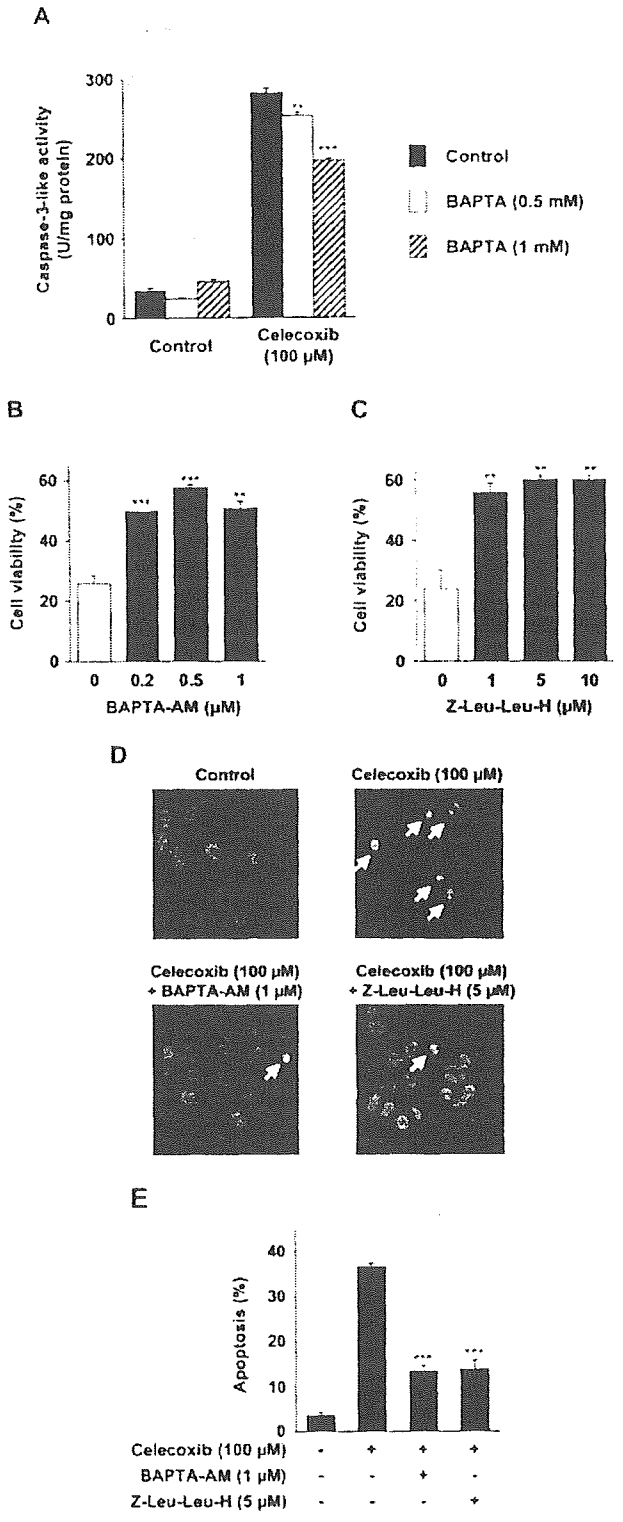

FIG. 6. **Effect of Ca^{2+} chelators or an inhibitor of calpain on celecoxib-induced apoptosis.** Cultured guinea pig gastric mucosal cells were preincubated with indicated concentrations of BAPTA-AM (*B*, *D*, and *E*) or Z-Leu-Leu-H (*C–E*) for 1 h. Cells were further incubated with or without 100 μM celecoxib for 6 h (*A*) or 16 h (*B–E*) under the same conditions as the preincubation step (medium containing BAPTA was used for *A*). Caspase-3-like activity was measured using fluorogenic peptide substrates (acetyl-DEVD-methylcoumarin amide) (*A*). Cell viability was determined by the MTT method (*B* and *C*). Values are mean ± S.E. ($n = 3$). ***, $p < 0.001$; **, $p < 0.01$ (*A–C*). After staining with Ho 342, cells were observed using a fluorescence microscope. The *arrows* show condensed chromatin (*D*). The numbers of apoptotic cells with condensed chromatin were counted from representative photomicrographs and are expressed as a percentage of total cell number ($n = 100$). Values are mean ± S.E. ($n = 3$). ***, $p < 0.001$ (*E*).

Therefore, the increase in intracellular Ca^{2+} levels caused by celecoxib seems to be involved in celecoxib-induced apoptosis. BAPTA-AM did not affect the celecoxib-dependent stimulation of K^+ efflux (Fig. 5*D*), suggesting that the membrane permeabilization by celecoxib is located upstream of increase in intracellular Ca^{2+} level and induction of apoptosis by celecoxib.

We also examined mitochondrial dysfunction in the presence of celecoxib. As shown in Fig. 7*A*, treatment of cells with celecoxib increased the amount of cytochrome *c* in the cytosolic fractions, suggesting that celecoxib stimulated the release of cytochrome *c* from mitochondria. The addition of BAPTA-AM partially suppressed this increase in the amount of cytochrome *c* (Figs. 7, *A* and *B*), suggesting that the celecoxib-dependent release of cytochrome *c* from mitochondria is partly due to the increase in the intracellular Ca^{2+} level.

We also examined mitochondrial dysfunction by monitoring the mitochondrial membrane potential, using DePsipher, a mitochondrial dye. This mitochondrial dye normally exists in solution as a monomer emitting green fluorescence (530 nm) and forms a dimer emitting red fluorescence (590 nm) in a reaction that is driven by the mitochondrial membrane potential. Thus, a decrease in the mitochondrial membrane potential should reduce the red fluorescence. As shown in Fig. 7*E*, the red fluorescence was apparent in control cells (without celecoxib) but was markedly decreased in celecoxib-treated cells. However, when cells were treated with celecoxib in the presence of BAPTA-AM, the red fluorescence was partially recovered (Fig. 7, *E* and *F*), suggesting that the celecoxib-dependent decrease in mitochondrial membrane potential is partly due to the celecoxib-induced increase in the intracellular Ca^{2+} level. These results show that the observed increase in the intracellular Ca^{2+} level is involved in celecoxib-induced mitochondrial dysfunction.

Mechanism for Ca^{2+}-dependent Celecoxib-induced Apoptosis—Then we examined the pathway for celecoxib-induced apoptosis downstream of the increase in the intracellular Ca^{2+} level. First, we examined the contribution of the intracellular Ca^{2+} level to celecoxib-induced CHOP expression, which was shown to play an important role in NSAID-induced apoptosis (19). Treatment of cells with celecoxib increased the amount of CHOP, as was previously reported (19) (Fig. 8, *A* and *B*). However, when cells were treated with celecoxib in the presence of BAPTA-AM, this CHOP induction was partially inhibited, suggesting that CHOP induction by celecoxib is partly due to the celecoxib-induced increase in the intracellular Ca^{2+} level.

We also examined the participation of calpain in celecoxib-induced apoptosis. We found that the activity of calpain was increased in celecoxib-treated cells by use of cell-permeable calpain substrate (Fig. 9). This calpain activation was partially inhibited by pretreatment of cells with BAPTA-AM (Fig. 9), suggesting that this activation is partially due to an increase in the intracellular Ca^{2+} level in the presence of celecoxib. An inhibitor of calpain, Z-Leu-Leu-H, partially suppressed the celecoxib-dependent decrease in cell viability and apoptotic chromatin condensation (Fig. 6, *C–E*). At the concentrations used, Z-Leu-Leu-H did not affect cell viability in the absence of celecoxib (data not shown). These results suggest that calpain is involved in NSAID-induced apoptosis. As shown in Fig. 7, *C–F*, both the celecoxib-stimulated release of cytochrome *c* from mitochondria and the celecoxib-dependent decrease in mitochondrial membrane potential were also partially suppressed by the addition of Z-Leu-Leu-H. From these observations, it is likely that calpain also plays an important role in celecoxib-dependent mitochondrial dysfunction. In other words, activation of calpain by the celecoxib-induced increase in the intracellular Ca^{2+} level seems to damage the mitochondria. On the other hand, in

