Protein kinase C phosphorylates the inositol 1,4,5-trisphosphate receptor type 2 and decreases the mobilization of Ca^{2+} in pancreatoma AR4-2J cells

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Abstract

In non-excitable cells, the inositol 1,4,5-trisphosphate receptor (IP_3R) is an intracellular Ca^{2+} channel, which plays a major role in Ca^{2+} signalling. Three isoforms of IP_3R have been identified (IP_3R -1, IP_3R -2 and IP_3R -3) and most cell types express different proportions of each isoform. The differences between the pharmacological and functional properties of the various isoforms of IP_3R are poorly understood. AR4-2J cells, which express almost exclusively ($\sim 86\%$) the IP_3R -2, represent an interesting model to study this particular isoform. Here, we investigated a regulatory mechanism by which protein kinase C (PKC) influences IP_3R -2-mediated Ca^{2+} release. Using an immunoprecipitation approach, we confirmed that AR4-2J cells express almost exclusively the IP_3R -2 isoform. Using an *in vitro* phosphorylation assay, we showed that the

immunopurified IP_3R -2 was efficiently phosphorylated by exogenous PKC. In intact AR4-2J cells metabolically labelled with ^{32}P i, we showed that phorbol-12-myristate-13-acetate (PMA) and Ca^{2+} mobilizing agonists cause the phosphorylation of IP_3R -2. In saponin-permeabilized AR4-2J cells, IP_3 -induced Ca^{2+} release was reduced after a pre-treatment with PMA or with exogenous PKC. PMA also reduced the Ca^{2+} response of intact AR4-2J cells stimulated with carbachol and epidermal growth factor, two agonists that use different receptor types to activate phospholipase C. These results demonstrate that PKC decreases the Ca^{2+} mobilizing activity of IP_3R -2 and thus exerts a negative feedback on the agonists-induced Ca^{2+} response of AR4-2J cells.

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Introduction

Inositol 1,4,5-trisphosphate receptors (IP_3Rs) are intracellular Ca^{2+} channels that are activated by the second messenger IP_3 , generated from the hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP_2) by phospholipase C in response to Ca^{2+} -mobilizing hormones (Mikoshiba 1997, Patel *et al.* 1999). IP_3Rs are located on the endoplasmic reticulum and their opening liberates stored Ca^{2+} that diffuses in the cytosol and triggers Ca^{2+} -dependent cellular responses. IP_3Rs play major roles in agonists-induced intracellular Ca^{2+} release and also in store operated Ca^{2+} entry, a process whereby the depletion of intracellular Ca^{2+} store causes the opening of Ca^{2+} channels in the plasma membrane (for review see Smyth *et al.* 2006). The elevation of cytosolic free Ca^{2+} concentration is a dynamic signal regulating a variety of important cellular functions such as secretion, contraction, metabolism and gene transcription (Berridge *et al.* 2003).

In mammalian cells, three subtypes of IP_3Rs (IP_3R -1, IP_3R -2 and IP_3R -3), derived from three distinct genes, have been identified but the actual role of each subtype in physiological Ca^{2+} signalling remains to be established

(Patterson *et al.* 2004, Bezprozvanny 2005, Futatsugi *et al.* 2005). IP_3Rs subtypes are highly homologous and have the same overall structure, including an N-terminal ligand-binding domain, a C-terminal channel domain and an intervening coupling domain or regulatory domain containing binding sites for Ca^{2+} , nucleotides, calmodulin, diverse proteins and modulatory factors, as well as putative phosphorylation sites for several protein kinases (Patel *et al.* 1999, Patterson *et al.* 2004). IP_3Rs associate in tetrameric structures that form functional Ca^{2+} channels. Individual cell types can express more than one IP_3R subtype, which may form different populations of homo- and hetero-tetrameric channels (Joseph *et al.* 1995, Monkawa *et al.* 1995, Wojcikiewicz & He 1995, Nucifora *et al.* 1996). The relatively poor homology between the regulatory domains of the three IP_3Rs subtypes is likely to confer specific pharmacological and functional properties to each subtype. The functional significance of subtype diversity has not been clearly established for homo-tetrameric channels and much less so for hetero-tetrameric channels. It is therefore important to characterize the specific pharmacological properties and the regulatory mechanisms of each IP_3R subtype.

