Journal of Cellular Biochemistry 81:93–101 (2001)

Role of Pertussis Toxin-Sensitive G-proteins in Intracellular Ca^{2+} Release and Apoptosis Induced by Inhibiting Cystic Fibrosis Transmembrane Conductance Regulator (CFTR) Cl^- Channels in HepG2 Human Hepatoblastoma Cells

Jung-Ae Kim,[1] Young Shin Kang,[2] Sun Hee Lee,[2] Eun-Hee Lee,[1] and Yong Soo Lee[2]*

[1]College of Pharmacy, Yeungnam University, Korea
[2]Department of Physiology, College of Medicine, Kwandong University, Korea

Abstract Previously, we have reported that inhibition of cystic fibrosis transmembrane conductance regulator (CFTR) Cl^- channels by glibenclamide induced intracellular Ca^{2+} release from IP_3-sensitive stores and apoptosis in HepG2 human hepatoblastoma cells (Kim JA, Kang YS, Lee SH, Lee EH, Yoo BH, Lee YS. 1999. *Biochem Biophys Res Commun* 261:682–688). In this study we investigated the upstream signals involved in the mechanism of these actions of glibenclamide. Treatment with glibenclamide initiated production of inositol 1,4,5-trisphosphate (IP_3) in a dose- and time-dependent manner. The glibenclamide-induced formation of IP_3 was significantly inhibited by CFTR activators (levamisole and bromotetramisole). The intracellular Ca^{2+} release and apoptosis induced by glibenclamide were significantly suppressed by treatment with phospholipase C (PLC) inhibitors (U-73122 and manoalide) or by pretreatment with pertussis toxin (PTx). In addition, PTx-catalyzed ADP-ribosylation of GTP-binding proteins (G-proteins) was markedly enhanced by treatment with glibenclamide in a time-dependent manner. Taken together, these results suggest that PTx-sensitive G-proteins coupled to PLCβ may mediate the intracellular Ca^{2+} release and apoptosis induced by inhibiting CFTR Cl^- channels in HepG2 cells. These results further suggest that the PTx-sensitive G-proteins may be a valuable target for the therapeutic intervention of human hepatomas. J. Cell. Biochem. 81:93–101, 2001.

Key words: G-proteins; phospholipase C; cystic fibrosis transmembrane conductance regulator; apoptosis; intracellular Ca^{2+}; glibenclamide; pertussis toxin

Apoptosis is a highly organized cell death process characterized by early and prominent condensation of nuclear chromatin, loss of plasma membrane phospholipid asymmetry, activation of proteases and endonucleases, enzymatic cleavage of the DNA into oligonucleosomal fragments, and segmentation of the cells into membrane-bound apoptotic bodies [Kidd, 1998]. Apoptosis plays an important role in the maintenance of tissue homeostasis by the selective elimination of excessive cells [Song and Steller, 1999]. Particularly, genetic mutations resulting in the disruption of apoptosis or derangement of apoptosis-signaling pathways are likely to be critical components of carcinogenesis [Wang, 1999; Lowe and Lin, 2000]. In addition, apoptosis induction of cancer cells appears to be useful for cancer treatment [Kornblau, 1998] including chemotherapy [Kamesaki, 1998] and radiation therapy [Crompton, 1998]. However, signaling pathways for the induction of apoptosis are not completely understood.

Cystic fibrosis transmembrane conductance regulator (CFTR) proteins are expressed in epithelial cells that line organs such as the

Grant sponsor: Korea Research Foundation; Grant number: 1999-015-DP0341.

*Correspondence to: Yong Soo Lee, Ph.D., Department of Physiology, College of Medicine, Kwandong University, Kangnung 210-701, Korea. E-mail: yslee@mail.kwandong.ac.kr

Received 17 March 2000; Accepted 30 August 2000

This article published online in Wiley InterScience, December XX, 2000.

lungs and intestines, and act as Cl^- channels, thereby regulating the water and salt balance in these cells [Kelley et al., 1992]. CFTR is mutated in cystic fibrosis (CF) patients, which results in malfunction in Cl^- secretions, leading to numerous complications including serious lung infections, and ultimately death of these patients [Aitken, 1996]. Recently, we have reported that inhibition of CFTR Cl^- channels by glibenclamide [Sheppard and Robinson, 1997] induces apoptosis in HepG2 human hepatoblastoma cells [Kim et al., 1999a]. In previous research done by our laboratory [Kim et al., 1999a], we have also demonstrated that the apoptosis induced by glibenclamide is due to a sustained increase in the level of intracellular Ca^{2+}. The source of Ca^{2+} mediating the apoptotic events following glibenclamide treatment was only the internal release from inositol 1,4,5-trisphosphate (IP_3)-sensitive stores. This conclusion was based upon the fact that the Ca^{2+} increase was not altered by EGTA, an extracellular Ca^{2+} chelator, but completely inhibited by dantrolene or TMB-8, blockers of Ca^{2+} release from IP_3-dependent pools. In addition, these blockers completely suppressed the apoptosis induced by glibenclamide. However, the mechanisms by which CFTR inhibition perturbs intracellular Ca^{2+} homeostasis, and in turn induces apoptosis, are currently unknown.

Thus, the main purpose of the present study was to determine the upstream mechanism of intracellular Ca^{2+} changes by inhibition of CFTR Cl^- channels. To this end, we investigated (1) whether phospholipase C (PLC) whose activation releases IP_3 [Exton, 1997], is involved in the glibenclamide-induced intracellular Ca^{2+} release, and (2) whether GTP-binding proteins (G-proteins) are possibly involved in the PLC-Ca^{2+} signaling pathway activated by glibenclamide in HepG2 cells.