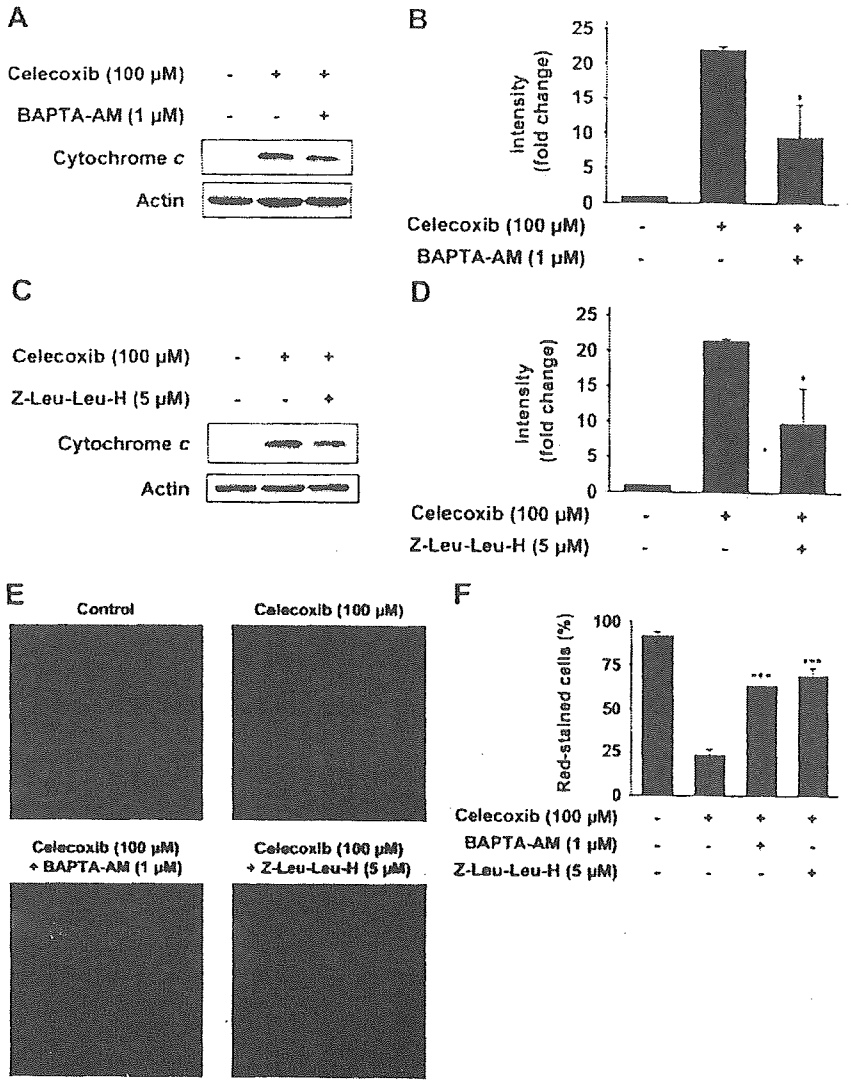

FIG. 7. **Effect of an intracellular Ca^{2+} chelator or an inhibitor of calpain on celecoxib-dependent mitochondrial dysfunction.** Cultured guinea pig gastric mucosal cells were preincubated with indicated concentrations of BAPTA-AM (*A*, *B*, *E*, and *F*) or Z-Leu-Leu-H (*C*–*F*) for 1 h. Cells were further incubated with or without 100 μM celecoxib for 16 h under the same conditions as the preincubation step. After subcellular fractionation, cytosolic fractions were analyzed by immunoblotting with an antibody against cytochrome *c* or actin (*A* and *C*). After normalization by the use of actin bands, the amounts of cytochrome *c* relative to control (without celecoxib and BAPTA-AM or Z-Leu-Leu-H) were determined and expressed as intensity. Values are mean ± S.E. ($n = 3$). *, $p < 0.05$ (*B* and *D*). Mitochondrial membrane potential was analyzed as described under "Experimental Procedures" (*E*). The numbers of *red*-stained cells with DePsipher were counted from representative photomicrographs and are expressed as a percentage of total cell number ($n = 100$). Values are mean ± S.E. ($n = 3$). ***, $p < 0.001$ (*F*).

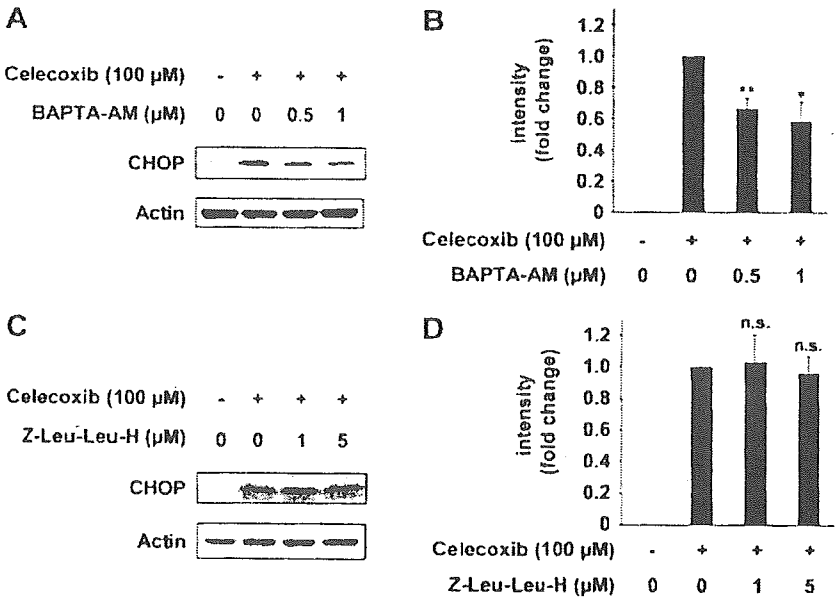

FIG. 8. **Effect of an intracellular Ca^{2+} chelator or an inhibitor of calpain on celecoxib-dependent CHOP induction.** Cultured guinea pig gastric mucosal cells were preincubated with indicated concentrations of BAPTA-AM (*A* and *B*) or Z-Leu-Leu-H (*C* and *D*) for 1 h. Cells were further incubated with or without 100 μM celecoxib under the same conditions as the preincubation step. Whole cell extracts were analyzed by immunoblotting with an antibody against CHOP or actin (*A* and *C*). The intensity of the actin bands was used to correct for differences in sample loading, and the relative amounts of CHOP to control (with celecoxib but without BAPTA-AM or Z-Leu-Leu-H) are expressed as intensity. Values are mean ± S.E. ($n = 3$). **, $p < 0.01$; *, $p < 0.05$; *n.s.*, not significant (*B* and *D*).

contrast to BAPTA-AM, addition of Z-Leu-Leu-H did not significantly affect CHOP induction in the presence of celecoxib (Fig. 8, *C* and *D*), suggesting that induction of CHOP by celecoxib is independent of calpain.

DISCUSSION

In this study, we aimed to identify the pathway for celecoxib-induced apoptosis using primary cultures of gastric mucosal cells. First, using 10 different NSAIDs, we demonstrated the

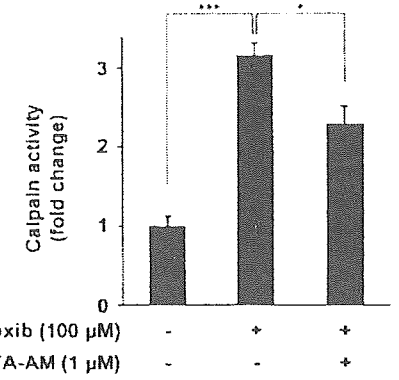

FIG. 9. **Activation of calpain by celecoxib.** Cultured guinea pig gastric mucosal cells were preincubated with or without 1 μM BAPTA-AM for 1 h. Cells were further incubated with or without 100 μM celecoxib for 16 h under the same conditions as the preincubation step. Calpain activity was measured using fluorogenic peptide substrates (succinyl-L-leucyl-L-leucyl-L-valyl-L-tyrosine 4-methylcoumaryl-7-amide). Values are mean ± S.E. (n = 3). ***, $p < 0.001$; *, $p < 0.05$.

close relationship between their apoptosis-inducing and membrane permeabilization activities. Therefore, it seems that membrane phospholipids are the primary target of NSAIDs for the induction of apoptosis. To investigate the mechanism by which the membrane permeabilization activity of NSAIDs causes apoptosis, we focused on the intracellular Ca^{2+} level, because permeabilization of cytoplasmic membranes increases the intracellular Ca^{2+} level by stimulating Ca^{2+} influx, and the intracellular Ca^{2+} level is a key regulator of apoptosis. We have concluded that intracellular Ca^{2+} levels are involved in celecoxib-induced apoptosis, since each of the NSAIDs that we tested increased the intracellular Ca^{2+} level, this Ca^{2+}-increasing activity of NSAIDs (except etodolac) correlated with their membrane permeabilization activity, and an intracellular Ca^{2+} chelator (BAPTA-AM) partially inhibited celecoxib-dependent apoptosis and mitochondrial dysfunction. To elucidate the mechanism by which the increase in the intracellular Ca^{2+} level causes apoptosis, we focused on calpain, because Ca^{2+} activates this enzyme, and activation of this enzyme can induce apoptosis. We have demonstrated the involvement of calpain in celecoxib-induced apoptosis by showing that an inhibitor of calpain (Z-Leu-Leu-H) partially inhibited celecoxib-dependent apoptosis and mitochondrial dysfunction. Cleavage by calpain activates and inhibits Bax and Bid and Bcl-2 and Bcl-X_L, respectively. Since Bax and Bid cause mitochondrial membrane permeabilization (stimulate release of cytochrome c from the mitochondria and decrease the mitochondrial membrane potential) (25, 30–32), whereas Bcl-2 and Bcl-X_L protect mitochondrial membrane from permeabilization, activation of calpain by increasing the intracellular Ca^{2+} level seems to play an important role in celecoxib-induced mitochondrial dysfunction. Calpain cleaves and thereby activates procaspase-12 and apoptosis-inducing factor and cleaves and thereby inhibits the X-linked inhibitor of apoptosis (26, 27, 41). These events may be involved in celecoxib-induced apoptosis.

Suppression of celecoxib-induced apoptosis by BAPTA-AM was partial, suggesting that a Ca^{2+}-independent pathway is also involved in this process. One candidate is the death receptor signaling pathway. It was recently reported that celecoxib induces expression of death receptors, especially DR5, and small interfering RNA for DR5 partially suppresses celecoxib-induced apoptosis in lung cancer cells (42). Induction of death receptors causes activation of caspase-8, and it has been shown that celecoxib activates caspase-8 and that an inhibitor of caspase-8 partially suppresses celecoxib-dependent apoptosis (42). We previously reported that various NSAIDs activate caspase-8 in cultured gastric mucosal cells (11). This DR5-dependent pathway may be involved in celecoxib-induced apoptosis in cultured gastric mucosal cells. As described in the Introduction, inactivation of phosphatidylinositol 3-kinase/3-phosphoinositide-dependent kinase-1/Akt and mitogen-activated protein kinase/extracellular signal-regulated kinase signal transduction systems may also be involved in NSAID-induced apoptosis (20–22). However, at present, it is not clear whether these pathways are Ca^{2+}-dependent or Ca^{2+}-independent, because upstream signals for these systems have not yet been revealed.