The protein kinase C (PKC) activator diacylglycerol is co-generated with IP₃ from the cleavage of PIP₂ by phospholipase C. The regulatory domains of IP₃Rs contain several putative phosphorylation sites (S/TXR/K) for PKC. Previous studies showed that PKC increases IP₃-induced Ca²⁺ release in tissues expressing in large majority the subtype IP₃R-1 (Matter *et al.* 1993, Poirier *et al.* 2001), but little is known about the effect of PKC on IP₃R-2 and IP₃R-3. The rat pancreatoma cell line AR4-2J expresses IP₃R-2 almost exclusively and thus constitutes a good model for the study of this particular IP₃R subtype. The purpose of the present study was therefore to verify whether IP₃R-2 is a substrate for PKC and, if so, to determine the functional consequences of IP₃R-2 phosphorylation by PKC. Using *in vitro* and *in cellulo* phosphorylation assays, we demonstrated that IP₃R-2 is phosphorylated by PKC. Spectrofluorometric Ca²⁺ measurement assays revealed that PKC decreases IP₃-induced Ca²⁺ release. We conclude that in AR4-2J cells, that express predominantly the IP₃R-2 subtype, PKC is exerting a negative regulatory role on intracellular Ca²⁺ mobilization.

Materials and Methods

Materials

Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS) and penicillin-streptomycin-glutamine were from Gibco Life Technologies. IP₃ was from Alexis Biochemicals (San Diego, CA, USA). Anti-IP₃R-1 antibody was obtained from Affinity Bioreagent (Golden, CO, USA). Anti-IP₃R-3 was obtained from BD Biosciences Transduction Laboratories (Mississauga, ON, USA). Anti-IP₃R-2 antibody was obtained by immunizing New Zealand rabbits with the C-terminus of IP₃R-2 coupled with keyhole limpet haemocyanin (Poitras *et al.* 2000). Protein AG-agarose was from Santa Cruz Technology (Santa Cruz, CA, USA), ATP, BSA, creatine phosphokinase, phosphocreatine, diolein, phosphatidylserine, poly-L-lysine hydrobromide, saponin and thapsigargin were from Sigma-Aldrich. Carbachol (CCh), free acid (fura2), fura2/AM, bisindolylmaleimide I (GF 1), phorbol 12-myristate 13-acetate (PMA), okadaic acid, cyclosporine A (CSA), PKC isozyme sampler set and phosphatase inhibitor cocktail set II were from Calbiochem (San Diego, CA, USA). Proteases inhibitors cocktail (Complete) was from Roche Molecular Biochemicals (Laval, QC, Canada). PKC was obtained from Promega. [γ -³²P]-ATP and [³²P]Pi (orthophosphate) were from Perkin-Elmer (Boston, MA, USA).

Cell culture

The rat pancreatoma AR4-2J cell line was cultured in complete DMEM (supplemented with 15% heat-inactivated FBS, 2 mM L-glutamine, 100 IU/ml penicillin and

100 µg/ml streptomycin) at 37 °C in a humidified atmosphere containing 5% CO₂. When 80% confluent, cells were subcultured (1:8 dilution) every 6–7 days using 0.25% trypsin, 1 mM EDTA and fed every other day with fresh DMEM.

In vitro phosphorylation of IP₃R-2

AR4-2J cells (2 × 10⁷ cells/ml) were solubilized for 1 h at 4 °C, under gentle agitation, in lysis buffer (50 mM Tris-HCl pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100) supplemented with the proteases inhibitors cocktail Complete (1X). The solubilized material was centrifuged at 30 000 × g for 30 min and the supernatant was immunoprecipitated for 16 h, at 4 °C, under gentle agitation, with anti-IP₃R-2 antibody and 50 µl protein AG-agarose beads. The immunoprecipitated IP₃Rs were phosphorylated for different time periods at 30 °C in phosphorylation buffer (25 mM Tris-HCl pH 7.4, 1.8 mM CaCl₂, 10 mM MgCl₂) containing 10 µCi [γ -³²P]ATP, 50 µM non-radioactive ATP, 500 µg/ml phosphatidylserine, 50 µg/ml diolein and 0.15 U of PKC in a final volume of 50 µl. Immune complexes were then washed twice with 1 ml ice-cold PBS (137 mM NaCl, 2.8 mM KCl, 1.5 mM KH₂PO₄ and 8 mM Na₂HPO₄) supplemented with 1 mM EGTA and resuspended in 45 µl of 1 × Laemmli buffer. Samples were heated for 5 min at 95 °C and loaded onto a 5% SDS-PAGE gel that was run at 20 mA for 120 min. IP₃R-2 protein was stained with Coomassie blue and gels were vacuum-dried for 1 h at 80 °C and exposed for 24 h on a BioMax MR film with an intensifying screen. Bands intensities were determined by densitometry using a Hewlett Packard Scan Jet 5100c (Hewlett-Packard Co.) and integrated peak areas were determined using the gel analysis Quantity One software (version 4.2; Bio-Rad Laboratories).