MATERIALS AND METHODS

Materials

The powders for Eagle's minimum essential medium (MEM) and Earle's basal salt solution (EBSS), trypsin solution, levamisole, manoalide, sodium pyruvate, probenecid, propidium iodide (PI), ribonuclease A and all salt powders were obtained from Sigma Chemical Co. (St. Louis, MO). Bromotetramisole was from Alex Bichem. (San Diego, CA). Glibenclamide, diazoxide, pinacidil, pertussis toxin (PTx) and 1-(6-((17β-3-methoxyestra-1,3,5(10)-trien-17-yl) amino)hexyl)-1H-pyrrole-2,5,-dione (U-73122) were from RBI (Natick, MA). 1-(2,5-Carboxyoxazol-2-yl-6-aminobenzfuran-5-oxyl)-2-(2′-amino- methylphenoxy)-ethane-*N,N,N′,N′*-tetraacetoxylmethyl ester (Fura-2/AM) was from Molecular Probes, Inc. (Eugene, OR). [α-^{32}P]Nicotinamide adenine dinucleotide (NAD) and *myo*-[2-^{3}H]inositol were from DuPont NEN (Boston, MA) and Amersham Pharmacia Biotech, respectively. Fetal bovine serum (FBS) and antibiotics (penicillin and streptomycin mixture) were purchased from GIBCO (Grand Island, NY). The stock solutions of drugs were sterilized by filtration through 0.2 μm disc filters (Gelman Sciences: Ann Arbor, MI).

Cell Lines and Cell Culture

The HepG2 human hepatoblastoma cell line was purchased from American Type Culture Collection (Rockville, MA). Cells were grown at 37°C in a humidified incubator under 5% CO_2/95% air in a MEM supplemented with 10% FBS, 1 mM sodium pyruvate and 200 IU/ml penicillin and 200 μg/ml of streptomycin. The culture medium was replaced every other day. After attaining confluence the cells were subcultured following trypsinization with 0.25% trypsin–EDTA solution.

Determination of Inositol Trisphosphate

Cells were labeled by incubating with MEM containing *myo*-[2-^{3}H]inositol (1 μCi/ml) for 48 h. Thereafter, the medium was removed and the cells were washed twice with HEPES-MED to remove free [^{3}H]inositol. Cells were incubated for 30 min in HEPES-MEM containing 10 mM LiCl. The cells were further incubated for a designated time in the absence or the presence of drugs. Reaction was stopped by the addition of 0.5 ml ice-cold methanol. After cells were harvested, phosphoinositides were separated using ion exchange chromatography [Berridge et al., 1983]; inositol monophosphate (IP_1), inositol diphosphate (IP_2) and inositol trisphosphate (IP_3) were serially eluted with 0.1 M formic acid, 0.2 M ammonium formate; 0.1 M formic acid, 0.5 M ammonium formate; and 0.1 M formic acid, and 1 M ammonium formate, respectively. Production of [^{3}H]IP_3 was measured by liquid scintillation counting.

Flow Cytometry Assays

For flow cytometry analysis, HepG2 cells were collected and washed twice with PBS buffer (pH 7.4). After fixing in 80% ethanol for 30 min, cells were washed twice, and resuspended in PBS buffer (pH 7.4) containing 0.1% Triton X-100, 5 μg/ml PI and 50 μg/ml ribonuclease A for DNA staining. Cells were then analyzed by a FACScan (BIO-RAD, Hercules, CA). At least 20,000 events were evaluated. All histograms were analyzed using WinBryte software (BIO-RAD) to determine the percentage of nuclei with hypodiploid content indicative of apoptosis [Bombeli et al., 1997].

Intracellular Ca^{2+} Measurement

Aliquots of the HepG2 cells were washed in EBSS. Then, 5 μM Fura-2/AM was added, and the cells were incubated for 30 min at 37°C. Unloaded Fura-2/AM was removed by centrifugation at $150 \times g$ for 3 min. The cells were resuspended at a density of 2×10^6 cells/ml in Krebs–Ringer buffer containing 125 mM NaCl, 5 mM KCl, 1.3 mM $CaCl_2$, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4$, 5 mM $NaHCO_3$, 25 mM HEPES, 6 mM glucose, and 2.5 mM probenecid (pH 7.4). Fura-2/AM-loaded cells were maintained at 25°C for 90 min before fluorescence measurement. For each experiment, a 0.5 ml aliquot of Fura-2/AM-loaded cells was equilibrated to 37°C in a stirred quartz cuvette. Fluorescence emission (510 nm) was monitored with the excitation wavelength cycling between 340 and 380 nm using a Hitachi F4500 fluorescence spectrophotometer. At the end of an experiment, fluorescence maximum and minimum values at each excitation wavelength were obtained by lysis of cells with 20 μg/ml digitonin (maximum) and then adding 10 mM EGTA (minimum). With the maximum and minimum values, the 340:380 nm fluorescence ratios were converted into free Ca^{2+} concentrations using a software, F-4500 Intracellular Cation Measurement System, provided by Hitachi.

Preparation of Plasma Membranes

For the preparation of plasma membranes, cells were harvested, washed twice with PBS (pH 7.4), and homogenized with a hand-held type homogenizer in ice-cold TE buffer (25 mM Tris–HCl, 0.1 mM EDTA, pH 7.5) containing protease inhibitors (1 mM PMSF, 10 μg/ml pepstatin A, 10 μg/ml leupeptin and 1 μg/ml aprotinin) at 4°C. The homogenate suspension was centrifuged at $500 \times g$ for 10 min, and the pellet containing nuclei and unbroken cells was discarded. The supernatant was spun at $40{,}000 \times g$ for 10 min. The supernatant and the homogenized pellet were recombined, centrifuged at $40{,}000 \times g$ for 60 min, and the pellet was resuspended in 25 mM Tris buffer containing the protease inhibitors to a final protein concentration between 2 and 4 mg/ml, and stored at −80°C.