Suppression of celecoxib-induced apoptosis by Z-Leu-Leu-H was also partial, and this implies that a calpain-independent pathway is involved in this process. Of course, logically, the Ca^{2+}-independent pathways discussed above may be responsible for the calpain-independent pathway. However, we have considered the contribution of Ca^{2+}-dependent but calpain-independent pathways to celecoxib-induced apoptosis. One candidate for such a pathway is the ER stress response, in particular the induction of CHOP. We recently reported that NSAID-induced apoptosis is suppressed in cultured guinea pig gastric mucosal cells by expression of the dominant negative form of CHOP and in peritoneal macrophages from CHOP-deficient mice (19). In the current study, we have shown that celecoxib-dependent CHOP induction is suppressed by BAPTA-AM but not by Z-Leu-Leu-H, suggesting that in the celecoxib-induced apoptosis pathway, CHOP induction is located downstream of the increase in the intracellular Ca^{2+} level but not of calpain activation. Furthermore, various Ca^{2+}-dependent but calpain-independent signal pathways other than CHOP induction may be involved in celecoxib-induced apoptosis. For example, Ca^{2+}-dependent activation of protein kinase Cδ, a protein kinase C isoform, induces release of cytochrome c from mitochondria (43). Calcineurin, a Ca^{2+}-dependent serine-threonine phosphatase, dephosphorylates BAD (a proapoptotic member of the Bcl-2 family) and stimulates apoptosis (44). Opening of permeability transition pores in mitochondria and stimulation of reactive oxygen species synthesis are also possible Ca^{2+}-dependent but calpain-independent pathways for celecoxib-induced apoptosis (26–29).

We recently proposed that both COX inhibition at gastric mucosa and direct gastric mucosal cell damage (such as induction of apoptosis) by NSAIDs are required for the production of gastric lesions *in vivo*. Gastric lesions develop in a manner that depends on both an intravenously administered low dose of indomethacin (inhibition of COX activity at the gastric mucosa without direct gastric mucosal cell damage) and an orally administered COX-2-selective NSAID, such as celecoxib (causes direct gastric mucosal cell damage without inhibition of COX at gastric mucosa) (12). Therefore, based on the results in this study, it may be suggested that NSAIDs without membrane-permeabilizing activity and intracellular Ca^{2+} level-increasing activity may have reduced gastrointestinal side effects compared with other NSAIDs. An issue that was recently raised concerning the use of COX-2-selective NSAIDs, a class of gastrointestinally safe NSAIDs, is their potential risk for promoting cardiovascular thrombotic events (45). Prostacyclin, a potent anti-aggregator of platelets and a vasodilator, is mainly produced by COX-2 in vascular endothelial cells, whereas thromboxane A_2, a potent aggregator of platelets and a vasoconstrictor, is mainly produced by COX-1 in platelets (46, 47). Until recently, rofecoxib and celecoxib were leading COX-2-selective NSAIDs in the market. Rofecoxib was withdrawn from the market due to the risk of its promoting cardiovascular thrombotic events, and the United States Food and Drug Administration advised physicians to consider alternatives to

celecoxib due to the risk of its causing cardiovascular thrombotic events (48, 49). Based on our findings, NSAIDs that do not exhibit membrane permeabilization activity may be safe for the gastrointestinal tract even if they are not highly selective for COX-2. This type of NSAID may be of clinical benefit, because they are predicted to be safe for both the gastrointestinal tract and cardiovascular system.

On the other hand, much attention has also been given to the direct cytotoxicity of NSAIDs in relation to cancer therapy. Epidemiological studies have shown that prolonged use of aspirin or other NSAIDs reduces the risk of cancer (50, 51). Furthermore, several NSAIDs are presently under clinical development as anti-cancer drugs. A number of *in vivo* and *in vitro* studies have revealed that NSAIDs cause apoptosis (or stimulate apoptosis by anti-cancer reagents) in cancer cells and that these activities of NSAIDs are involved in their anti-cancer activity (52). PGs, such as PGE_2, inhibit apoptosis (53). Furthermore, overexpression of COX-2 has been reported in various tumor cells and tissues (54, 55). Therefore, the inhibition of COX by NSAIDs was previously thought to be solely responsible for their chemopreventive effect. However, several lines of evidence suggest that chemoprevention by NSAIDs also involves COX-independent mechanisms. Sulindac sulfone, a derivative of the NSAID sulindac, does not inhibit COX activity and has been shown to display anti-tumor activity *in vivo* as well as induce apoptosis and inhibit cell growth in tumor cells *in vitro* (56, 57). Moreover, NSAID induction of apoptosis and inhibition of cell growth in COX-null fibroblasts and tumor cells in which COX expression was absent have been reported (58, 59). Therefore, an understanding of COX-independent mechanisms for the induction of apoptosis by NSAIDs is very important in order to develop more effective NSAIDs for cancer therapy. If the results presented here can be applied to the case of cancer cells, NSAIDs with more potent membrane-permeabilizing and Ca^{2+}-increasing activity may have more potent anti-cancer effects than other NSAIDs.

