FK506 Blocks Intracellular Ca^{2+} Oscillations in Bovine Adrenal Glomerulosa Cells†

Stéphane N. Poirier,‡ Marc Poitras,‡ Alzbeta Chorvatova,§ Marcel-Daniel Payet,§ and Gaétan Guillemette*,‡

Department of Pharmacology and Department of Physiology and Biophysics, Faculty of Medicine, Université de Sherbrooke, Sherbrooke, Québec, Canada J1H 5N4

Received January 31, 2001; Revised Manuscript Received March 27, 2001

ABSTRACT: The inositol 1,4,5-trisphosphate ($InsP_3$) receptor is a ligand-gated Ca^{2+} channel playing an important role in the control of intracellular Ca^{2+}. In the study presented here, we demonstrate that angiotensin (AngII), phorbol ester (PMA), and FK506 significantly increase the level of $InsP_3$ receptor phosphorylation in intact bovine adrenal glomerulosa cells. With a back-phosphorylation approach, we showed that the $InsP_3$ receptor is a good substrate for protein kinase C (PKC) and that FK506 increases the level of PKC-mediated $InsP_3$ receptor phosphorylation. With a microsomal preparation from bovine adrenal cortex, we showed that PKC enhances the release of Ca^{2+} induced by a submaximal dose of $InsP_3$. We also showed that FK506 blocks intracellular Ca^{2+} oscillations in isolated adrenal glomerulosa cells by progressively increasing the intracellular Ca^{2+} concentration to a high plateau level. This effect is consistent with an inhibitory role of FK506 on calcineurin dephosphorylation of the $InsP_3$ receptor, thus keeping the receptor in a phosphorylated, high-conductance state. Our results provide further evidence for the crucial role of the $InsP_3$ receptor in the regulation of intracellular Ca^{2+} oscillations and show that FK506, by maintaining the phosphorylated state of the $InsP_3$ receptor, causes important changes in the Ca^{2+} oscillatory process.

Inositol 1,4,5-trisphosphate ($InsP_3$)[1] is a second messenger playing a crucial role in the complex mechanism of cellular Ca^{2+} regulation (*1*, *2*). $InsP_3$ is produced from the cleavage of phosphatidylinositol 4,5-bisphosphate by phospholipase C, in the response of a wide variety of cells to Ca^{2+}-mobilizing hormones. $InsP_3$ releases Ca^{2+} from certain sections of the endoplasmic reticulum by activating a specific receptor ($InsP_3$ receptor) (*3*, *4*) which constitutes a Ca^{2+} channel (*5*). $InsP_3$ receptors have been characterized in many different tissues (*6*−*10*), purified to homogeneity (*11*, *12*), and cloned and sequenced (*13*, *14*). To date, cloning experiments have identified three distinct but highly homologous types of $InsP_3$ receptors (*13*−*16*). The three types of $InsP_3$ receptors appear to be differentially distributed among tissues, but they all constitute functional Ca^{2+} channels. In isolated cells, Ca^{2+} level elevations often occur as oscillatory patterns or repetitive propagating waves (*17*). Among the models that were proposed to explain the mechanism of cellular Ca^{2+} oscillations, a strong emphasis has been put on the dual regulation of $InsP_3$ receptor activity by the ambient cytosolic Ca^{2+} concentration. The positive feedback induced by low Ca^{2+} concentrations and the negative feedback induced by high Ca^{2+} concentrations have been proposed to initiate and terminate short bursts of Ca^{2+} release even under conditions where the $InsP_3$ level remains constant (*18*−*20*). These dual effects of low and high Ca^{2+} concentrations could in part be mediated by activation of Ca^{2+}-dependent effector proteins such as kinases and phosphatases (*21*). The $InsP_3$ receptor is a known substrate of the Ca^{2+}-dependent enzyme protein kinase C (PKC) (*22*, *23*). It was also recently shown that the Ca^{2+}-dependent protein phosphatase calcineurin is physically and functionally associated with the $InsP_3$ receptor (*23*). Calcineurin was shown to dephosphorylate the PKC phosphorylation site on the $InsP_3$ receptor, thereby reducing $InsP_3$-mediated Ca^{2+} flux (*23*). These results suggested that Ca^{2+}-dependent phosphorylation−dephosphorylation processes might be involved in the regulation of the cellular Ca^{2+} concentration. The functional significance of $InsP_3$ receptor regulation by PKC and calcineurin has however not been thoroughly assessed in intact cells.

In the study presented here, we investigated the effect of FK506, a known calcineurin inhibitor, on the Ca^{2+} oscillatory patterns in intact bovine adrenal glomerulosa cells (BAG cells). We showed that FK506 increases the level of PKC-mediated $InsP_3$ receptor phosphorylation and stops intracellular Ca^{2+} oscillations by maintaining the cytosolic Ca^{2+} concentration at a high level. These results illustrate the dual effect of Ca^{2+}-dependent enzymes on the regulation of the Ca^{2+} concentration in intact cells.

MATERIALS AND METHODS

Materials. Cell culture media and protein A−agarose beads were purchased from Gibco (Burlington, ON). The

† This work is part of the Ph.D. thesis of S.N.P., and it was supported by a grant from the Medical Research Council of Canada. S.N.P. is the recipient of a studentship from the Fonds de la Recherche en Santé du Québec.

* To whom correspondence should be addressed: Département de Pharmacologie, Faculté de Médecine, Université de Sherbrooke, 3001, 12e Avenue Nord, Sherbrooke, Québec, Canada J1H 5N4. Telephone: (819) 564-5347. Fax: (819) 564-5400. E-mail: gguillem@courrier.usherb.ca.

‡ Department of Pharmacology.

§ Department of Physiology and Biophysics.

[1] Abbreviations: $InsP_3$, inositol 1,4,5-trisphosphate; AngII, angiotensin II; PMA, phorbol 12-myristate 13-acetate; BAG, bovine adrenal glomerulosa; DMEM, Dulbecco's modified Eagle's medium; PAGE, polyacrylamide gel electrophoresis; BSA, bovine serum albumin; M_r, apparent molecular mass.