ADP Ribosylation of G-proteins in Membranes

ADP-ribosylation of G-protein substrates was assayed using PTx-catalyzed incorporation of [^{32}P]-ADP-ribose from [^{32}P]NAD. PTx was preactivated by incubation of 50 mM Tris–HCl buffer (pH 7.6) containing 20 mM DTT and 0.1 mM ATP for 30 min at 37°C. Membranes (2 mg protein/ml) were resuspended in a 30 μl of ADP-ribosylation cocktail containing 20 μg/ml activated PTx, 20 mM Tris–HCl (pH 7.6), 2 mM ATP, 6 mM $MgCl_2$, 2 mM EDTA, 2 mM DTT, 2 μM NAD, 200 μM GTP, 20 mM thymidine, and 200 μCi/ml [α-^{32}P]NAD, and incubated for 60 min at 37°C. The ADP-ribosylation reactions were stopped by ice-cold 10% TCA. The mixture was centrifugated at $12{,}000 \times g$ at 4°C for 20 min. The pellet was resuspended in 10 μl of Laemmli buffer, boiled for 5 min and applied to 11% SDS-PAGE, and the gels were autoradiographed.

Data Analysis

All experiments were performed four times. Data were expressed as mean ± standard error of the mean (SEM) and were analyzed using one-way analysis of variance (ANOVA) and Student–Newman–Keul's test for individual comparisons. *P*-values less than 0.05 are considered statistically significant.

RESULTS

Glibenclamide Produces IP_3 Through Inhibition of CFTR Cl^- Channels

The effect of glibenclamide on the production of IP_3 in HepG2 cells was examined. Glibenclamide produced IP_3 in a concentration- and time-dependent manner as shown in Figure 1A and 1B, respectively. A significant increase in the IP_3 level compared to control was induced by glibenclamide at a concentration of 500 μM.

This effect of glibenclamide was most effective for the first 20 sec, and gradually declined after that time. These effects of glibenclamide seem to be mediated by its pharmacological actions, inhibition of ATP-sensitive K^+ (K_{ATP}) channels [Luzi and Pozza, 1997] and/or CFTR Cl^- channels [Sheppard and Robinson, 1997]. To verify which type of channels is involved, the reversing effects of agonists of these channels against the antagonistic activities of glibenclamide were evaluated. In these experiments diazoxide and pinacidil were used as agonists of K_{ATP} channels [Bray and Quast, 1992], and levamisole and bromotetramisole were used as agonists of CFTR Cl^- channels [Becq et al., 1996]. Treatment with CFTR Cl^- channel activators (1 mM levamisole or 1 mM bromotetramisole) significantly inhibited the IP_3 production induced by glibenclamide (1 mM), whereas K_{ATP} channel openers (500 μM diazoxide or 25 μM pinacidil) did not significantly alter the effects of glibenclamide, as shown in Figure 1C. These results indicate that glibenclamide increased IP_3 levels not through inhibition of K_{ATP} channels, but through inhibition of CFTR Cl^- channels in the HepG2 cells.

PLC Mediates the Glibenclamide-Induced Intracellular Ca^{2+} Release and Apoptosis

To examine the role of PLC in these actions of glibenclamide, the effects of PLC inhibitors on the glibenclamide-induced intracellular Ca^{2+} release and apoptosis were investigated. Treatment with either U-73122 (50 μM), a specific PLC inhibitor [Jin et al., 1994], or manoalide (20 μM), a non-specific inhibitor of PLC and phospholipase A_2 [Bennett et al., 1987], significantly suppressed the glibenclamide (1 mM)-induced intracellular Ca^{2+} release measured by Fure-2 fluorescence technique as illustrated in Figure 2A. These PLC inhibitors also completely prevented the apoptosis induced by glibenclamide (1 mM) assessed using flow cytometry, as depicted in Figure 2B. These results imply that PLC may mediate the glibenclamide-induced intracellular Ca^{2+} release through liberation of IP_3, which ultimately results in the induction of apoptosis.

PTx-Sensitive G-Proteins are Involved in the Glibenclamide-Induced Intracellular Ca^{2+} Release and Apoptosis

To determine the possible involvement of G-proteins in the glibenclamide-induced PLC