REFERENCES

1. Smalley, W. E., Ray, W. A., Daugherty, J. R., and Griffin, M. R. (1995) *Am. J. Epidemiol.* **141,** 539–545
2. Hawkey, C. J. (2000) *Gastroenterology* **119,** 521–535
3. Barrier, C. H., and Hirschowitz, B. I. (1989) *Arthritis Rheum.* **32,** 926–932
4. Gabriel, S. E., Jaakkimainen, L., and Bombardier, C. (1991) *Ann. Intern. Med.* **115,** 787–796
5. Fries, J. F., Miller, S. R., Spitz, P. W., Williams, C. A., Hubert, H. B., and Bloch, D. A. (1989) *Gastroenterology* **96,** 647–655
6. Kurata, J. H., and Abbey, D. E. (1990) *J. Clin. Gastroenterol.* **12,** 260–266
7. Vane, J. R., and Botting, R. M. (1996) *Scand. J. Rheumatol. Suppl.* **102,** 9–21
8. Miller, T. A. (1983) *Am. J. Physiol.* **245,** G601–G623
9. Ligumsky, M., Golanska, E. M., Hansen, D. G., and Kauffman, G. J. (1983) *Gastroenterology* **84,** 756–761
10. Ligumsky, M., Sestieri, M., Karmeli, F., Zimmerman, J., Okon, E., and Rachmilewitz, D. (1990) *Gastroenterology* **5,** 1245–1249
11. Tomisato, W., Tsutsumi, S., Rokutan, K., Tsuchiya, T., and Mizushima, T. (2001) *Am. J. Physiol.* **281,** C1092–C1100
12. Tomisato, W., Tsutsumi, S., Hoshino, T., Hwang, H. J., Mio, M., Tsuchiya, T., and Mizushima, T. (2004) *Biochem. Pharmacol.* **67,** 575–585
13. Tomisato, W., Tanaka, K., Katsu, T., Kakuta, H., Sasaki, K., Tsutsumi, S., Hoshino, T., Aburaya, M., Li, D., Tsuchiya, T., Suzuki, K., Yokomizo, K., and Mizushima, T. (2004) *Biochem. Biophys. Res. Commun.* **323,** 1032–1039
14. Kaufman, R. J. (1999) *Genes Dev.* **13,** 1211–1233
15. Mori, K. (2000) *Cell* **101,** 451–454
16. Liu, H., Bowes, R. C., III, van de Water, B., Sillence, C., Nagelkerke, J. F., and Stevens, J. L. (1997) *J. Biol. Chem.* **272,** 21751–21759
17. Reddy, R. K., Lu, J., and Lee, A. S. (1999) *J. Biol. Chem.* **274,** 28476–28483
18. Zinszner, H., Kuroda, M., Wang, X., Batchvarova, N., Lightfoot, R. T., Remotti, H., Stevens, J. L., and Ron, D. (1998) *Genes Dev.* **12,** 982–995
19. Tsutsumi, S., Gotoh, T., Tomisato, W., Mima, S., Hoshino, T., Hwang, H. J., Takenaka, H., Tsuchiya, T., Mori, M., and Mizushima, T. (2004) *Cell Death Differ.* **11,** 1009–1016
20. Ding, H., Han, C., Zhu, J., Chen, C. S., and D'Ambrosio, S. M. (2005) *Int. J. Cancer* **113,** 803–810
21. Zhang, Z., Lai, G. H., and Sirica, A. E. (2004) *Hepatology* **39,** 1028–1037
22. Hsu, A. L., Ching, T. T., Wang, D. S., Song, X., Rangnekar, V. M., and Chen, C. S. (2000) *J. Biol. Chem.* **275,** 11397–11403
23. Jiang, S., Chow, S. C., Nicotera, P., and Orrenius, S. (1994) *Exp. Cell Res.* **212,** 84–92
24. Gwag, B. J., Canzoniero, L. M., Sensi, S. L., Demaro, J. A., Koh, J. Y., Goldberg, M. P., Jacquin, M., and Choi, D. W. (1999) *Neuroscience* **90,** 1339–1348
25. Gil-Parrado, S., Fernandez-Montalvan, A., Assfalg-Machleidt, I., Popp, O., Bestvater, F., Holloschi, A., Knoch, T. A., Auerswald, E. A., Welsh, K., Reed, J. C., Fritz, H., Fuentes-Prior, P., Spiess, E., Salvesen, G. S., and Machleidt, W. (2002) *J. Biol. Chem.* **277,** 27217–27226
26. Orrenius, S., Zhivotovsky, B., and Nicotera, P. (2003) *Nat. Rev. Mol. Cell. Biol.* **4,** 552–565
27. Rizzuto, R., Pinton, P., Ferrari, D., Chami, M., Szabadkai, G., Magalhaes, P. J., Di Virgilio, F., and Pozzan, T. (2003) *Oncogene* **22,** 8619–8627
28. Szabadkai, G., and Rizzuto, R. (2004) *FEBS Lett.* **567,** 111–115
29. Hajnoczky, G., Davies, E., and Madesh, M. (2003) *Biochem. Biophys. Res. Commun.* **304,** 445–454
30. Mandic, A., Viktorsson, K., Strandberg, L., Heiden, T., Hansson, J., Linder, S., and Shoshan, M. C. (2002) *Mol. Cell. Biol.* **22,** 3003–3013
31. Wood, D. E., Thomas, A., Devi, L. A., Berman, Y., Beavis, R. C., Reed, J. C., and Newcomb, E. W. (1998) *Oncogene* **17,** 1069–1078
32. Nakagawa, T., and Yuan, J. (2000) *J. Cell Biol.* **150,** 887–894
33. Nozaki, S., Sledge Jr., G. W., and Nakshatri, H. (2001) *Oncogene* **20,** 2178–2185
34. Johnson, A. J., Hsu, A. L., Lin, H. P., Song, X., and Chen, C. S. (2002) *Biochem. J.* **366,** 831–837
35. Tomisato, W., Hoshino, T., Tsutsumi, S., Tsuchiya, T., and Mizushima, T. (2002) *Dig. Dis. Sci.* **47,** 2125–2133
36. Tsutsumi, S., Tomisato, W., Takano, T., Rokutan, K., Tsuchiya, T., and Mizushima, T. (2002) *Biochim. Biophys. Acta* **1589,** 168–180
37. Kao, J. P., Harootunian, A. T., and Tsien, R. Y. (1989) *J. Biol. Chem.* **264,** 8179–8184
38. Katsu, T., Kobayashi, H., Hirota, T., Fujita, Y., Sato, K., and Nagai, U. (1987) *Biochim. Biophys. Acta* **899,** 159–170
39. Vane, J. (1994) *Nature* **367,** 215–216
40. Wang, J. L., Lin, K. L., Chen, J. S., Lu, Y. C., Jiann, B. P., Chang, H. T., Hsu, S. S., Chen, W. C., Huang, J. K., Ho, C. M., and Jan, C. R. (2004) *Biochem. Pharmacol.* **67,** 1123–1130
41. Polster, B. M., Basanez, G., Etxebarria, A., Hardwick, J. M., and Nicholls, D. G. (2005) *J. Biol. Chem.* **280,** 6447–6454
42. Liu, X., Yue, P., Zhou, Z., Khuri, F. R., and Sun, S. Y. (2004) *J. Natl. Cancer Inst.* **96,** 1769–1780
43. Majumder, P. K., Pandey, P., Sun, X., Cheng, K., Datta, R., Saxena, S., Kharbanda, S., and Kufe, D. (2000) *J. Biol. Chem.* **275,** 21793–21796
44. Wang, H. G., Pathan, N., Ethell, I. M., Krajewski, S., Yamaguchi, Y., Shibasaki, F., McKeon, F., Bobo, T., Franke, T. F., and Reed, J. C. (1999) *Science* **284,** 339–343
45. Mukherjee, D. (2002) *Biochem. Pharmacol.* **63,** 817–821
46. McAdam, B. F., Catella, L. F., Mardini, I. A., Kapoor, S., Lawson, J. A., and FitzGerald, G. A. (1999) *Proc. Natl. Acad. Sci. U. S. A.* **96,** 272–277
47. Belton, O., Byrne, D., Kearney, D., Leahy, A., and Fitzgerald, D. J. (2000) *Circulation* **102,** 840–845
48. Singh, D. (2004) *Br. Med. J.* **329,** 816
49. Ray, W. A., Griffin, M. R., and Stein, C. M. (2004) *N. Engl. J. Med.* **351,** 2767
50. Giovannucci, E., Rimm, E. B., Stampfer, M. J., Colditz, G. A., Ascherio, A., and Willett, W. C. (1994) *Ann. Intern. Med.* **121,** 241–246
51. Baron, J. A. (2003) *Prog. Exp. Tumor Res.* **37,** 1–24
52. Chan, T. A. (2002) *Lancet Oncol.* **3,** 166–174
53. Hoshino, T., Tsutsumi, S., Tomisato, W., Hwang, H. J., Tsuchiya, T., and Mizushima, T. (2003) *J. Biol. Chem.* **278,** 12752–12758
54. Eberhart, C. E., Coffey, R. J., Radhika, A., Giardiello, F. M., Ferrenbach, S., and DuBois, R. N. (1994) *Gastroenterology* **107,** 1183–1188
55. Ristimaki, A., Honkanen, N., Jankala, H., Sipponen, P., and Harkonen, M. (1997) *Cancer Res.* **57,** 1276–1280
56. Piazza, G. A., Alberts, D. S., Hixson, L. J., Paranka, N. S., Li, H., Finn, T., Bogert, C., Guillen, J. M., Brendel, K., Gross, P. H., Sperl, G., Ritchie, J., Burt, R. W., Ellsworth, L., Ahnen, D. J., and Pamukcu, R. (1997) *Cancer Res.* **57,** 2909–2915
57. Reddy, B. S., Kawamori, T., Lubet, R. A., Steele, V. E., Kelloff, G. J., and Rao, C. V. (1999) *Cancer Res.* **59,** 3387–3391
58. Zhang, X., Morham, S. G., Langenbach, R., and Young, D. A. (1999) *J. Exp. Med.* **190,** 451–459
59. Hanif, R., Pittas, A., Feng, Y., Koutsos, M. I., Qiao, L., Staiano-Coico, L., Shiff, S. I., and Rigas, B. (1996) *Biochem. Pharmacol.* **52,** 237–245

0026-895X/05/6804-1156–1161$20.00
MOLECULAR PHARMACOLOGY
Copyright © 2005 The American Society for Pharmacology and Experimental Therapeutics
Mol Pharmacol 68:1156–1161, 2005

Vol. 68, No. 4
15784/3056065
Printed in U.S.A.

Geranylgeranylacetone Protects Membranes against Nonsteroidal Anti-Inflammatory Drugs

Hironori Ushijima, Ken-Ichiiro Tanaka, Miho Takeda, Takashi Katsu, Shinji Mima, and Tohru Mizushima

Graduate School of Medical and Pharmaceutical Sciences, Kumamoto University, Kumamoto, Japan (H.U., K.-I.T., M.T., S.M., T.M.); and Graduate School of Medicine, Dentistry, and Pharmaceutical Sciences, Okayama University, Okayama, Japan (T.K.)

Received June 17, 2005; accepted July 26, 2005

ABSTRACT

Direct gastric mucosal cell damage mediated by nonsteroidal anti-inflammatory drugs (NSAIDs) is involved in the formation of NSAID-induced gastric lesions. We recently suggested that this direct cytotoxicity of NSAIDs is caused by their membrane-permeabilization activity. Geranylgeranylacetone (GGA), a clinically used antiulcer drug, can protect gastric mucosa against lesion formation mediated by NSAIDs. However, the mechanism by which this occurs is not fully understood. In this study, we show that GGA acts to stabilize membranes against NSAIDs. GGA suppressed NSAID-induced permeabilization of calcein-loaded liposomes and NSAID-induced stimulation of K^+-efflux across the cytoplasmic membrane in cells. GGA was effective even when coadministered with NSAIDs and was also able to restore membrane fluidity that had been compromised by NSAIDs. This mechanism seems to play an important role in the antiulcer activity of GGA.

Nonsteroidal anti-inflammatory drugs (NSAIDs) are of significant clinical value, accounting for nearly 5% of all prescribed medications (Smalley et al., 1995). Nonetheless, NSAID use is often associated with gastrointestinal complications (Hawkey, 2000), with approximately 15 to 30% of long-term users experiencing gastrointestinal ulcers and bleeding (Barrier and Hirschowitz, 1989; Fries et al., 1989; Kurata and Abbey, 1990; Gabriel et al., 1991). In the United States alone, approximately 16,500 people per year die as a result of these complications (Singh, 1998). Therefore, in general, antiulcer drugs are prescribed in combination with NSAIDs to prevent the NSAID-induced side effects.

Geranylgeranylacetone (GGA) was developed in Japan and has become the leading antiulcer drug on the Japanese market (Murakami et al., 1981). In both preclinical and clinical studies, it has been shown to protect the gastric mucosa against the development of lesions induced by various irritants, including NSAIDs, without affecting gastric acid secretion (Murakami et al., 1981; Terano et al., 1986; Pappas et al., 1987). Various mechanisms have been proposed for this protective effect of GGA. First, it stimulates the synthesis of mucus (Terano et al., 1986; Bilski et al., 1987; Rokutan et al., 2000) and increases mucosal blood flow, an important factor in maintaining the integrity of the mucosa (Kunisaki and Sugiyama, 1992). It has also been reported recently that GGA induces heat shock proteins (HSPs), a novel activity of GGA that has been shown to be involved in its ability to protect the gastric mucosa against NSAIDs (Hirakawa et al., 1996; Mizushima et al., 1999; Tomisato et al., 2001b; Takano et al., 2002). However, the rapid anti-ulcer activity of GGA against NSAIDs observed in clinical situations cannot be fully explained by these indirect actions of GGA, given that this ameliorating effect is observed even when GGA is coadministered with NSAIDs. Therefore, GGA is also believed to have unknown direct actions.