10.1021/bi010207k CCC: $20.00
Published on Web 04/17/2001

silver stain kit plus was obtained from Bio-Rad (Mississauga, ON). Protease inhibitor cocktail (Complete) was purchased from Roche Molecular Biochemicals (Laval, PQ). [γ-^{32}P]-ATP, $^{32}P_i$ (orthophosphate), and [^{35}S]methionine/[^{35}S]cysteine Pro Mix were from Amersham Corp. (Oakville, ON). $InsP_3$ (trilithium salt) was obtained from LC Services Corp. (Woburn, MA). Angiotensin II (AngII) was obtained from Bachem (Torrance, CA). Fluo-3/AM, Fura-2 (free acid), bisindolylmaleimide I (GF I), and phorbol 12-myristate 13 acetate (PMA) were obtained from Calbiochem (La Jolla, CA). Protein kinase C was obtained from Promega (Madison, WI). All the other chemicals were from Sigma (St. Louis, MO). FK506 was a gift from Fujisawa Inc. (Deerfield, IL).

Cell Preparation. Bovine adrenal glands were obtained at a nearby slaughterhouse. BAG cells were prepared as previously described (*24*). Briefly, outer 0.5 mm slices of bovine adrenal cortex were minced into 1 mm × 1 mm fragments and digested with 2 mg/mL collagenase and 0.2 mg/mL DNase I, followed by a mechanical dispersion. This procedure was repeated five times. After two washes, BAG cells were purified on a Percoll gradient (20%) previously prepared by centrifugation at 35000*g* for 30 min at 4 °C. BAG cells were poured on top of the Percoll gradient and centrifuged at 500*g* for 15 min at 4 °C. BAG cells were washed and resuspended in DMEM supplemented with 10% fetal bovine serum, 1% GIBCO ITSX (serum supplement containing 1 g/L insulin, 0.55 g/L transferrin, and 0.7 mg/L selenium), 50 units/mL penicillin, 60 mg/mL streptomycin, and 2 mM L-glutamine. Cells were plated at a density of 2 × 10^6 cells per 100 mm dish for the phosphorylation experiments. They were plated at a density of 2 × 10^5 cells on cover slips for the single-cell experiments. Cells were cultured in a humidified atmosphere of 5% CO_2 in air at 37 °C for 3−4 days for Ca^{2+} measurements in a single cell and were grown to confluence for the phosphorylation experiments (7−10 days).

Metabolic Labeling of the $InsP_3$ Receptor with [^{35}S]-Methionine/[^{35}S]Cysteine Pro Mix. BAG cells grown to 60−75% confluence were metabolically labeled with [^{35}S]-methionine/[^{35}S]cysteine Pro Mix (250 μCi) for 40 h. Cells were washed twice with ice-cold PBS (phosphate-buffered saline) and frozen at −80 °C. Cells were then warmed at 37 °C and scraped with a rubber policeman. After centrifugation at 35000*g* for 15 min, the supernatant was discarded and the pellet was solubilized in ice-cold lysis buffer [50 mM Tris, 150 mM NaCl, 1% Triton X-100, and 1 mM EDTA (pH 7.4)] containing a protease inhibitor cocktail (1×). After 30 min on ice, the solubilized material was centrifuged at 35000*g* for 30 min. The supernatant was then incubated for 1 h at 4 °C with anti-type 1 $InsP_3$ receptor antibodies (obtained by immunization of a rabbit against the peptide Lys-Met-Asn-Val-Asn-Pro-Gln-Gln-Pro-Ala that corresponds to the C-terminal portion of the rat type 1 $InsP_3$ receptor). Immune complexes were incubated for an additional 1 h with 60 μL of 50% protein A−agarose beads. Immune complexes were then precipitated by centrifugation at 15000*g* for 5 min and washed three times with ice-cold lysis buffer. Immune complexes were desorbed from protein A−agarose beads by incubation in Laemmli buffer (*25*) for 5 min at 95 °C. Samples were loaded onto a 7.5% SDS−PAGE gel that was run for the first 15 min at 100 V and at 200 V for 75 min. $InsP_3$ receptor protein content was evaluated by silver staining. The gel was then dried and autoradiographed with X-OMAT film (Kodak).

$InsP_3$ Binding Assay of the Immunoprecipitated $InsP_3$ Receptor. Bovine adrenal cortex membranes were prepared as previously described (*26*). Membranes (1 mg of protein) were solubilized, and the $InsP_3$ receptor was immunoprecipitated as described in the previous section. The immunopurified $InsP_3$ receptor was incubated with appropriate concentrations of [^{3}H]$InsP_3$ at 4 °C for 30 min in 500 μL of binding medium containing 25 mM Tris-HCl buffered at pH 8.5, 5 mM KH_2PO_4, 1 mM EDTA, and protease inhibitor cocktail. Nonspecific binding was assessed in the presence of 1 μM $InsP_3$. The reaction was stopped by centrifugation at 15000*g* for 5 min. Receptor-bound radioactivity was analyzed by liquid scintillation spectrometry.

$InsP_3$ Receptor Phosphorylation in Intact Cells. Confluent BAG cells were washed three times with phosphate-free DMEM and incubated in the same buffer for 4 h at 37 °C in the presence of 150 μCi/mL $^{32}P_i$. Cells were then washed three times in KRH buffer [118 mM NaCl, 2.4 mM KCl, 1.8 mM $CaCl_2$, 0.8 mM $MgCl_2$, 10 mM glucose, 0.1% (w/v) BSA, and 20 mM Hepes (pH 7.4)] and were incubated in the same medium for 10 min at 37 °C. Cells were then stimulated with different kinase activators for the appropriate periods of time, washed twice with ice-cold PBS buffer (containing 1 mM Na_3VO_4, 50 mM NaF, 1 mM phenylmethanesulfonyl fluoride, 100 nM okadaic acid, and protease inhibitor cocktail) (PBS+) and frozen at −80 °C. The phosphorylated receptor was solubilized, immunoprecipitated (as described above), and analyzed by SDS−PAGE. $InsP_3$ receptor protein content of these preparations was then evaluated by silver staining and scanning densitometry. Radioactivity associated with electrophoresed $InsP_3$ receptors was initially quantified with a GS-250 Molecular Imager (Bio-Rad), and then the dried gel was autoradiographed with Bio-Max film (Kodak).