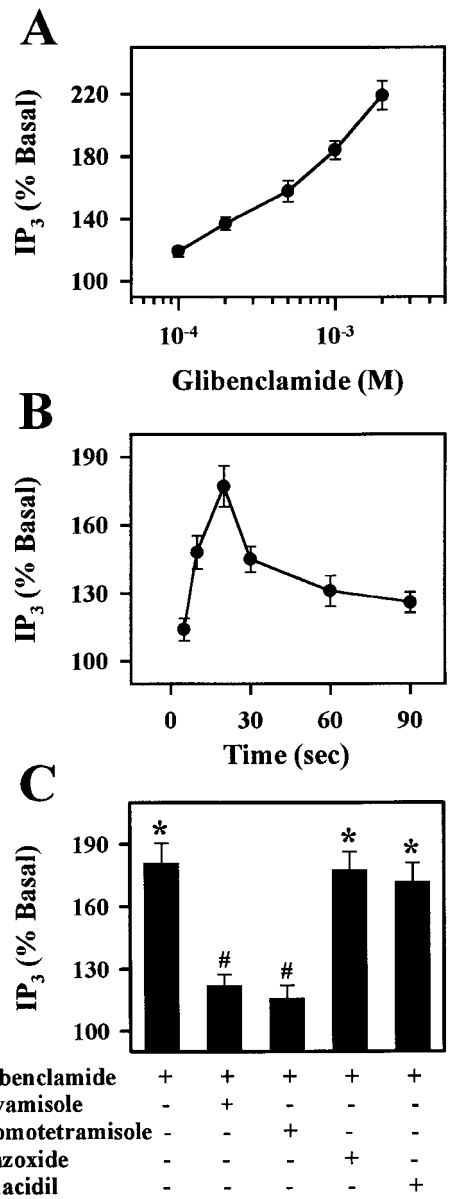

Fig. 1. Glibenclamide, an inhibitor of CFTR Cl^- channels, increases IP_3 production in a concentration- (**A**) and time-dependent manner (**B**) in HepG2 human hepatoblastoma cells. Cells were labeled with *myo*-[^{3}H]inositol for 48 h, followed by treatment with or without glibenclamide (1 mM). Then, production of [^{3}H]IP_3 was measured as described in Materials and Methods. The results are expressed as the percentage increase in [^{3}H]IP_3 for treated cells above basal levels obtained from untreated cells. In the experiments of (A) and (B), glibenclamide was treated at each concentration for 20 sec, and at the concentration of 1 mM each time, respectively. In the experiments of (**C**) activators of CFTR Cl^- channels (1 mM levamisole and 1 mM bromotetramisole) and K_{ATP} channel activators (500 μM diazoxide and 25 μM pinacidil) were treated simultaneously with glibenclamide (1 mM) for 20 sec. Data represent the mean values of four replications with bars indicating SEM. $^*P < 0.05$ compared to control condition in which the cells were incubated without glibenclamide. $^{\#}P < 0.05$ compared to glibenclamide alone.

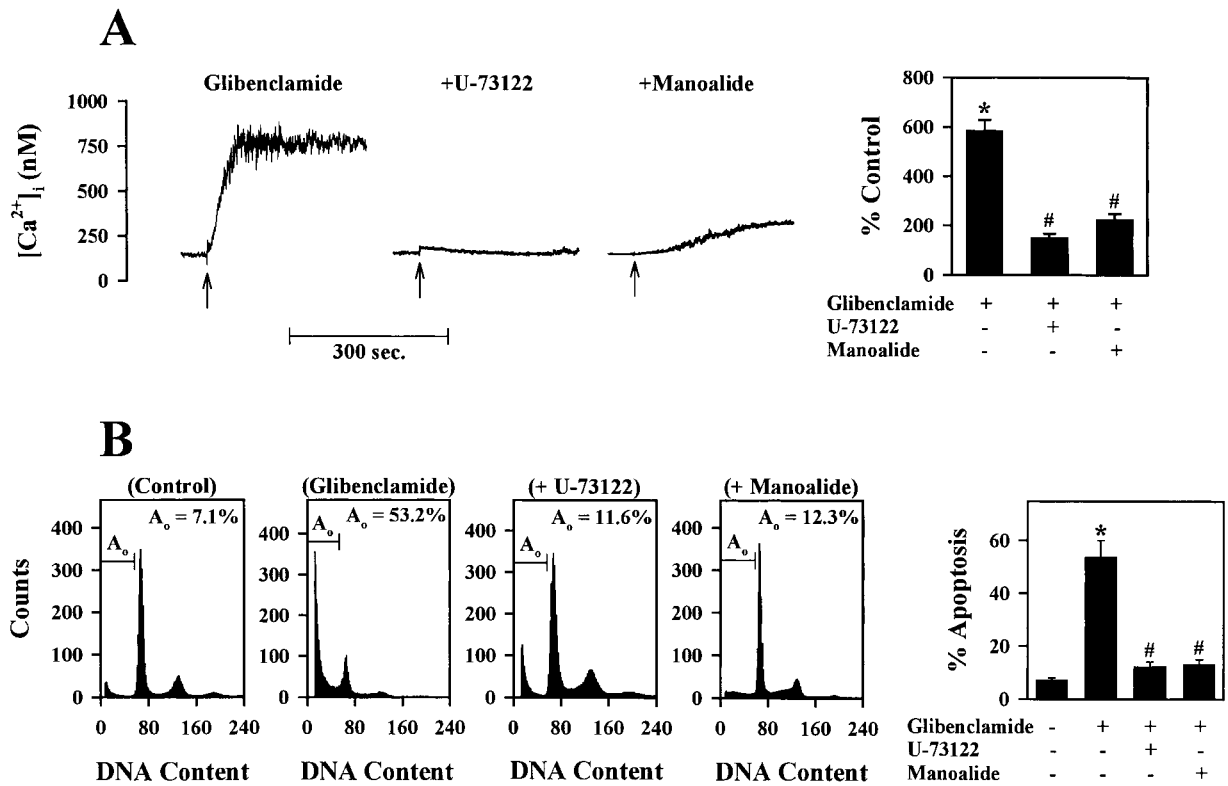

Fig. 2. PLC mediates the glibenclamide-induced intracellular Ca^{2+} release (**A**) and apoptosis (**B**) in HepG2 human hepatoblastoma cells. Intracellular Ca^{2+} concentration and apoptosis were assessed by Fura-2 fluorescence technique and flow cytometry, respectively. In the experiments of (A) PLC inhibitors, U-73122 (50 μM) and manoalide (20 μM) were added 5 min before glibenclamide application. The data represent intracellular Ca^{2+} changes with time. The arrows show the time points for addition of glibenclamide (1 mM). In the experiments of (B), glibenclamide (1 mM) was treated for 4 h. U-73122 (50 μM) and manoalide (20 μM) were added 30 min before glibenclamide treatment. In bar graphs the data represent the mean values of four replications with bars indicating SEM. $^*P < 0.05$ compared to control. $^{\#}P < 0.05$ compared to glibenclamide alone.