The anti-inflammatory action of NSAIDs is mediated through their inhibitory effect on cyclooxygenase (COX) activity. COX is an enzyme essential for the synthesis of prostaglandins (PGs), which have a strong capacity to induce inflammation. The inhibition of COX was believed to be the sole explanation for the gastric complications of NSAIDs, given that PGs exert a strong protective effect on gastric mucosa (Miller, 1983; Vane and Botting, 1996). However, it is now believed that the induction of gastric lesions by NSAIDs involves additional mechanisms, because the increased inci-

This work was supported by Grants-in-Aid for Scientific Research from the Ministry of Health, Labor, and Welfare of Japan, the Suzuken Memorial Foundation, the Tokyo Biochemical Research Foundation, Kumamoto Technology and Industry Foundation, and the Japan Research Foundation for Clinical Pharmacology

Article, publication date, and citation information can be found at http://molpharm.aspetjournals.org.
doi:10.1124/mol.105.015784.

ABBREVIATIONS: NSAID, nonsteroidal anti-inflammatory drug; GGA, geranylgeranylacetone; HSP, heat shock protein; COX, cyclooxygenase; PG, prostaglandin; PC, phosphatidylcholine.

dence of gastric lesions and the decrease in PG levels induced by NSAIDs do not always occur in parallel (Ligumsky et al., 1983, 1990). We have demonstrated previously that NSAIDs induce in vitro cell death (apoptosis and necrosis) independent of COX inhibition and have suggested that both COX inhibition and NSAID-induced cell death are required to produce gastric lesions in vivo (Tomisato et al., 2001a, 2004b). Furthermore, we have shown recently that all of the NSAIDs tested have membrane-permeabilization activity, which seems to be responsible for the NSAID-induced apoptosis and necrosis (Tomisato et al., 2004a). In this study, we have found that GGA protects membranes from permeabilization by NSAIDs. This is the first report showing that a clinically used antiulcer drug has membrane-stabilization activity in the presence of NSAIDs. We have also demonstrated that GGA restores the membrane fluidity that is compromised by NSAIDs.

Materials and Methods

Chemicals, Media, and Animals. Fetal bovine serum was purchased from Invitrogen (Carlsbad, CA). RPMI 1640 medium was obtained from Nissui (Tokyo, Japan). Indomethacin was purchased from Wako Pure Chemicals (Tokyo, Japan), whereas ibuprofen, diclofenac, mefenamic acid, flufenamic acid, and ketoprofen came from Sigma-Aldrich (Tokyo, Japan). Nimesulide and flurbiprofen were obtained from Cayman Chemical (Ann Arbor, MI), and egg phosphatidylcholine (PC) was from Kanto Chemicals Co. (Tokyo, Japan). GGA was kindly provided by Eisai Co. (Tokyo, Japan). Celecoxib was purchased from LKT Laboratories, Inc. (St. Paul, MN). Etodolac was a gift, kindly provided by Nippon Shinyaku Co. (Kyoto, Japan).

Treatment of Cells with NSAIDs. Human gastric carcinoma (AGS) cells were cultured in RPMI 1640 medium containing 10% fetal bovine serum. Cells were exposed to NSAIDs by replacement of the entire bathing medium with fresh medium containing the NSAID under investigation. NSAIDs were dissolved in dimethyl sulfoxide; control experiments (without NSAIDs) were performed in the presence of the same concentration of dimethyl sulfoxide.

Membrane Permeability Assay. Permeabilization of calcein-loaded liposomes was assayed as described previously (Tomisato et al., 2004a), with some modifications. Liposomes were prepared using the reversed-phase evaporation method. Egg PC (10 μmol, 7.7 mg) was dissolved in chloroform/methanol (1:2; v/v), dried, dissolved in 1.5 ml of diethyl ether, and added to 1 ml of 100 mM calcein-NaOH, pH 7.4. The mixture was then sonicated to obtain a homogenous emulsion. The diethyl ether solvent was removed, and the resulting suspension of liposomes was centrifuged and washed twice with fresh buffer A [10 mM phosphate buffer (Na_2HPO_4-NaH_2PO_4, pH 6.8) containing 150 mM NaCl] to remove untrapped calcein. The final liposome precipitate was resuspended in 5 ml of buffer A. A 30-μl aliquot of this suspension was diluted with buffer A up to 20 ml, and 400 μl of this diluted suspension was then incubated at 30°C for 10 min in the presence of the NSAID under investigation. The release of calcein from liposomes was determined by measuring fluorescence intensity at 520 nm (excitation at 490 nm).

Assay for K^+ Efflux from Cells. K^+ efflux from cells was monitored as described previously (Katsu et al., 1987), with some modifications. Cells were washed twice with buffer A and then suspended in fresh buffer A (2.4×10^6 cells/ml). After incubation with NSAIDs for 10 min at 37°C, K^+ efflux from the cells was measured with a K^+ ion-selective electrode.

Fluorescence Polarization. Membrane fluidity was measured using the fluorescence polarization technique (Makise et al., 2002). Diphenylhexatriene (1%, mol/mol, of egg PC) was used as a fluorescence probe. Liposomes were prepared using a reversed-phase evaporation method similar to that used in the membrane-permeability experiments, except for the addition of 1 ml of buffer A instead of 100 mM calcein-NaOH. Measurements were carried out using a Hitachi F-4500 fluorospectrophotometer (Hitachi Software Engineering, Yokohama, Japan). The degree of polarization (P) was calculated according to the following equation: $P = (I_{VV} - C_f I_{VH})/(I_{VV} + C_f I_{VH})$, where I is the fluorescence intensity and subscripts V and H refer, respectively, to the vertical and horizontal orientations of the excitation (first) and emission (second) polarizers. C_f ($= I_{HV}/I_{HH}$) is a correction factor.

Statistical Analyses. All results were expressed as the mean ± S.E.M. One-way analysis of variance followed by Scheffe's multiple comparison was used for the evaluation of differences between the groups. A Student's t test for unpaired results was performed to evaluate differences between two groups. Differences were considered significant for values of $P < 0.05$.

Results

GGA Suppresses NSAID-Induced Membrane Permeabilization. We have reported recently that some NSAIDs (celecoxib, mefenamic acid, flufenamic acid, nimesulide, and flurbiprofen) cause membrane permeabilization in calcein-loaded liposomes (Tomisato et al., 2004a). In this study, we first confirmed the membrane-permeabilization activity of a number of NSAIDs using the same assay. Calcein fluoresces very weakly at high concentrations caused by self-quenching, so the addition of membrane-permeabilizing drugs to a medium containing calcein-loaded liposomes should cause an increase in fluorescence by diluting the calcein (Tomisato et al., 2004a). As shown in Fig. 1, each of the NSAIDs tested increased the calcein fluorescence in a dose-dependent manner, indicating that they have membrane-permeabilization activity. Results for some NSAIDs were consistent with our previous reports (Tomisato et al., 2004a). Indomethacin, diclofenac, and celecoxib were selected for further study because their membrane-permeabilization activity was higher than that of the other NSAIDs.

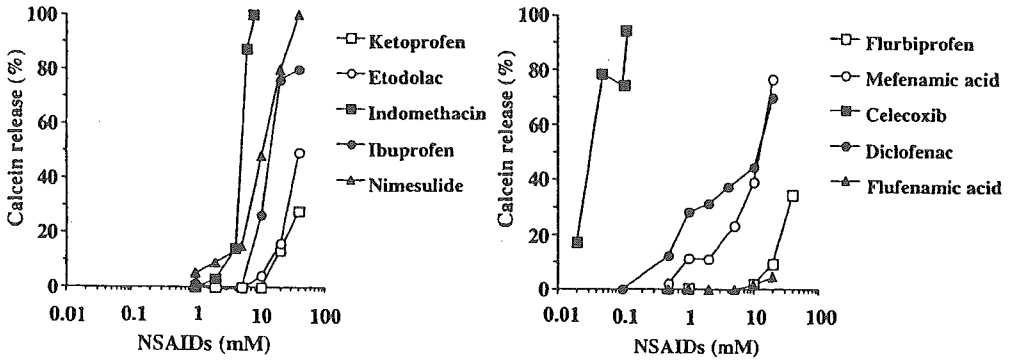

Fig. 1. Membrane permeabilization by NSAIDs. Calcein-loaded liposomes were incubated for 10 min with varying concentrations of each NSAID. The release of calcein from the liposomes was then determined by measuring fluorescence intensity. Melittin (10 μM) was used to establish the 100% level of membrane permeabilization (Katsu et al., 1987).

The effect of GGA on indomethacin-induced membrane permeabilization is illustrated in Fig. 2A. GGA decreased the calcein fluorescence in a dose-dependent manner in the presence of 6 or 8 mM indomethacin. Treatment with GGA had no effect on fluorescence when calcein-loaded liposomes were studied in the absence of indomethacin (data not shown). Furthermore, GGA did not directly affect calcein fluorescence (data not shown). These results suggested that GGA was protecting the liposome membranes from permeabilization by indomethacin. As shown in Fig. 2, B and C, GGA also protected liposome membranes against diclofenac and celecoxib, although relatively higher concentrations of GGA (greater than 10^{-5} M) were required in the case of celecoxib.

In vivo, gastric mucosa can be exposed not only to NSAIDs but also to various other lesion-inducing irritants (such as ethanol, gastric acid, and reactive oxygen species) against which GGA provides protection. We therefore examined the membrane-permeabilization activity of these irritants using the same assay. Ethanol, but not hydrochloric acid or hydrogen peroxide, showed membrane-permeabilization activity under our assay conditions (data not shown). As shown in Fig. 2D, GGA protected liposome membranes from permeabilization by 10 or 20% ethanol, suggesting that the effect of GGA is nonspecific.