$InsP_3$ Receptor Back-Phosphorylation by PKA and PKC. Confluent unlabeled BAG cells were preincubated for 10 min at 37 °C in KRH buffer. BAG cells were then stimulated with AngII or other stimuli for appropriate periods of time, to activate endogenous protein kinases that (in vivo) phosphorylate their specific substrates with endogenous nonradioactive ATP. The incubations were then stopped by washing the cells twice with ice-cold PBS+ buffer and freezing the cells at −80 °C. The $InsP_3$ receptor was then solubilized in ice-cold lysis buffer and immunoprecipitated as described above. The immunoprecipitated $InsP_3$ receptor was then phosphorylated (in vitro) for 15 min at 30 °C in phosphorylation buffer [25 mM Tris, 1.8 mM $CaCl_2$, 10 mM $MgCl_2$, 1× protease inhibitor cocktail, and 100 μM DTT (pH 7.4)] containing 10 μCi of [γ-^{32}P]ATP, 50 μM nonradioactive ATP, 10 μM FK506, and either the catalytic subunit of PKA (100 units/mL) or 80 ng of PKC with 500 μg/mL phosphatidylserine and 50 μg/mL diolein in a final volume of 50 μL. The reaction was stopped by centrifugation at 15000*g* for 5 min. Immune complexes were then washed twice with 1 mL of phosphorylation buffer and analyzed by SDS−PAGE. $InsP_3$ receptor protein content of these preparations was then evaluated by silver staining and autoradiography. In the back-phosphorylation procedure, an important in vivo phosphorylation of the $InsP_3$ receptor in

treated BAG cells will markedly diminish the level of incorporation of $^{32}P_i$ in the in vitro phosphorylation assay.

Microsomal Ca^{2+} Release Measurements. Bovine adrenal cortex microsomes (8−10 mg of protein) were incubated in a medium containing 20 mM Tris-HCl buffered at pH 7.2, 110 mM KCl, 10 mM NaCl, 5 mM KH_2PO_4, 2 mM $MgCl_2$, 40 mM phosphocreatine, and 20 units/mL creatine kinase in a final volume of 1.5 mL. Under our experimental conditions, the Ca^{2+} in the medium was exclusively contaminating Ca^{2+}. Ca^{2+} uptake was initiated by the addition of ATP (2 mM) to the bathing medium containing the microsomes. The Ca^{2+} releasing effect of $InsP_3$ was measured shortly after ATP-dependent Ca^{2+} sequestering activity had reached a steady state. The free Ca^{2+} concentration of the medium was monitored with Fura-2 (free acid, 1 μM) on a Hitachi F-2000 spectrofluorometer. The excitation wavelength was 340 nm (slit width of 10 nm), and the emission was recorded at 510 nm (slit width of 10). Incubations were performed at 37 °C. Each record was calibrated by the addition of a known amount of Ca^{2+} ($CaCl_2$) to the mixture. The actual free Ca^{2+} concentration of the medium was calculated from the F_{max} and F_{min} values obtained by adding excess Ca^{2+} and EGTA (at pH 8.5), respectively, after treatment with 1 μM ionomycin. The equation that was used was $[Ca^{2+}] = 224\ nM \times [(F - F_{min})/(F_{max} - F)]$.

Cytosolic [Ca^{2+}] Measurements. Cells cultured on coverslips were loaded with 1 μM Fluo-3/AM for 45 min at room temperature in a modified Krebs-Ringer buffer solution [KRBS; 118 mM NaCl, 2.42 mM KCl, 1.8 mM $CaCl_2$, 1.18 mM KH_2PO_4, 0.8 mM $MgSO_4$, 20 mM Hepes (pH 7.4), 5 mM $NaHCO_3$, 10 mM glucose, and 1 mg/mL BSA]. The loading buffer was replaced with fresh KRBS for a further 45 min incubation period at room temperature. Coverslips were then transferred to a 35 mm diameter chamber on the stage of a Nikon DM 400 microscope and examined using a 40× objective. $[Ca^{2+}]_i$ was determined by microfluorometry where the dye was excited at 488 nm and emission signals at >515 nm were collected. Increases in $[Ca^{2+}]_i$ were expressed as a percentage of baseline fluorescence (*27*). All stimulations and pharmacological treatments of the cells were performed at room temperature by the addition of small volume of the substance (5 μL) in a final volume of 1 mL.

Data Analysis. Experimental procedures were performed at least three times. When needed, data were analyzed by a Student's *t* test. *P* values of <0.05 were considered to be statistically significant.

RESULTS

Immunoprecipitation of the $InsP_3$ Receptor. BAG cells were metabolically labeled with [^{35}S]methionine/[^{35}S]cysteine Pro Mix, and the $InsP_3$ receptor was immunoprecipitated as described in Materials and Methods. The receptor migrated on a 7.5% SDS−PAGE gel as a single radioactive band with an M_r of ~230 kDa (Figure 1A, lane 2). This protein was not immunoprecipitated with a preimmune serum (Figure 1A, lane 1). Silver staining of the same gel indicated that the $InsP_3$ receptor was immunoprecipitated only with the immune serum (Figure 1B). Silver staining was used in further experiments to ensure that equal amounts of $InsP_3$ receptors were loaded on each gel lane. [^{3}H]$InsP_3$ dose-displacement binding assays of the immunoprecipitated $InsP_3$ receptor from

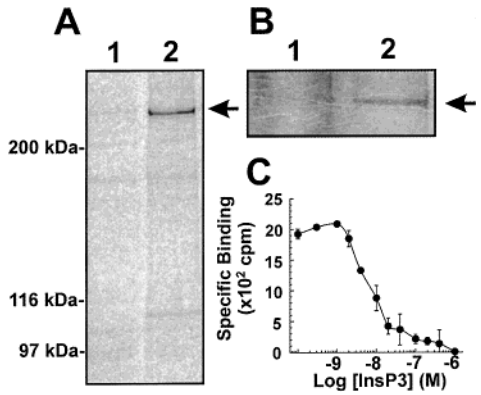

FIGURE 1: $InsP_3$ receptor immunoprecipitation. BAG cells were metabolically labeled for 40 h with ^{35}S. After solubilization with Triton X-100, the $InsP_3$ receptor was immunoprecipitated with the anti-type 1 $InsP_3$ receptor antibody (lane 2) or with preimmune serum (lane 1) and analyzed by SDS−PAGE. Panels A and B show the autoradiography and the silver-stained correlation, respectively. The arrow indicates the $InsP_3$ receptor migration position. Panel C shows the results of a [^{3}H]$InsP_3$ (22 000 cpm, 2.0 nM) dose-displacement binding experiment on the immunoprecipitated $InsP_3$ receptor from solubilized bovine adrenal cortex.