activation and intracellular Ca^{2+} release, we observed the effects of preincubation of PTx (500 μM) for 24 h. This experimental protocol can effectively inhibit the coupling actions of PTx-sensitive G-proteins to the effector proteins, such as PLC [Post and Brown, 1996]. The results showed that pretreatment with PTx significantly inhibited the glibenclamide-induced intracellular Ca^{2+} release as depicted in Figure 3A. Under these conditions the glibenclamide-induced apoptosis was also profoundly prevented as shown in Figure 3B. These results indicate that the PTX-sensitive G-proteins may couple the glibenclamide-induced inhibition of CFTR to the activation of PLC, which is responsible for intracellular Ca^{2+} release and apoptosis.

Glibenclamide Inhibits PTx-Catalyzed ADP Ribosylation of G-Proteins

To verify the direct involvement of PTX-sensitive G-proteins in these glibenclamide-induced actions, we examined whether PTx-catalyzed ADP-ribosylation of G-proteins is sensitive to treatment with glibenclamide. As shown in Figure 4, in a basal condition in which the cells were incubated without glibenclamide, ~41 kDa proteins were ribosylated by PTx (500 μM). The size of these proteins is matched with inhibitory G-proteins (G_i) in human hepatoma cells [McKillop et al., 1998]. Treatment with glibenclamide markedly enhanced the PTx-catalyzed ADP-ribosylation of the G-proteins in a time-dependent manner,

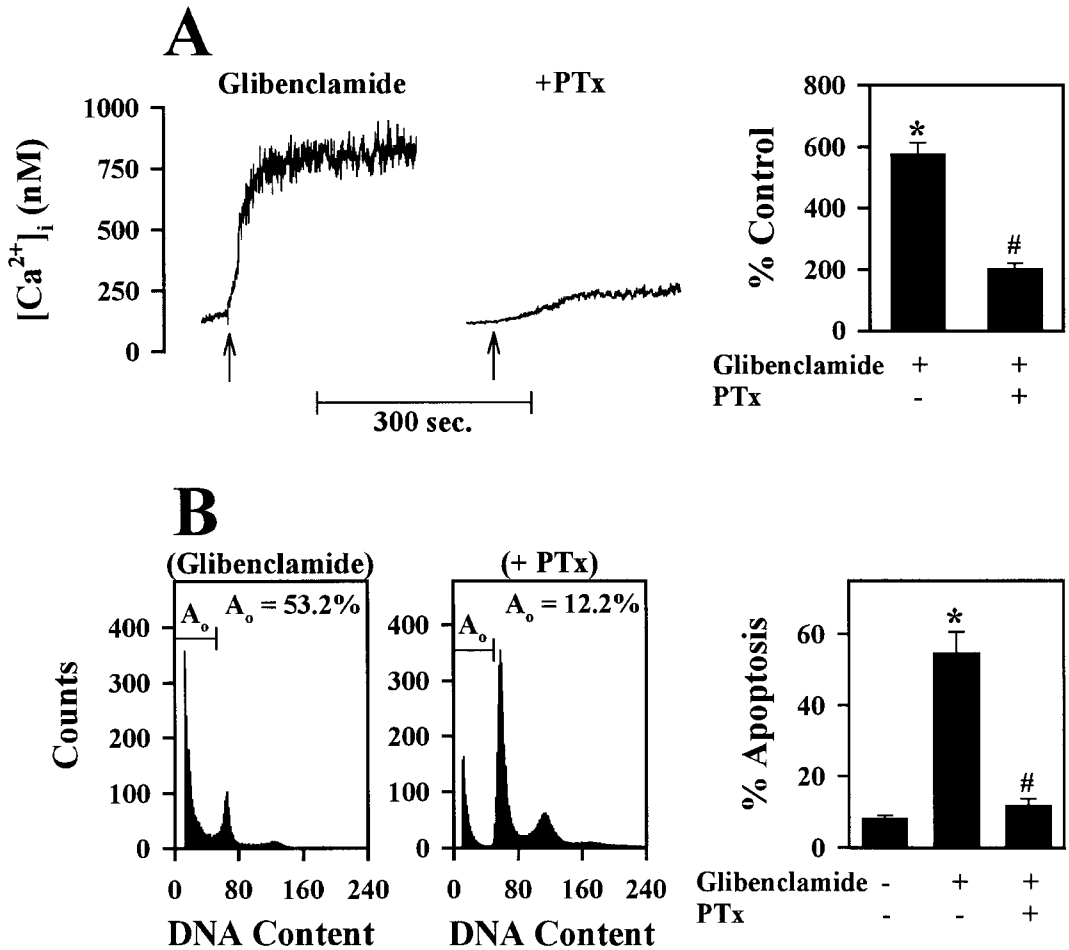

Fig. 3. Pretreatment with PTx inhibits the glibenclamide-induced intracellular Ca^{2+} release (**A**) and apoptosis (**B**) in HepG2 human hepatoblastoma cells. Data presentation is the same as in Fig. 2. In these experiments PTx (500 μM) was preincubated for 24 h to inhibit the action of G-proteins. In the experiments of (A) PTx was added 5 min before glibenclamide application. The arrows show the time points for addition of glibenclamide (1 mM). In the experiments of (B) the cells were treated with glibenclamide (1 mM) for 4 h. PTx were added 24 h before glibenclamide application. In bar graphs the data represent the mean values of four replications with bars indicating SEM. $^{*}P < 0.05$ compared to control. $^{\#}P < 0.05$ compared to glibenclamide alone.