GGA Protects Membranes against NSAIDs even when Coadministered. As for the experiments described above, calcein-loaded liposomes were preincubated with GGA and were subsequently treated with various NSAIDs in the presence of the same concentration of GGA, as in the preincubation step. As shown in Fig. 3A, GGA suppressed the indomethacin-induced membrane permeabilization under these conditions ("pretreated" in Fig. 3A). However, a similar result was obtained even when GGA was added simultaneously with indomethacin ("cotreated" in Fig. 3A). Treatment with GGA and either diclofenac or celecoxib (Fig. 3, B and C) also produced a similar outcome. These results showed that GGA very rapidly protects liposome membranes against NSAIDs. In contrast, GGA did not significantly affect the calcein fluorescence when it was added after NSAID-treatment ("post-treated" in Fig. 3), again supporting the notion that the activity of GGA in this paradigm cannot be explained by its direct effect on calcein fluorescence.

GGA Protects Cell Membranes from NSAID-Mediated K^+ Efflux. We next examined whether GGA can protect cell membranes against NSAIDs. Permeabilization of cytoplasmic membranes should stimulate K^+ efflux from cells. Here we examined the effect of various NSAIDs on K^+ efflux from AGS cells. The K^+ concentration in the medium increased depending on the dose of not only celecoxib but also indomethacin or diclofenac (Fig. 4), showing that each of these NSAIDs stimulated K^+ efflux from the cells or, in other words, permeabilized the cytoplasmic membranes. As shown in Fig. 5, the increase in K^+ concentration in the medium was not as great in the presence of GGA. GGA alone had no effect (data not shown). These findings suggest that GGA protects the cytoplasmic membrane from permeabilization by NSAIDs.

GGA Increases Membrane Fluidity. We have recently reported that some NSAIDs (celecoxib, mefenamic acid, flufenamic acid, nimesulide, and flurbiprofen) decrease membrane fluidity (Tomisato et al., 2004a). Here, we examined the effect of GGA on membrane fluidity in the presence or

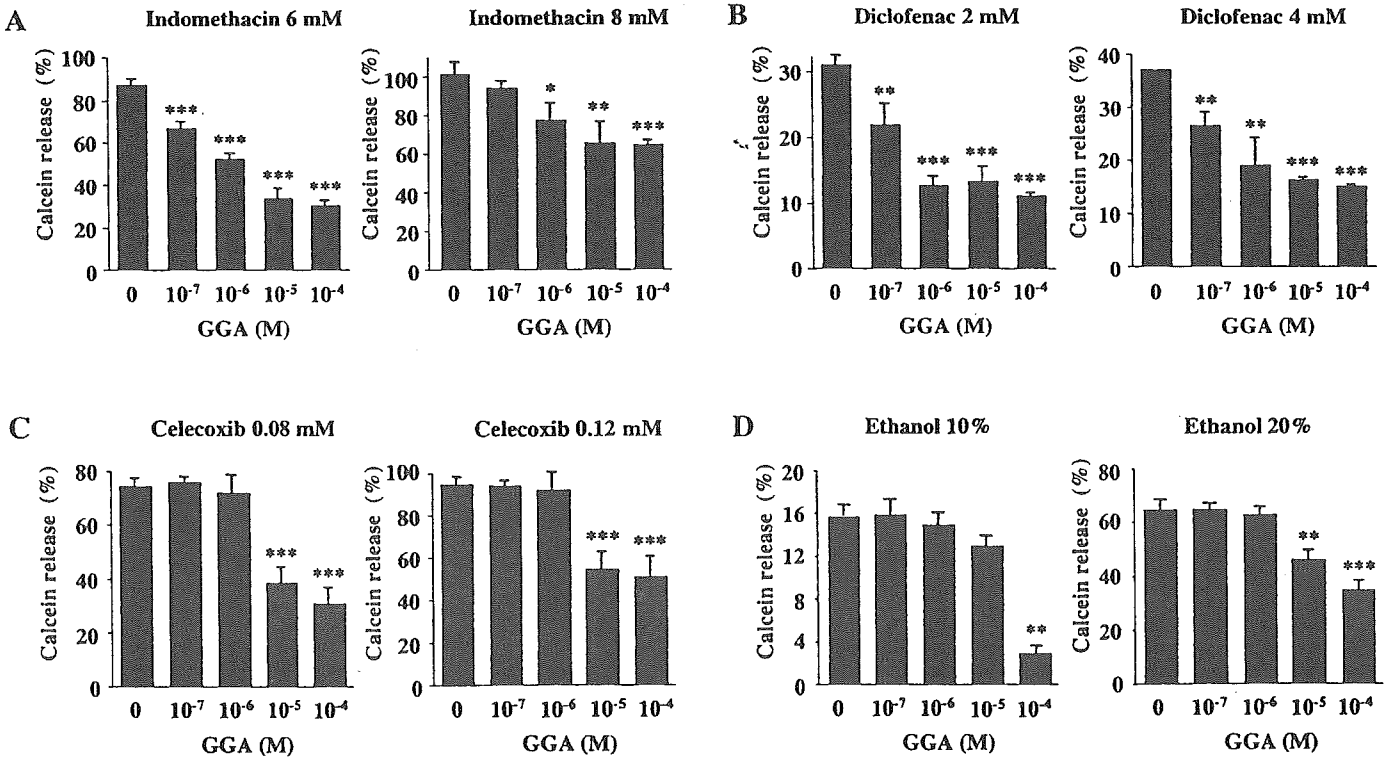

Fig. 2. Effect of GGA on membrane permeabilization. Calcein-loaded liposomes were preincubated with varying concentrations of GGA for 10 min and then treated with NSAID (A–C) or ethanol (D) in the presence of the same concentration of GGA. The release of calcein from liposomes was determined and expressed as described in the legend of Fig. 1. Values shown are mean ± S.E.M. (n = 3). ***, $P < 0.001$; **, $P < 0.01$; *, $P < 0.05$.

absence of NSAIDs using the fluorescence polarization technique. In such experiments, the higher the calculated *P* value, the lower the membrane fluidity. We first examined the effect of various NSAIDs on the membrane fluidity of PC liposomes. As described previously (Tomisato et al., 2004a), celecoxib increased the *P* value (i.e., decreased the membrane fluidity) (Table 1). Indomethacin and diclofenac had a similar effect, although the extent of the decrease differed between NSAIDs (Table 1). In contrast, GGA decreased the *P* value in a dose-dependent manner, reflecting an increase in membrane fluidity (Table 1).

We next examined the effect of GGA on membrane fluidity in the presence of NSAIDs. Membrane fluidity in the presence of various concentrations of celecoxib was restored by GGA in a dose-dependent manner (Table 1). GGA (10^{-4} M) also partially restored membrane fluidity in the presence of 2 mM indomethacin or diclofenac but had no effect in the presence of 1 mM indomethacin or 5 or 10 mM diclofenac.

Discussion

In this study, we have shown that GGA suppresses NSAID-induced K^+ efflux from cells, suggesting that GGA protects the cytoplasmic membranes from permeabilization. Because a similar effect was observed in calcein-loaded liposomes, which consist only of phospholipids (without membrane proteins), the membrane stabilization activity of GGA seems to be mediated by its direct interaction with phospholipids, a conclusion supported by the observation that GGA increases the membrane fluidity of PC liposomes. This is the first report that a clinically used antiulcer drug protects membranes from permeabilization by NSAIDs and other gastric irritants (ethanol). GGA is clinically used at 150 mg/day. The maximum serum concentration under this administration is approximately 5 μM (5×10^{-6} M) in patients (data from interview form from the manufacturer). The maximum concentration of GGA at gastric mucosa should be higher,

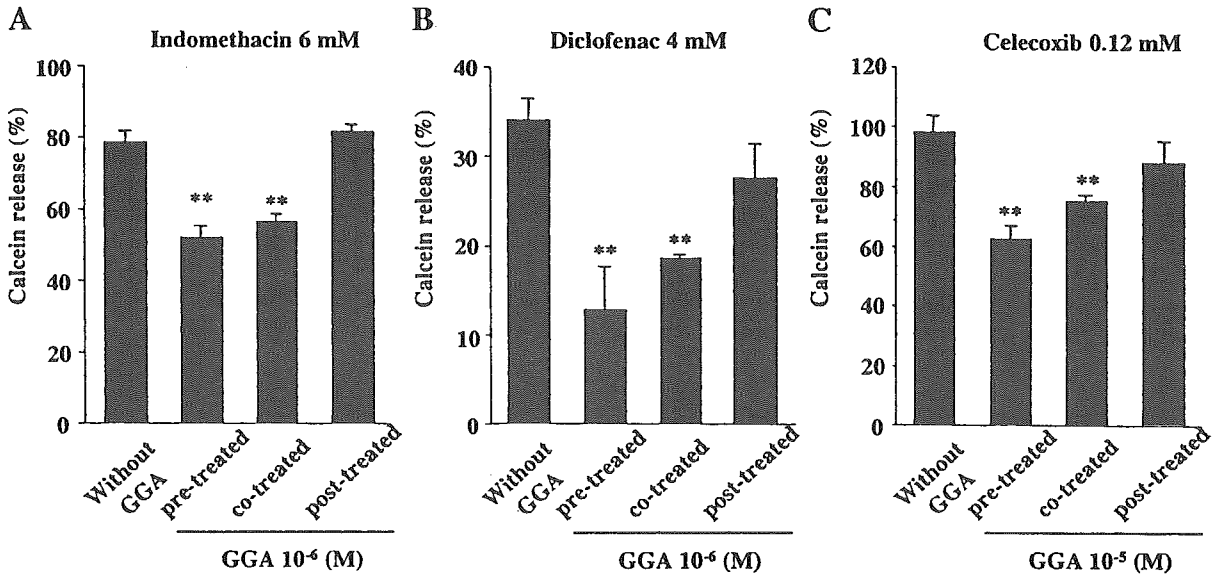

Fig. 3. Rapid protection of membranes by GGA. Calcein-loaded liposomes were either preincubated with GGA for 10 min and then incubated with NSAID in the presence of GGA (pretreated), simultaneously incubated with GGA and NSAID (cotreated), or preincubated with NSAID for 10 min and then treated with GGA in the presence of NSAID (post-treated). The release of calcein from liposomes was determined and expressed as described in the legend to Fig. 1. Values shown are mean ± S.E.M. (n = 3). **, $P < 0.01$.