bovine adrenal cortex membranes revealed an IC_{50} of 7.8 ± 2.9 nM which corresponds to the affinity of the $InsP_3$ receptor found in intact adrenal cortex membranes (*28*) (Figure 1C). Furthermore, the total amount of specific binding sites for $InsP_3$ found in the immunoprecipitate (280 ± 88 fmol/mg of protein) demonstrates that under our conditions, a large proportion of $InsP_3$ receptors is immunoprecipitated with our anti-type 1 $InsP_3$ receptor antibody. We previously showed that the type 1 $InsP_3$ receptor is the most abundant in the bovine adrenal cortex and that it is present either alone (homotetramers) or together with the low-abundance type 2 and type 3 receptors (heterotetramers) in most $InsP_3$ receptor−Ca^{2+} channel tetrameric complexes (*29*). The anti-type 1 $InsP_3$ receptor antibody can thus immunoprecipitate almost all the detergent soluble $InsP_3$ binding activity of bovine adrenal cortex.

$InsP_3$ Receptor Phosphorylation. After solubilization with Triton X-100, the $InsP_3$ receptor was immunoprecipitated and phosphorylated in vitro as described in Materials and Methods. Figure 2 shows that the $InsP_3$ receptor from adrenal cortex is a good substrate for PKC (Figure 2, lanes 1 and 2) and PKA (Figure 2, lanes 3 and 4). In intact BAG cells, angiotensin II (AngII) and phorbol 12-myristate 13-acetate (PMA) increased the level of $InsP_3$ receptor phosphorylation by ~2-fold above basal level (Figure 3A, lanes 2 and 4). FK506 also caused an important increase in the level of $InsP_3$ receptor phosphorylation (Figure 3A, lane 3). Quantitative phospho-imager analysis of ^{32}P-labeled bands indicated that the levels of phosphorylation of the $InsP_3$ receptor in cells treated with AngII, FK506, and PMA were increased by 1.96-, 1.82-, and 1.70-fold, respectively, above the basal level found in untreated cells (Figure 3B). These results clearly indicate that PKC can phosphorylate the $InsP_3$ receptor, and they suggest that AngII-activated $InsP_3$ receptor phosphorylation is mediated by PKC. They also show that FK506 is able to elevate the phosphorylation level of the $InsP_3$ receptor in BAG cells. The concentration of FK506 needed to increase the level of $InsP_3$ receptor phosphorylation is in the same range as the concentration of FK506 needed to dissociate the phosphoprotein phosphatase calcineurin from

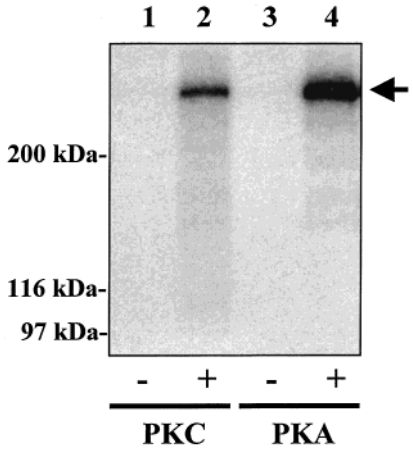

FIGURE 2: In vitro InsP3 receptor phosphorylation. Bovine adrenal cortex microsomes (1 mg of protein) were solubilized with Triton X-100, and the $InsP_3$ receptor was immunoprecipitated and phosphorylated in vitro with either PKC or the catalytic subunit of PKA in the presence of [γ-^{32}P]ATP as described in Materials and Methods. The immunoprecipitates were analyzed by 7.5% SDS−PAGE and autoradiography. The arrow indicates the $InsP_3$ receptor migration position.

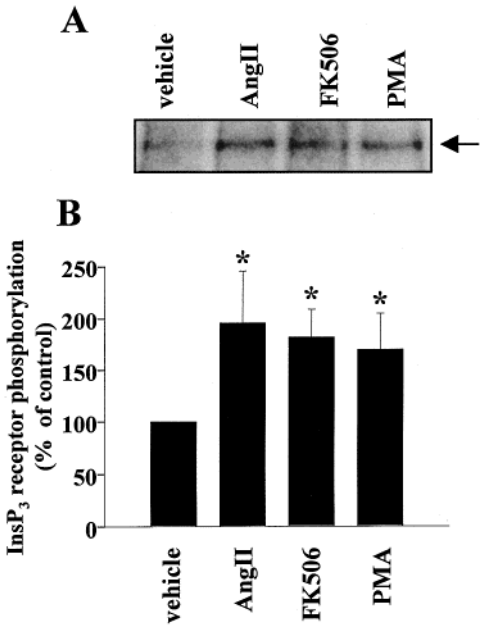

FIGURE 3: $InsP_3$ receptor phosphorylation in intact BAG cells. After being labeled with $^{32}P_i$ for 4 h, BAG cells were incubated for 5 min in the absence (control) or presence of AngII (1 μM), FK506 (10 μM), or PMA (2 μM). After solubilization with Triton X-100, the $InsP_3$ receptor was immunoprecipitated, analyzed on a 7.5% SDS−PAGE gel, and silver stained as described in Materials and Methods. Panel A shows the autoradiography of the gel (the arrow indicates the $InsP_3$ receptor migration position). In panel B, the radioactivity associated with the phosphorylated receptor was quantified by phosphoimaging. Data are shown as a percentage of the control value (100%) and represent the means ± standard deviation of four separate experiments (asterisks denote $p < 0.05$ vs control).

the $InsP_3$ receptor (*23*). These results suggest that under our basal experimental conditions, there is an equilibrium between PKC phosphorylation and calcineurin dephosphorylation of the $InsP_3$ receptor.