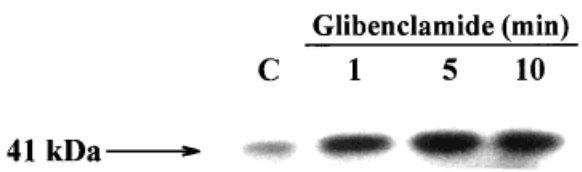

Fig. 4. Effect of glibenclamide on PTx-catalyzed [^{32}P]ADP ribosylation of G-proteins (~41 kDa) in membrane preparation of HepG2 human hepatoblastoma cells. Membranes were extracted from the cells incubated in the absence or presence of glibenclamide (1 mM) at each time, and then further incubated with PTx and [^{32}P]NAD as described in Materials and Methods section. The proteins were separated by SDS-PAGE and processed by autoradiography. A representative autoradiograph of three experiments performed is shown.

and implies that PTx-sensitive G-proteins may be directly involved in these glibenclamide-induced effects.

DISCUSSION

Intracellular Ca^{2+} has been shown to act as a common mediator of apoptosis [McConkey and Orrenius, 1996]. Particularly, in HepG2 human hepatoblastoma cells, an intracellular Ca^{2+} signal appears to play an essential role in the mechanism of apoptosis induced by a variety of agents including tamoxifen [Kim et al., 1999b], 4-aminopyridine (a K^{+} channel inhibitor) [Kim et al., 2000a], and *t*-butyl

hydroperoxide (an organic peroxide) [Kim et al., 2000b]. Moreover, inhibition of CFTR Cl^- channels by glibenclamide resulted in the apoptosis of the HepG2 cells in which intracellular Ca^{2+} was also involved in the mechanism of its action [Kim et al., 1999a]. Although further studies are needed for complete understanding, the downstream signals of elevated intracellular Ca^{2+} leading to apoptosis have been previously characterized. One of the targets of increased intracellular Ca^{2+} is the activation of the Ca^{2+}-dependent protein kinases and phosphatases [Bonnefoy-Berard et al., 1994] which has been frequently seen during apoptosis [Shibasaki et al., 1997]. Direct activation of enzymes, such as Ca^{2+}-dependent proteinase [Squier and Cohen, 1997], Ca^{2+}/Mg^{2+}-dependent endonuclease [Cohen and Duke, 1984; Wyllie et al., 1984], and Ca^{2+}-dependent transglutaminase [Fesus et al., 1987; Melino et al., 1994], may represent another target for intracellular Ca^{2+} action in apoptosis. However, the exact source of intracellular Ca^{2+} elevation associated with the induction of apoptosis was different depending on the agents used, either from Ca^{2+} influx [Kim et al., 1999b, 2000a, 2000b] or intracellular Ca^{2+} release [Kim et al., 1999a]. These findings suggest that the routes of Ca^{2+} increase may not be important for the induction of apoptosis.

In the present study we tried to determine the detailed mechanism of elevation of intracellular Ca^{2+} tightly linked to apoptosis induced by the inhibition of CFTR Cl^- channels in the HepG2 cells [Kim et al., 1999a]. The intracellular Ca^{2+} release through inhibition of CFTR by glibenclamide appears to be due to the activation of the IP_3-sensitive intracellular Ca^{2+} release channels [Kim et al., 1999a]. Indeed, glibenclamide increased IP_3 levels in a dose- and time-dependent manner (Fig. 1A, B). In addition, the glibenclamide-induced IP_3 production was significantly inhibited not by activators of K_{ATP} channels (diazoxide or pinacidil), but by activators of CFTR Cl^- channels (levamisole or bromotetramisole) (Fig. 1C). These results further demonstrate that in spite of multiple pharmacological actions of glibenclamide [Luzi and Pozza, 1997], the glibenclamide-induced IP_3 liberation may be the result of the inhibition of CFTR Cl^- channels.

IP_3 is produced by members of the PLC family of enzymes which hydrolyze phosphatidylinositol (4,5)-bisphosphate (PIP_2), generating IP_3 and diacylglycerol [Singer et al., 1997]. The glibenclamide-induced intracellular Ca^{2+} increase was completely prevented by PLC inhibitors (U-73122 or manoalide) (Fig. 2A), indicating that PLC is definitely involved in the mechanism of glibenclamide-induced intracellular Ca^{2+} mobilization. In addition, significant inhibition of the glibenclamide-induced apoptosis by these PLC inhibitors (Fig. 2B), suggests that PLC activation may be necessary for this apoptotic process.

The PLC family is further classified into three subgroups: β, γ, and δ. PLCγ is most commonly activated by protein tyrosine kinases (receptor kinases or cytoplasmic kinases), and PLCβ by heterotrimeric G-proteins [Singer et al., 1997], which are composed of three subunits ($\alpha\beta\gamma$) and which include four families: G_s, G_i, G_q, and G_{12} [Sternweis and Smrcka, 1993]. PLCβ can be activated by the α subunits of G_q family, which includes four members: G_q, G_{11}, G_{14}, and G_{15}/G_{16}, or by the $\beta\gamma$ subunits associated with any of the α subunit families [Singer et al., 1997]. In this study the glibenclamide-induced Ca^{2+} release was completely inhibited by pretreatment with PTx (Fig. 3A), indicating that this action of glibenclamide may be mediated by PLCβ coupled to G_i family members G_i and G_o, which are sensitive to PTx [Simon et al., 1991; Birnbaumer, 1993]. Interestingly, in the HepG2 cells used in this study, only G_i, not G_o proteins were identified by immunoblotting [Schaak et al., 1997]. Therefore, the PTx-sensitive G_i proteins may be involved in these actions of glibenclamide. In fact, G_i proteins couple to PLCβ in many types of cells [Mattera et al., 1998; Murthy and Makhlouf, 1998]. Additionally, pretreatment with PTx significantly suppressed the glibenclamide-induced apoptosis (Fig. 3B), further supporting the fact that PTx-sensitive G_i proteins play an essential role in the apoptotic mechanism of glibenclamide.