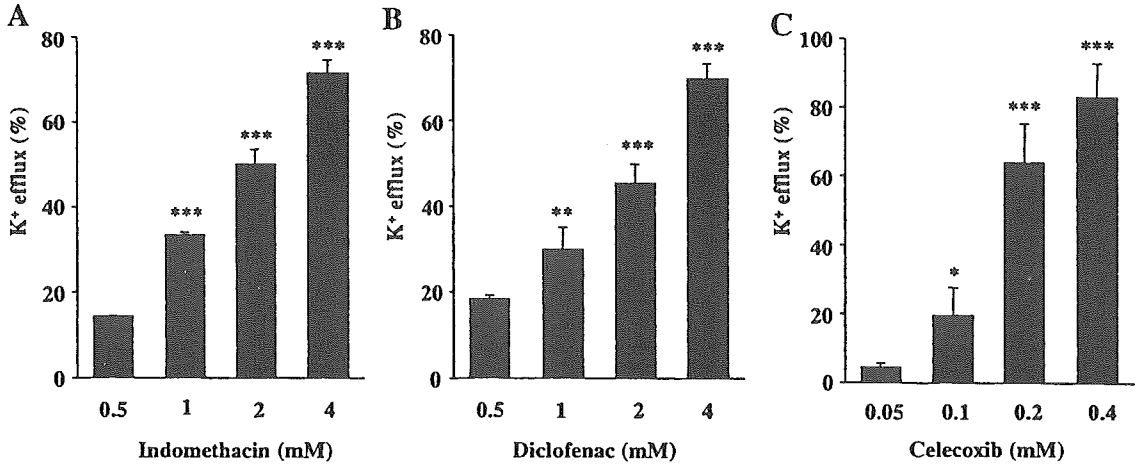

Fig. 4. Stimulation of K^+ efflux from cells by NSAIDs. AGS cells were incubated with varying concentrations of each NSAID for 10 min, and the level of K^+-efflux was measured using a K^+ ion-selective electrode. Melittin (10 μM) was used to establish the 100% level of K^+ efflux (Katsu et al., 1987). Values shown are mean ± S.E.M. (n = 3). *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

suggesting that concentrations of GGA used in this study are clinically significant.

From our previous studies (see below), we consider that this novel activity of GGA is involved in its antiulcer activity against NSAIDs. We recently proposed that both COX inhibition at the gastric mucosa and direct gastric mucosal cell damage (necrosis and apoptosis in gastric mucosal cells) are required for the production of gastric lesions by NSAIDs in vivo; in this experimental paradigm, gastric lesions developed in a manner that depended on both an intravenously administered low dose of indomethacin (inhibition of COX activity at the gastric mucosa without direct gastric mucosal cell damage) and an orally administered cytotoxic COX-2–selective NSAID, such as celecoxib (direct gastric mucosal cell damage without inhibition of COX) (Tomisato et al., 2004b). We subsequently suggested that the direct gastric mucosal cell damage is caused by the membrane permeabilization activity of NSAIDs; the ED_{50} values of the 10 NSAIDs for gastric mucosal cell death (concentrations of NSAID required for 50% inhibition of cell viability by necrosis or apoptosis) correlated well with the ED_{20} values for membrane permeabilization (concentration of NSAID required for 20% release of calcein); plotting ED_{50} values for necrosis or apoptosis versus ED_{20} values for membrane permeabilization yielded an r^2 value of 0.94 or 0.93, respectively (Tomisato et al., 2004a; Tanaka et al., 2005). We therefore consider that the membrane stabilization activity of GGA causes suppression of NSAID-induced direct gastric mucosal cell damage, conferring protection against the development of ulcers. This raises the possibility that the membrane stabilization assay can be used as a rapid screening technique for potential new antiulcer drugs.

As outlined in the Introduction, GGA has a number of pharmacological activities that are believed to be involved in its antiulcer activity. These include stimulating the synthesis of gastric mucus, increasing gastric mucosal blood flow, and inducing HSPs in gastric mucosal cells (Terano et al., 1986; Bilski et al., 1987; Kunisaki and Sugiyama, 1992; Hirakawa et al., 1996; Mizushima et al., 1999; Tomisato et al., 2000; Takano et al., 2002). However, these activities cannot be

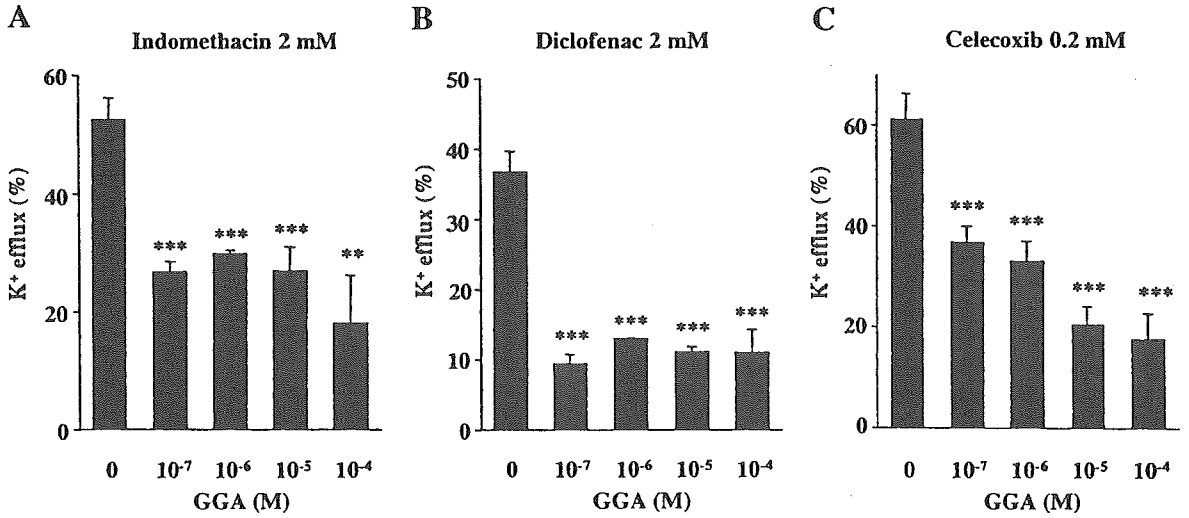

Fig. 5. Effect of GGA on K^+ efflux from cells in the presence of NSAIDs. AGS cells were preincubated with varying concentrations of GGA and then treated with NSAID in the presence of the same concentrations of GGA. The level of K^+ efflux was measured and expressed as described in the legend to Fig. 4. Values shown are mean ± S.E.M. (n = 3). **, $P < 0.01$; ***, $P < 0.001$.

TABLE 1

Effect of GGA on membrane fluidity in the presence or absence of various NSAIDs

The degree of polarization of PC liposomes in the presence of GGA and/or various NSAIDs was measured as described under *Materials and Methods*. The final lipid concentration was adjusted to 30 μ M. Fluorescence polarization was measured by excitation at 360 nm and emission at 430 nm using a Hitachi F-4500 fluorospectrophotometer equipped with polarizers and thermoregulated cells. Values shown are mean ± S.E.M. (n = 3).

NSAIDs	Degree of Polarization		
	PC	+ GGA 10^{-5}	+ GGA 10^{-4}
	M		
Control	0.121 ± 0.007	0.097 ± 0.005**	0.071 ± 0.003***
Indomethacin			
1 mM	0.172 ± 0.033	0.165 ± 0.037	0.170 ± 0.020
2 mM	0.217 ± 0.017	0.177 ± 0.021	0.165 ± 0.024*
Diclofenac			
2 mM	0.149 ± 0.012	0.153 ± 0.008	0.114 ± 0.007*
5 mM	0.151 ± 0.012	0.152 ± 0.010	0.154 ± 0.010
10 mM	0.159 ± 0.015	0.167 ± 0.010	0.160 ± 0.014
Celecoxib			
0.1 mM	0.133 ± 0.006	0.113 ± 0.004**	0.080 ± 0.007***
0.5 mM	0.210 ± 0.006	0.175 ± 0.005***	0.148 ± 0.010***
1 mM	0.210 ± 0.009	0.187 ± 0.005*	0.159 ± 0.006***

* $P < 0.05$.
** $P < 0.01$.
*** $P < 0.001$.

measured experimentally without an initial incubation period (for example, induction of HSPs by GGA requires at least 1 h incubation both in vitro and in vivo) (Hirakawa et al., 1996). In contrast, in clinical situations, GGA can suppress gastric lesions even when administered simultaneously with NSAIDs, suggesting a more direct protective mechanism, such as the membrane stabilization proposed here. Nonetheless, longer-term indirect actions of GGA may also play a role in its antiulcer activity and that the different time courses of these effects could confer a clinical advantage.