$InsP_3$ Receptor Back-Phosphorylation by PKA and PKC. To determine which kinase was responsible for the phosphorylation of the $InsP_3$ receptor upon stimulation of BAG

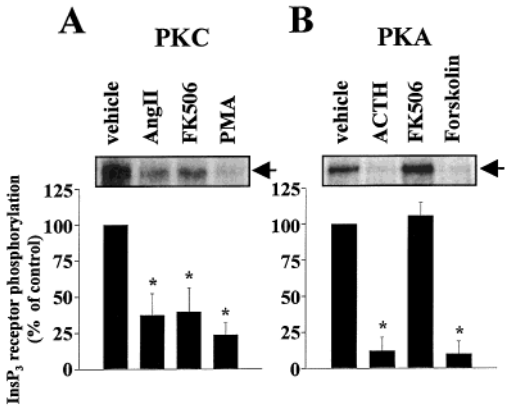

FIGURE 4: $InsP_3$ receptor back-phosphorylation. The $InsP_3$ receptor from control or pretreated BAG cells was solubilized in Triton X-100, immunoprecipitated, and then phosphorylated with either PKC or the catalytic subunit of PKA in the presence of [γ-^{32}P]-ATP as described in Materials and Methods. The back-phosphorylated immunoprecipitates were then analyzed on a 7.5% SDS−PAGE gel, silver stained, and autoradiographed. Panel A shows the autoradiography and the histograms of the $InsP_3$ receptor back-phosphorylated with PKC after pretreatment of BAG cells with vehicle (lane 1), 1 μM AngII (lane 2), 10 μM FK506 (lane 3), or 2 μM PMA (lane 4). Panel B shows the autoradiography and the histograms of the $InsP_3$ receptor back-phosphorylated with PKA after pretreatment of BAG cells with vehicle (lane 1), 1 μM ACTH (lane 2), 10 μM FK506 (lane 3), or 10 μM forskolin (lane 4). The arrow indicates the $InsP_3$ receptor migration position. The histograms show phosphoimaging quantification as a percentage of vehicle value (100%) and represent the means ± standard deviation of four separate experiments (asterisks denote $p < 0.05$ vs vehicle).

cells, we utilized a back-phosphorylation procedure. In a first step, intact BAG cells were stimulated with different agents that can activate endogenous protein kinases. After solubilization with Triton X-100, the $InsP_3$ receptor was immunoprecipitated and phosphorylated in vitro with exogenously added PKC or PKA in the presence of [^{32}P]ATP. Figure 4A shows a robust PKC-mediated phosphorylation of the $InsP_3$ receptor immunoprecipitated from nontreated BAG cells, demonstrating a low phosphorylation level under basal conditions within the cells (Figure 4A, lane 1). After a pretreatment with AngII, FK506, or PMA, the level of back-phosphorylation with PKC was strongly decreased (Figure 4A, lanes 2−4). These results demonstrate that AngII, PMA, and FK506 increase the level of phosphorylation of the $InsP_3$ receptor in intact BAG cells. Quantitative phospho-imager analysis of ^{32}P-labeled bands indicated that the level of back-phosphorylation of the $InsP_3$ receptor from cells pretreated with AngII, FK506, or PMA was at least 3 times lower than the level of back-phosphorylation of the $InsP_3$ receptor from nontreated cells (Figure 4A, histograms). The same approach was used to verify whether FK506 was able to modulate PKA-mediated $InsP_3$ receptor phosphorylation. Figure 4B shows that a pretreatment of BAG cells with ACTH or forskolin strongly decreased the level of PKA-mediated $InsP_3$ receptor back-phosphorylation, whereas a pretreatment of BAG cells with FK506 did not interfere with the back-phosphorylation. Quantitative phospho-imager analysis is shown in Figure 4B (histograms). These results clearly demonstrate that the $InsP_3$ receptor is a substrate for PKC and for PKA in intact BAG cells. Furthermore, they show that FK506 selectively increases the level of PKC-mediated

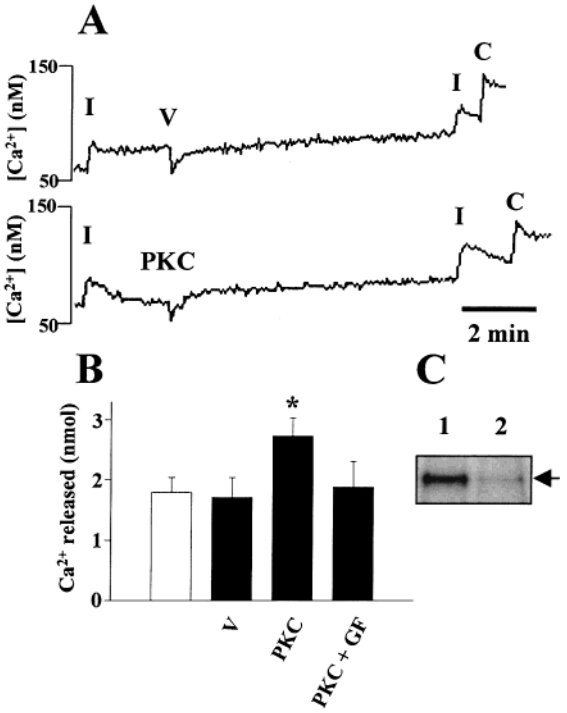

FIGURE 5: Effect of PKC on $InsP_3$-induced Ca^{2+} release activity. Microsomes (8−10 mg of proteins) were incubated at 37 °C, and their Ca^{2+} release activity was monitored using Fura-2 (free acid, 1 μM) under the conditions described in Materials and Methods. The sequestered Ca^{2+} was partially released by $InsP_3$. In panel A, the release of Ca^{2+} by a submaximal dose of $InsP_3$ (0.3 μM) is evaluated before and after an 8 min treatment with vehicle or with PKC: (I) 0.3 μM $InsP_3$, (C) 3 nmol of Ca^{2+}, (V) vehicle, and (PKC) 80 ng. In panel B, the empty column illustrates the amount of Ca^{2+} released by $InsP_3$ before treatment whereas the dark columns illustrate the amount of Ca^{2+} released by $InsP_3$ after treatment with vehicle, PKC or PKC in the presence of its selective inhibitor GF I (0.5 μM). In panel C, microsomes were treated with vehicle (lane 1) or with PKC (lane 2) for 8 min and solubilized with Triton X-100. The $InsP_3$ receptor was then immunoprecipitated and back-phosphorylated with PKC in the presence of [γ-^{32}P]ATP as described in Materials and Methods. The immunoprecipitate was analyzed by 7.5% SDS−PAGE and autoradiography. The arrow indicates the $InsP_3$ receptor migration position. Data are the means ± standard deviation of three to six separate experiments and were reproduced with at least four different microsomal preparations [asterisks denote $p < 0.05$ vs $InsP_3$-induced Ca^{2+} release observed before treatment (empty column)].

phosphorylation of the $InsP_3$ receptor without affecting the PKA-mediated phosphorylation of the $InsP_3$ receptor.