The results of this study that PTx-catalyzed ADP-ribosylation of G_i-proteins was markedly enhanced by glibenclamide (Fig. 4), further demonstrate that these types of G-proteins may be directly involved in the effects of glibenclamide. Activation of G_i proteins as an upstream signal of the PLC–IP_3 mechanisms may occur via their interaction with CFTR Cl^- channels, rather than nonspecific actions of glibenclamide, since the glibenclamide-induced increase in IP_3 levels was completely prevented

by the activators of CFTR Cl^- channels (Fig. 1C). However, the exact mechanism by which the glibenclamide-induced inhibition of CFTR Cl^- channels interact with the PTx-sensitive G_i proteins, is currently unknown, and remains to be determined. Although speculated, the activation of G-proteins may be the result of altered intracellular Cl^- concentration, because intracellular Cl^- levels appear to influence the activity of G-proteins [Toyoshige et al., 1996; Lenz et al., 1997]. Indeed, glibenclamide-induced inhibition of CFTR Cl^- channels induced increase in intracellular Cl^- concentration [Kim et al., 1999a]. Another possibility may also exist. Glibenclamide-induced increase in intracellular Cl^- concentration shifted membrane potential to more negative [Kim et al., 1999a]. Since alteration of membrane potential has also been associated with the activation of G-proteins [Anis et al., 1999], the glibenclamide-induced hyperpolarization may possibly activate G_i. In addition, CFTR proteins have been shown to regulate the activity of outwardly rectified epithelial Cl^- channels in association with PTx-sensitive G-proteins [Ismailov et al., 1996]. Thus, the possibility that CFTR proteins bound to glibenclamide directly interact with G_i, regardless of changes in intracellular Cl^- concentration or membrane potential, cannot be excluded. However, these possibilities should be tested in the future studies for an understanding of the exact mechanism.

In conclusion, PTX-sensitive G_i proteins coupled to PLCβ may mediate the intracellular Ca^{2+} release and apoptosis induced by inhibiting CFTR Cl^- channels in HepG2 cells. These results further suggest that the PTx-sensitive G_i proteins may be a valuable target for the induction of apoptosis that is importantly involved in cancer treatment.

REFERENCES

Aitken ML. 1996. Cystic fibrosis. Curr Opin Pulm Med 2:435–438.

Anis Y, Nurnberg B, Visochek L, Reiss N, Naor Z, Cohen-Armon M. 1999. Activation of G_o proteins by membrane depolarization traced by in situ photoaffinity labeling of $G\alpha_o$-proteins with [α^{32}P]GTP-azidoanilide. J Biol Chem 274:7431–7440.

Becq F, Verrier B, Chang XB, Riordan JR, Hanrahan JW. 1996. cAMP- and Ca^{2+}-independent activation of cystic fibrosis transmembrane conductance regulator channels by phenylimidazothiazole drugs. J Biol Chem 271: 16171–16179.

Bennett CF, Mong S, Wu HL, Clark MA, Wheeler L, Crooke ST. 1987. Inhibition of phosphoinositide-specific phospholipase C by manoalide. Mol Pharmacol 32:587–593.

Berridge MJ, Dawson RM, Downes CP, Heslop JP, Irvine RF. 1983. Changes in the levels of inositol phosphates after agonist-dependent hydrolysis of membrane phosphoinositides. Biochem J 212:473–482.

Birnbaumer L. 1993. Heterotrimeric G proteins. Molecular diversity and functional correlates. J Recept Res 13:19–26.

Bombeli T, Karsan A, Tait JF, Harlan JM. 1997. Apoptotic vascular endothelial cells become procoagulant. Blood 89:2429–2442.

Bonnefoy-Berard N, Genestier L, Flacher M, Revillard JP. 1994. The phosphoprotein phosphatase calcineurin controls calcium-dependent apoptosis in B cell lines. Eur J Immunol 24:325–329.

Bray KM, Quast U. 1992. A specific binding site for K^+ channel openers in rat aorta. J Biol Chem 267:11689–11692.

Cohen JJ, Duke RC. 1984. Glucocorticoid activation of a calcium-dependent endonuclease in thymocyte nuclei leads to cell death. J Immunol 132:38–42.

Crompton NE. 1998. Programmed cellular response to ionizing radiation damage. Acta Oncol 37:1–49.

Exton JH. 1997. Regulation of phosphoinositide phospholipases by G-proteins. Adv Exp Med Biol 400A:3–8.

Fesus L, Thomazy V, Falus A. 1987. Induction and activation of tissue transglutaminase during programmed cell death. FEBS Lett 224:104–108.

Ismailov II, Jovov B, Fuller CM, Berdiev BK, Keeton DA, Benos DJ. 1996. G-protein regulation of outwardly rectified epithelial chloride channels incorporated into planar bilayer membranes. J Biol Chem 271:4776–4780.

Jin W, Lo TM, Loh HH, Thayer SA. 1994. U73122 inhibits phospholipase C-dependent calcium mobilization in neuronal cells. Brain Res 642:237–243.