In the present study, we also demonstrated that GGA restores membrane fluidity that has been compromised by NSAIDs. At present, it is not certain that this activity of GGA underpins its membrane-stabilizing ability. Nor is the relationship between decreased membrane fluidity and NSAID-induced membrane permeabilization clear, given that we have shown previously that most but not all of the NSAIDs tested (mefenamic acid, flufenamic acid, celecoxib, and nimesulide, but not flurbiprofen) decrease membrane fluidity, and that cholesterol, which ameliorates the NSAID-induced decrease in membrane fluidity, renders liposomes resistant to some but not all NSAIDs (Tomisato et al., 2004a). Restoration of membrane fluidity by GGA also differed between NSAIDs (Table 1), suggesting that this effect cannot fully explain the membrane stabilization activity of GGA. However, if holes develop in membranes, such holes become more stable (in other words, the membrane becomes more permeable) when membrane fluidity decreases. It is also possible that a GGA-mediated increase in membrane fluidity is involved in the maintenance of surface hydrophobicity at the gastric mucosa, which is believed to be important for maintaining mucosal integrity. Lichtenberger and his coworkers have proposed that NSAIDs disrupt the hydrophobic barrier properties of the gastric mucosal surface, rendering it susceptible to attack by luminal acid. They showed that NSAIDs cause a marked decrease in surface hydrophobicity and observed a close relationship between a decrease in gastric surface hydrophobicity and gastric lesion score in rats (Darling et al., 2004; Lichtenberger et al., 1995). They also suggested that an NSAID-induced decrease in membrane fluidity is related to the decrease in surface hydrophobicity mediated by NSAIDs (Giraud et al., 1999). However, further studies are necessary to elucidate the effect of GGA on membrane fluidity and its influences on antiulcer activity.

References

Barrier CH and Hirschowitz BI (1989) Controversies in the detection and management of nonsteroidal antiinflammatory drug-induced side effects of the upper gastrointestinal tract. *Arthritis Rheum* **32**:926–932.

Bilski J, Sarosiek J, Murty VL, Aono M, Moriga M, Slomiany A, and Slomiany BL (1987) Enhancement of the lipid content and physical properties of gastric mucus by geranylgeranylacetone. *Biochem Pharmacol* **36**:4059–4065.

Darling RL, Romero JJ, Dial EJ, Akunda JK, Langenbach R, and Lichtenberger LM (2004) The effects of aspirin on gastric mucosal integrity, surface hydrophobicity and prostaglandin metabolism in cyclooxygenase knockout mice. *Gastroenterology* **127**:94–104.

Fries JF, Miller SR, Spitz PW, Williams CA, Hubert HB, and Bloch DA (1989) Toward an epidemiology of gastropathy associated with nonsteroidal antiinflammatory drug use. *Gastroenterology* **96**:647–655.

Gabriel SE, Jaakkimainen L, and Bombardier C (1991) Risk for serious gastrointestinal complications related to use of nonsteroidal anti-inflammatory drugs. A meta-analysis. *Ann Intern Med* **115**:787–796.

Giraud MN, Motta C, Romero JJ, Bommelaer G, and Lichtenberger LM (1999) Interaction of indomethacin and naproxen with gastric surface-active phospholipids: a possible mechanism for the gastric toxicity of nonsteroidal anti-inflammatory drugs (NSAIDs). *Biochem Pharmacol* **57**:247–254.

Hawkey CJ (2000) Nonsteroidal anti-inflammatory drug gastropathy. *Gastroenterology* **119**:521–535.

Hirakawa T, Rokutan K, Nikawa T, and Kishi K (1996) Geranylgeranylacetone induces heat shock proteins in cultured guinea pig gastric mucosal cells and rat gastric mucosa. *Gastroenterology* **111**:345–357.

Katsu T, Kobayashi H, Hirota T, Fujita Y, Sato K, and Nagai U (1987) Structure-activity relationship of gramicidin S analogues on membrane permeability. *Biochim Biophys Acta* **899**:159–170.

Kunisaki C and Sugiyama M (1992) Effect of teprenone on acute gastric mucosal lesions induced by cold-restraint stress. *Digestion* **53**:45–53.

Kurata JH and Abbey DE (1990) The effect of chronic aspirin use on duodenal and gastric ulcer hospitalizations. *J Clin Gastroenterol* **12**:260–266.

Lichtenberger LM, Wang ZM, Romero JJ, Ulloa C, Perez JC, Giraud MN, and Barreto JC (1995) Non-steroidal anti-inflammatory drugs (NSAIDs) associate with zwitterionic phospholipids: insight into the mechanism and reversal of NSAID-induced gastrointestinal injury. *Nat Med* **1**:154–158.

Ligumsky M, Golanska EM, Hansen DG, and Kauffman GJ (1983) Aspirin can inhibit gastric mucosal cyclo-oxygenase without causing lesions in rat. *Gastroenterology* **84**:756–761.

Ligumsky M, Sestieri M, Karmeli F, Zimmerman J, Okon E, and Rachmilewitz D (1990) Rectal administration of nonsteroidal antiinflammatory drugs. Effect on rat gastric ulcerogenicity and prostaglandin E2 synthesis. *Gastroenterology* 1245–1249.

Makise M, Mima S, Katsu T, Tsuchiya T, and Mizushima T (2002) Acidic phospholipids inhibit the DNA-binding activity of DnaA protein, the initiator of chromosomal DNA replication in Escherichia coli. *Mol Microbiol* **46**:245–256.

Miller TA (1983) Protective effects of prostaglandins against gastric mucosal damage: current knowledge and proposed mechanisms. *Am J Physiol* **245**:G601–G623.

Mizushima T, Tsutsumi S, Rokutan K, and Tsuchiya T (1999) Suppression of ethanol-induced apoptotic DNA fragmentation by geranylgeranylacetone in cultured guinea pig gastric mucosal cells. *Dig Dis Sci* **44**:510–514.

Murakami M, Oketani K, Fujisaki H, Wakabayashi T, and Ohgo T (1981) Antiulcer effect of geranylgeranylacetone, a new acyclic polyisoprenoid on experimentally induced gastric and duodenal ulcers in rats. *Arzneimittelforschung* **31**:799–804.

Pappas TN, Mulvihill SJ, Goto Y, and Debas HT (1987) Advances in drug therapy for peptic ulcer disease. *Arch Surg* **122**:447–450.

Rokutan K, Teshima S, Kawai T, Kawahara T, Kusumoro K, Mizushima T, and Kishi K (2000) Geranylgeranylacetone stimulates mucin synthesis in cultured guinea pig gastric pit cells by inducing a neuronal nitric oxide synthase. *J Gastroenterol* **35**:673–681.

Singh G (1998) Recent considerations in nonsteroidal anti-inflammatory drug gastropathy. *Am J Med* **105**:31S–38S.

Smalley WE, Ray WA, Daugherty JR, and Griffin MR (1995) Nonsteroidal anti-inflammatory drugs and the incidence of hospitalizations for peptic ulcer disease in elderly persons. *Am J Epidemiol* **141**:539–545.

Takano T, Tsutsumi S, Tomisato W, Hoshino T, Tsuchiya T, and Mizushima T (2002) Geranylgeranylacetone protects guinea pig gastric mucosal cells from gastric stressor-induced apoptosis. *Dig Dis Sci* **47**:1546–1553.

Tanaka K, Tomisato W, Hoshino T, Ishihara T, Namba T, Aburaya M, Katsu T, Suzuki K, Tsutsumi S, and Mizushima T (2005) Involvement of intracellular Ca^{2+} levels in nonsteroidal anti-inflammatory drug-induced apoptosis. *J Biol Chem* **280**:31059–31067.

Terano A, Hiraishi H, Ota S, and Sugimoto T (1986) Geranylgeranylacetone, a novel anti-ulcer drug, stimulates mucus synthesis and secretion in rat gastric cultured cells. *Digestion* **33**:206–210.

Tomisato W, Takahashi N, Komoto C, Rokutan K, Tsuchiya T, and Mizushima T (2000) Geranylgeranylacetone protects cultured guinea pig gastric mucosal cells from indomethacin. *Dig Dis Sci* **45**:1674–1679.

Tomisato W, Tanaka K, Katsu T, Kakuta H, Sasaki K, Tsutsumi S, Hoshino T, Aburaya M, Li D, Tsuchiya T, Suzuki K, et al. (2004a) Membrane permeabilization by non-steroidal anti-inflammatory drugs. *Biochem Biophys Res Commun* **323**:1032–1039.

Tomisato W, Tsutsumi S, Hoshino T, Hwang HJ, Mio M, Tsuchiya T, and Mizushima T (2004b) Role of direct cytotoxic effects of NSAIDs in the induction of gastric lesions. *Biochem Pharmacol* **67**:575–585.

Tomisato W, Tsutsumi S, Rokutan K, Tsuchiya T, and Mizushima T (2001a) NSAIDs induce both necrosis and apoptosis in guinea pig gastric mucosal cells in primary culture. *Am J Physiol* **281**:G1092–G1100.

Tomisato W, Tsutsumi S, Tsuchiya T, and Mizushima T (2001b) Geranylgeranylacetone protects guinea pig gastric mucosal cells from gastric stressor-induced necrosis by induction of heat-shock proteins. *Biol Pharm Bull* **24**:887–891.

Vane JR and Botting RM (1996) Mechanism of action of anti-inflammatory drugs. *Scand J Rheumatol Suppl* **102**:9–21.

Address correspondence to: Dr. Tohru Mizushima, Graduate School of Medical and Pharmaceutical Sciences, Kumamoto University, 5-1 Oe-honmachi, Kumamoto 862-0973, Japan. E-mail: mizu@gpo.kumamoto-u.ac.jp