In cellulo phosphorylation of IP₃R-2

AR4-2J cells (5 × 10⁶ cells) were washed twice with phosphate-free DMEM and incubated in the same buffer for 5 h at 37 °C in the presence of 70 µCi/ml ³²Pi. Cells were stimulated for different time periods with 2 µM PMA or other agonists, washed and immediately solubilized with lysis buffer supplemented with 100 nM CSA, 100 nM okadaic acid, (1X) phosphatases inhibitors cocktail set II and (1X) proteases inhibitors cocktail complete. Insoluble material was precipitated by centrifugation at 13 000 × g for 25 min at 4 °C. IP₃R-2 was immunoprecipitated for 1 h at 4 °C with anti-IP₃R-2 antibody and with 50 µl protein AG-agarose beads. Immune complexes were washed thrice with ice-cold lysis buffer and resuspended in 45 µl Laemmli buffer (1X). Samples were loaded onto a 4% SDS-PAGE gel that was subjected to a constant current of 15 mA for 150 min. The gel was vacuum dried for 1 h at 80 °C and subjected to autoradiography with Biomax MR film (Kodak).

Dynamic video imaging of cytosolic Ca²⁺

Fluorescence from fura2-loaded cells was monitored as previously described (Auger-Messier *et al.* 2004). Briefly, AR4-2J cells grown on glass coverslips were washed twice with 4-(2-hydroxyethyl)-1-piperazineethansulfonic acid (HEPES)-buffered physiological saline solution (HBSS: 20 mM HEPES pH 7.4, 120 mM NaCl, 5.3 mM KCl, 0.8 mM MgSO₄, 1.8 mM CaCl₂ and 11.1 mM dextrose) and loaded with 0.1 μM fura2/AM for 20 min at room temperature in the dark. Cells were then washed and bathed in fresh HBSS for 20 min to ensure complete hydrolysis of the fura2/AM before inserting the coverslip into a circular open-bottom chamber that was mounted on the stage of a Zeiss Axiovert microscope fitted with an Attofluor Digital Imaging and Photometry System (Attofluor Inc., Rockville, MD, USA). The system allows simultaneous data acquisition from up to 99 user-defined variably sized regions of interest per field of view. Fluorescence from isolated fura2-loaded cells was monitored by videomicroscopy using alternative excitatory wavelengths of 334 and 380 nm and recording emitted fluorescence at 510 nm. All experiments were done at room temperature and the data expressed as intracellular free Ca²⁺ concentration (nM) calculated from 334/380 fluorescence ratio according to Grynkiewicz *et al.* (1985). Data acquisition was typically at 3 s intervals and lasted for 600 s.

IP₃-induced Ca²⁺ release

AR4-2J cells (50 × 10⁶ cells grown in 10-cm dishes) were resuspended in 2 ml cytosol-like buffer (20 mM Tris-HCl pH 7.4, 110 mM KCl, 10 mM NaCl, 5 mM KH₂PO₄ and 2 mM MgCl₂), supplemented with 0.5 μM fura2 acid, 20 units creatine kinase, 20 mM phosphocreatine, 1 mM ATP, 100 nM okadaic acid and 100 nM CSA. Cells were pre-treated for 10 min with vehicle or with 2 μM PMA and permeabilized with 100 μg/ml saponin. Ambient Ca²⁺ concentrations were monitored at 37 °C using a Hitachi F-2000 spectrofluorometer (Hitachi Scientific Instruments Inc.) with alternative excitation wavelengths of 340 and 380 nm and with emission wavelength of 510 nm. Each record was calibrated by the addition of a known amount of CaCl₂ to the mixture. At the end of each recording, maximal fluorescence ratio (*R*_{max}) and minimal fluorescence ratio (*R*_{min}) were determined by adding successively an excess of CaCl₂ (1.8 mM) and 30 mM EGTA. Free Ca²⁺ concentrations were calculated according to Grynkiewicz *et al.* (1985).