PKC Increases the Level of $InsP_3$-Induced Ca^{2+} Release from Adrenal Cortex Microsomes. Because FK506 increased the level of PKC-mediated phosphorylation, it was important to verify the functional significance of $InsP_3$ receptor phosphorylation by PKC on $InsP_3$-induced Ca^{2+} release. Figure 5A shows a typical experiment where the ambiant Ca^{2+} concentration was measured by Fura-2 fluorescence with a microsomal preparation of bovine adrenal cortex. After ATP-induced Ca^{2+} uptake, the ambiant Ca^{2+} concentration reached a low nanomolar level (~50 nM). A submaximal dose of $InsP_3$ (0.3 μM) released a significant amount of Ca^{2+} that was quantified by the addition of a known amount of exogenous Ca^{2+} (3 nmol). The upper trace of Figure 5A shows that after an 8 min treatment with vehicle, a second dose of 0.3 μM $InsP_3$ released an amount of Ca^{2+} (1.5 nmol) similar to that released by the first dose (1.6 nmol). The lower trace of Figure 5A shows that after an 8 min treatment with PKC, the second dose of $InsP_3$ released much more Ca^{2+} (2.4 nmol) than the first dose (1.8 nmol). Figure 5B illustrates the results of several experiments in which $InsP_3$ released 2.7 ± 0.3 nmol of Ca^{2+} from PKC-treated microsomes as compared to 1.8 ± 0.3 nmol of Ca^{2+} from untreated microsomes. From microsomes treated with PKC in the presence of 0.5 μM GF I, a specific inhibitor of PKC, $InsP_3$ released only 1.9 ± 0.4 nmol of Ca^{2+}. Figure 5C shows a decrease in the level of PKC-mediated back-phosphorylation of the $InsP_3$ receptor immunoprecipitated from microsomes that had been pretreated with PKC (lane 2) as compared with the level of PKC-mediated back-phosphorylation of the $InsP_3$ receptor immunoprecipitated from microsomes that had been pretreated with the vehicle (lane 1). These results demonstrate that under our experimental conditions for $InsP_3$-induced Ca^{2+} release, PKC could effectively phosphorylate the $InsP_3$ receptor.

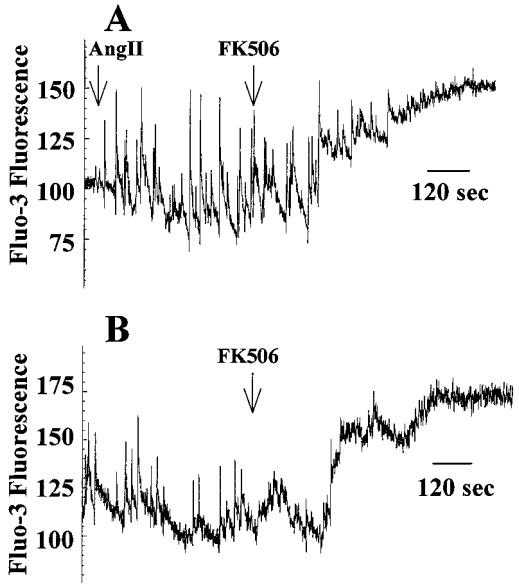

FIGURE 6: Intracellular Ca^{2+} oscillations. BAG cells were loaded with Fluo-3, and their intracellular Ca^{2+} concentration was monitored as described in Materials and Methods. Panel A shows a typical trace in which the Ca^{2+} concentration within a single cell starts oscillating after addition of 150 pM AngII. Addition of 4 μM FK506 caused an alteration in the Ca^{2+} oscillatory pattern by increasing the intracellular Ca^{2+} concentration to a high level. Panel B shows a typical trace obtained with a cell in which the intracellular Ca^{2+} concentration was spontaneously oscillating. Addition of 4 μM FK506 similarly altered the intracellular Ca^{2+} oscillations. These typical traces are representative of several experiments performed with 43 different cells from at least three different cell preparations. Of the 43 cells that were analyzed, 14 cells were spontaneously oscillating, 21 cells produced stable oscillations in response to AngII, and 8 cells did not respond to AngII or produced unstable oscillations in response to AngII. FK506 produced the same effect (as depicted in panels A and B) on all the cells that were oscillating either spontaneously or in response to AngII.

Effect of FK506 on Spontaneous or AngII-Induced Ca^{2+} Oscillations in BAG Cells. To investigate the functional importance of the effect of FK506 on the phosphorylation level of the $InsP_3$ receptor, we monitored the cytoplasmic Ca^{2+} concentration in intact single BAG cells. As shown in Figure 6A, the stimulation of a single BAG cell with a relatively small dose of AngII (150 pM) induced a typical pattern of intracellular Ca^{2+} oscillations. These oscillations

could be maintained for several minutes until the addition of FK506, which caused a continuous increase in the basal level of Ca^{2+} toward a high level corresponding to the oscillation peaks. About 5 min after FK506 addition, the Ca^{2+} oscillatory pattern stopped and the Ca^{2+} concentration was maintained at a high level. Similar results were obtained upon addition of FK506 to cells showing a spontaneous Ca^{2+} oscillatory pattern (without addition of AngII) (Figure 6B).

DISCUSSION

In the study presented here, we demonstrated that AngII and PMA (a PKC activator) increase the level of $InsP_3$ receptor phosphorylation in intact BAG cells. With a back-phosphorylation approach, we further demonstrated that AngII-induced $InsP_3$ receptor phosphorylation in intact BAG cells is mediated by PKC. Our results are in agreement with previous in vitro studies showing that the $InsP_3$ receptor is a good substrate for PKC (*22*, *23*, *30*). We also demonstrated that PKC-mediated $InsP_3$ receptor phosphorylation enhances its Ca^{2+} release activity. Together, these results suggest that in BAG cells, AngII enhances $InsP_3$ receptor activity through PLC-mediated $InsP_3$ production and PKC-mediated $InsP_3$ receptor phosphorylation.