Kamesaki H. 1998. Mechanisms involved in chemotherapy-induced apoptosis and their implications in cancer chemotherapy. Int J Hematol 68:29–43.

Kelley KA, Stamm S, Kozak CA. 1992. Expression and chromosome localization of the murine cystic fibrosis transmembrane conductance regulator. Genomics 13:381–388.

Kidd VJ. 1998. Proteolytic activities that mediate apoptosis. Annu Rev Physiol 60:533–573.

Kim JA, Kang YS, Lee SH, Lee EH, Yoo BH, Lee YS. 1999a. Glibenclamide induces apoptosis through inhibition of cystic fibrosis transmembrane conductance regulator (CFTR) Cl^- channels and intracellular Ca^{2+} release in HepG2 human hepatoblastoma cells. Biochem Biophys Res Commun 261:682–688.

Kim JA, Kang YS, Jung MW, Lee SH, Lee YS. 1999b. Involvement of Ca^{2+} influx in the mechanism of tamoxifen-induced apoptosis in HepG2 human hepatoblastoma cells. Cancer Lett 147:115–123.

Kim JA, Kang YS, Jung M, Kang G, Lee SH, Lee YS. 2000a. Ca^{2+} influx mediates apoptosis induced by 4-aminopyridine, a K^+ channel blocker, in HepG2 human hepatoblastoma cells. Pharmacology 60:74–81.

Kim JA, Kang YS, Lee SH, Lee YS. 2000b. Inhibitors of Na^+/Ca^{2+} exchanger prevent oxidant-induced intracellular Ca^{2+} increase and apoptosis in a human hepatoma cell line. Free Radic Res (in press).

Kornblau SM. 1998. The role of apoptosis in the pathogenesis, prognosis, and therapy of hematologic malignancies. Leukemia 12:S41–S46.

Lenz RA, Pitler TA, Alger BE. 1997. High intracellular Cl^- concentrations depress G-protein-modulated ionic conductances. J Neurosci 17:6133–6141.

Lowe SW, Lin AW. 2000. Apoptosis in cancer. Carcinogenesis 21:485–495.

Luzi L, Pozza G. 1997. Glibenclamide: an old drug with a novel mechanism of action? Acta Diabetol 34:239–244.

Mattera R, Hayek S, Summers BA, Grove DL. 1998. Agonist-specific alterations in receptor-phospholipase coupling following inactivation of $G_{i2}\alpha$ gene. Biochem J 332:263–271.

McConkey DJ, Orrenius S. 1996. The role of calcium in the regulation of apoptosis. J Leukoc Biol 59:775–783.

McKillop IH, Wu Y, Cahill PA, Sitzmann JV. 1998. Altered expression of inhibitory guanine nucleotide regulatory proteins (G_i-proteins) in experimental hepatocellular carcinoma. J Cell Physiol 175:295–304.

Melino G, Annicchiarico-Petruzzeli M, Piredda L, Candi E, Gentile V, Davies PJ, Piacentini M. 1994. Tissue transglutaminase and apoptosis: sense and antisense transfection studies with human neuroblastoma cells. Mol Cell Biol 14:6584–6596.

Murthy KS, Makhlouf GM. 1998. Coexpression of ligand-gated P_{2X} and G protein-coupled P_{2Y} receptors in smooth muscle. Preferential activation of P_{2Y} receptors coupled to phospholipase C (PLC)-β1 via $G\alpha_{q/11}$ and to PLC-β3 via $G\beta\gamma_{i3}$. J Biol Chem 273:4695–4704.

Post GR, Brown JH. 1996. G protein-coupled receptors and signaling pathways regulating growth responses. FASEB J 10:741–749.

Schaak S, Cayla C, Blaise R, Quinchon F, Paris H. 1997. HepG2 and SK-N-MC: two human models to study α-2 adrenergic receptors of the α-2C subtype. J Pharmacol Exp Ther 281:983–991.

Sheppard DN, Robinson KA. 1997. Mechanism of glibenclamide inhibition of cystic fibrosis transmembrane conductance regulator Cl^- channels expressed in a murine cell line. J Physiol (Lond) 503:333–346.

Shibasaki F, Kondo E, Akagi E, McKeon F. 1997. Suppression of signaling through NF-AT by interactions between calcineurin and BCL-2. Nature 386:728–731.

Simon MI, Strathmann MP, Gautam N. 1991. Diversity of G proteins in signal transduction. Science 252:802–808.

Singer WD, Brown HA, Sternweis PC. 1997. Regulation of eukaryotic phosphatidylinositol-specific phospholipase C and phospholipase D. Annu Rev Biochem 66: 475–509.

Song Z, Steller H. 1999. Death by design: mechanism and control of apoptosis. Trends Cell Biol 9:M49–M52.

Squier MKT, Cohen JJ. 1997. Calpain, an upstream regulator of thymocyte apoptosis. J Immunol 158:3690–3697.

Sternweis PC, Smrcka AV. 1993. G proteins in signal transduction: the regulation of phospholipase C. Ciba Found Symp 176:96–106.

Toyoshige M, Basi NS, Rebois RV. 1996. Chloride effects on G_s subunit dissociation. Fluoroaluminate binding to G_s does not cause subunit dissociation in the absence of chloride ion. J Biol Chem 271:8791–8795.

Wang XW. 1999. Role of p53 and apoptosis in carcinogenesis. Anticancer Res 19:4759–4771.

Wyllie AH, Morris RG, Smith AL, Dunlop D. 1984. Chromatin cleavage in apoptosis: association with condensed chromatin morphology and dependence on macromolecular synthesis. J Pathol 142:67–67.