In this study, we also showed that FK506 increases the level of $InsP_3$ receptor phosphorylation in intact BAG cells. Interestingly, in crude cerebellar membrane homogenates, PKC-mediated $InsP_3$ receptor phosphorylation was barely detectable unless the activity of the phosphoprotein phosphatase calcineurin was inhibited (*23*). With the back-phosphorylation procedure, we clearly demonstrated that FK506 increases the level of $InsP_3$ receptor phosphorylation selectively on the PKC site. These results are consistent with those of a previous in vitro study showing that FK506 disrupts the calcineurin−FKBP12 complex from the $InsP_3$ receptor, thus selectively regulating the phosphorylation status of the PKC site on the receptor and enhancing $InsP_3$-mediated Ca^{2+} flux (*23*). From these studies, one could predict that in intact BAG cells, by preventing the calcineurin-mediated dephosphorylation of the $InsP_3$ receptor on the PKC site, FK506 will enhance $InsP_3$ receptor activity.

We showed that the stimulation of Fluo-3-loaded BAG cells with 0.15 nM AngII elicits a cytosolic Ca^{2+} spiking behavior generally identified as Ca^{2+} oscillations. Similar results were obtained by Rossig et al. (*31*), who demonstrated that the stimulation of BAG cells with a low concentration of AngII elicits Ca^{2+} oscillations that are governed by the regulation of $InsP_3$ receptor activity. Ca^{2+} oscillations occur in most excitable and nonexcitable cells upon stimulation with low hormone concentrations, as most often encountered under physiological conditions (*2*). It is generally accepted that the basic mechanism of Ca^{2+} oscillations is dependent on the $InsP_3$-gated Ca^{2+} release from intracellular stores through the $InsP_3$ receptor and its subsequent reuptake into the same stores (*18*, *19*). In the study presented here, we showed that FK506 stops AngII-induced Ca^{2+} oscillations in BAG cells. In tracheal epithelial cells, Kanoh et al. (*32*) previously reported that FK506 gradually attenuated and abolished ATP-induced intracellular Ca^{2+} oscillations. They also showed that FK506 gradually increased basal Ca^{2+} levels in airway epithelial cells. Although they did not evaluate the phosphorylation state and the Ca^{2+} release activity of the $InsP_3$ receptor, their results suggested a role of FK506 in $InsP_3$ receptor function. Another recent study demonstrated that calcineurin reduces the level of $InsP_3$-induced Ca^{2+} release in COS-7 cells and that FK506 reverses the effect of calcineurin (*33*). All these results are consistent with the proposed effect of FK506 on the differential regulation of $InsP_3$ receptor phosphorylation by PKC and calcineurin (*23*). FK506 was also shown to inhibit Ca^{2+} uptake by the SERCA Ca^{2+} pump in several different types of permeabilized cells (*34*). This last study also showed that FK506 did not affect $InsP_3$-induced Ca^{2+} release in permeabilized SH-SY5Y cells and concluded that FK506 has no direct effect on $InsP_3$-induced Ca^{2+} release. We do not agree with this conclusion mostly because under the described experimental conditions with permeabilized cells, there is no endogenous PKC activity, and therefore, the $InsP_3$ receptor is most probably not phosphorylated on its PKC site. Under these conditions, FK506 is not expected to have any effect on $InsP_3$-induced Ca^{2+} release. Nevertheless, this study demonstrated that beyond its calcineurin antagonist property, FK506 may also directly interfere with the function of other important Ca^{2+}-handling proteins. Interestingly, both effects of FK506, on the $InsP_3$ receptor and on the SERCA Ca^{2+} pump, promote a depletion of intracellular Ca^{2+} stores and an increase in the level of cytosolic Ca^{2+} which are compatible with the changes observed in the Ca^{2+} oscillatory pattern of BAG cells.

We occasionally observed spontaneous Ca^{2+} oscillations in nonstimulated BAG cells. This phenomenon was also observed by other laboratories in BAG cells (*31*) and in other types of nonexcitable cells (*35*, *36*). It is likely the reflection of a basal cellular activity. The effect of FK506 on spontaneously oscillating BAG cells was not distinguishable from that on AngII-stimulated cells. These results indicate that the effect of FK506 is related to the general mechanism of cellular Ca^{2+} regulation rather than to a selective AngII-associated pathway.

Our results indicated that both ACTH and forskolin increase the level of PKA-mediated $InsP_3$ receptor phosphorylation in intact BAG cells. Previous studies demonstrated that the $InsP_3$ receptor is a good substrate for PKA in intact cells (*37*, *38*). Some studies reported that PKA potentiates $InsP_3$-induced Ca^{2+} release (*23*, *37*, *39*, *40*), while other studies reported opposite effects (*41*−*43*). As previously observed by Cameron et al. (*23*), we showed that FK506 does not interfere with $InsP_3$ receptor phosphorylation by PKA. These results indicate that the effect of calcineurin is selective for the PKC site on the $InsP_3$ receptor.

In summary, the major findings presented in this study are that (i) the $InsP_3$ receptor is phosphorylated in intact BAG cells stimulated with an agonist promoting PKC activation, (ii) FK506 increases the level of PKC-mediated phosphorylation of the $InsP_3$ receptor in intact BAG cells, (iii) PKC enhances $InsP_3$ receptor activity, and (iv) FK506 alters intracellular Ca^{2+} oscillations by gradually increasing the intracellular Ca^{2+} concentration to a high plateau level. These results support the contention that the $InsP_3$ receptor plays a crucial role in the regulation of intracellular Ca^{2+} oscillations. They also suggest the implication of a phosphorylation−dephosphorylation process in the regulation of $InsP_3$ receptor activity. Furthermore, this study demonstrated that besides its known immunosuppressive effects, FK506 may influence

many cellular functions that are modulated by intracellular Ca^{2+}.

ACKNOWLEDGMENT

We thank Dr. Ihor Bekersky of Fujisawa Inc. for the generous gift of the compound FK506.

REFERENCES

1. Berridge, M. J. (1997) *J. Exp. Biol. 200*, 315−319.
2. Thomas, A. P., Bird, G. S., Hajnoczky, G., Robb-Gaspers, L. D., and Putney, J. W., Jr. (1996) *FASEB J. 10*, 1505−1517.
3. Mikoshiba, K. (1997) *Curr. Opin. Neurobiol. 7*, 339−345.
4. Joseph, S. K. (1996) *Cell. Signalling 8*, 1−7.
5. Ferris, C. D., Huganir, R. L., Supattapone, S., and Snyder, S. H. (1989) *Nature 342*, 87−89.
6. Spat, A., Bradford, P. G., McKinney, J. S., Rubin, R. P., and Putney, J. W., Jr. (1986) *Nature 319*, 514−516.
7. Guillemette, G., Balla, T., Baukal, A. J., Spat, A., and Catt, K. J. (1987) *J. Biol. Chem. 262*, 1010−1015.
8. Guillemette, G., Balla, T., Baukal, A. J., and Catt, K. J. (1988) *J. Biol. Chem. 263*, 4541−4548.
9. Worley, P. F., Baraban, J. M., Supattapone, S., Wilson, V. S., and Snyder, S. H. (1987) *J. Biol. Chem. 262*, 12132−12136.
10. Parys, J. B., Sernett, S. W., DeLisle, S., Snyder, P. M., Welsh, M. J., and Campbell, K. P. (1992) *J. Biol. Chem. 267*, 18776−18782.
11. Supattapone, S., Worley, P. F., Baraban, J. M., and Snyder, S. H. (1988) *J. Biol. Chem. 263*, 1530−1534.
12. Chadwick, C. C., Saito, A., and Fleischer, S. (1990) *Proc. Natl. Acad. Sci. U.S.A. 87*, 2132−2136.
13. Mignery, G. A., Sudhof, T. C., Takei, K., and De Camilli, P. (1989) *Nature 342*, 192−195.
14. Furuichi, T., Yoshikawa, S., Miyawaki, A., Wada, K., Maeda, N., and Mikoshiba, K. (1989) *Nature 342*, 32−38.
15. Sudhof, T. C., Newton, C. L., Archer, B. T., III, Ushkaryov, Y. A., and Mignery, G. A. (1991) *EMBO J. 10*, 3199−3206.
16. Blondel, O., Takeda, J., Janssen, H., Seino, S., and Bell, G. I. (1993) *J. Biol. Chem. 268*, 11356−11363.
17. Berridge, M. J. (1993) *Nature 361*, 315−325.
18. Bezprozvanny, I., Watras, J., and Ehrlich, B. E. (1991) *Nature 351*, 751−754.
19. Iino, M., and Endo, M. (1992) *Nature 360*, 76−78.
20. Stojilkovic, S. S., Kukuljan, M., Tomic, M., Rojas, E., and Catt, K. J. (1993) *J. Biol. Chem. 268*, 7713−7720.
21. Zhang, B. X., Zhao, H., and Muallem, S. (1993) *J. Biol. Chem. 268*, 10997−11001.
22. Matter, N., Ritz, M. F., Freyermuth, S., Rogue, P., and Malviya, A. N. (1993) *J. Biol. Chem. 268*, 732−736.
23. Cameron, A. M., Steiner, J. P., Roskams, A. J., Ali, S. M., Ronnett, G. V., and Snyder, S. H. (1995) *Cell 83*, 463−472.
24. Boulay, G., Servant, G., Luong, T. T., Escher, E., and Guillemette, G. (1992) *Mol. Pharmacol. 41*, 809−815.
25. Laemmli, U. K. (1970) *Nature 227*, 680−685.
26. Poitras, M., Ribeiro-Do-Valle, R. M., Poirier, S. N., and Guillemette, G. (1995) *Biochemistry 34*, 9755−9761.
27. Schlosser, S. F., Burgstahler, A. D., and Nathanson, M. H. (1996) *Proc. Natl. Acad. Sci. U.S.A. 93*, 9948−9953.
28. Poitras, M., Bernier, S., Servant, M., Richard, D. E., Boulay, G., and Guillemette, G. (1993) *J. Biol. Chem. 268*, 24078−24082.
29. Poitras, M., Poirier, S. N., Laflamme, K., Simoneau, M., Escher, E., and Guillemette, G. (2000) *Recept. Channels 7*, 41−52.
30. Ferris, C. D., Huganir, R. L., Bredt, D. S., Cameron, A. M., and Snyder, S. H. (1991) *Proc. Natl. Acad. Sci. U.S.A. 88*, 2232−2235.
31. Rossig, L., Zolyomi, A., Catt, K. J., and Balla, T. (1996) *J. Biol. Chem. 271*, 22063−22069.
32. Kanoh, S., Kondo, M., Tamaoki, J., Shirakawa, H., Aoshiba, K., Miyazaki, S., Kobayashi, H., Nagata, N., and Nagai, A. (1999) *Am. J. Physiol. 276*, L891−L899.
33. Bandyopadhyay, A., Shin, D. W., and Kim, D. H. (2000) *Biochem. J. 348*, 173−181.
34. Bultynck, G., De Smet, P., Weidema, A. F., Ver Heyen, M., Maes, K., Callewaert, G., Missiaen, L., Parys, J. B., and De Smedt, H. (2000) *J. Physiol. 15*, 681−693.
35. Villalobos, C., Faught, W. J., and Frawley, L. S. (1998) *Mol. Endocrinol. 12*, 87−95.
36. Ueda, S., Oiki, S., and Okada, Y. (1986) *J. Membr. Biol. 91*, 65−72.
37. Wojcikiewicz, R. J. H., and Luo, S. G. (1998) *J. Biol. Chem. 273*, 5670−5677.
38. Sharma, K., Wang, L. W., Zhu, Y. Q., Bokkala, S., and Joseph, S. K. (1997) *J. Biol. Chem. 272*, 14617−14623.
39. Burgess, G. M., Bird, G. S. J., Obie, J. G., and Putney, J. W., Jr. (1991) *J. Biol. Chem. 266*, 4772−4781.
40. Nakade, S., Rhee, S. K., Hamanaka, H., and Mikoshiba, K. (1994) *J. Biol. Chem. 269*, 6735−6742.
41. Supattapone, S., Danoff, S., Theibert, A., Joseph, S. K., Steiner, J., and Snyder, S. H. (1988) *Proc. Natl. Acad. Sci. U.S.A. 85*, 8747−8750.
42. Quinton, T. M., and Dean, W. L. (1992) *Biochem. Biophys. Res. Commun. 184*, 893−899.
43. Murthy, K. S., Severi, C., Grider, J. R., and Makhlouf, G. M. (1993) *Am. J. Physiol. 264*, G967−G974.

BI010207K