Statistical analysis

All experiments were performed at least thrice. Means ± s.d. were calculated based on three to five independent experiments. Data were analysed statistically using Student's *t*-test. Results were considered statistically significant when *P* < 0.05.

Results

IP₃R-2 is abundantly expressed in AR4-2J cells

Using an immunoprecipitation and Coomassie blue staining approach, we evaluated the relative expression level of IP₃Rs subtypes in AR4-2J cells. Anti-IP₃R-1 antibody immunoprecipitated a small amount of IP₃R-1 (higher band migrating with an *M*_r of 260 kDa) together with a similar amount of IP₃R-2 (lower band migrating with an *M*_r of 230 kDa; Fig. 1A, lane R1). Anti-IP₃R-2 antibody immunoprecipitated a large amount of IP₃R-2 together with a small amount of IP₃R-1 (Fig. 1A, lane R2). Anti-IP₃R-3 antibody did not immunoprecipitate any significant amount of IP₃R (data not shown). The identities of IP₃R subtypes were confirmed by immunoblotting with selective anti-IP₃R antibodies and by partial sequencing using a Q-TOF mass spectrometry approach (data not shown). Fig. 1B shows the quantitative densitometric evaluation of data shown in Fig. 1A. Given the relative intensities of the different bands, these results suggest that the whole population of IP₃R-1 forms hetero-tetrameric Ca²⁺ channels with IP₃R-2 and that the major proportion of IP₃R-2 forms homo-tetrameric channels. As previously described (Wojcikiewicz 1995), these results confirm that AR4-2J cells express in large majority the IP₃R-2 subtype and thus constitute a good model to study the pharmacological and functional properties of IP₃R-2.

Figure 1 IP₃Rs expressed in AR4-2J cells. (A) IP₃Rs from 20 × 10⁶ AR4-2J cells were solubilized with Triton X-100, immunoprecipitated (IP) with selective anti-IP₃R-1 (R1) or anti-IP₃R-2 (R2) antibodies, resolved by SDS-PAGE (4%) and stained with Coomassie blue. (B) Densitometric analysis of the bands shown in A. IP₃R-1 (solid bar), IP₃R-2 (open bar). These typical results are representative of three independent experiments.

In vitro phosphorylation of IP₃R-2

After solubilization in lysis buffer, IP₃Rs from AR4-2J cells were immunoprecipitated with anti-IP₃R-2 antibody and phosphorylated *in vitro* as described in Materials and Methods. The material was resolved on a 5% SDS-PAGE. Fig. 2A (upper panel) shows that IP₃R-2 was a good substrate for PKC. Phosphorylation of IP₃R-2 occurred in a time-dependent manner reaching a maximal level within 15 min. As expected, when PKC was omitted, no significant phosphorylation of IP₃R-2 occurred (Fig. 2A, upper panel, lane 1). Coomassie blue staining of the same gel indicates that an equal amount of IP₃R-2 was loaded in each lane (Fig. 2A, lower panel). Coomassie blue staining was routinely used in further experiments to ensure that equal amounts of IP₃Rs were loaded on each gel lane. To determine the stability of

IP₃R-2 phosphorylation under our *in vitro* conditions, after maximal phosphorylation with PKC, IP₃R-2 was washed and re-incubated in phosphorylation buffer without PKC. Under these conditions, no significant dephosphorylation of IP₃R-2 was detected for as long as 60 min (Fig. 2B). The *in vitro* phosphorylation assay was done with different PKC isoforms. Interestingly, the conventional PKC isoforms α , β I and γ were the most efficient to phosphorylate IP₃R-2 (Fig. 2C). These isoforms are activated by Ca²⁺ and diacylglycerol, two second messengers generated by activation of phospholipase C. Therefore, these PKC isoforms are the most susceptible to feedback on the IP₃Rs during cell stimulation with a Ca²⁺-mobilizing hormone. Together, these results clearly indicate that IP₃R-2 is a good substrate for conventional PKC isoforms.

Figure 2 *In vitro* phosphorylation of IP₃R-2. IP₃Rs from 20×10⁶ AR4-2J cells were solubilized with Triton X-100, immunoprecipitated with anti-IP₃R-2 antibody, incubated at 30 °C with PKC in the presence of [³²P]-ATP, and resolved by SDS-PAGE (5%). (A) The phosphorylation of IP₃R-2 for different time periods with a PKC preparation from rat brain (3 U/ml). In (B) IP₃R-2 was phosphorylated *in vitro* for 30 min with PKC, washed with phosphorylation buffer to remove PKC and incubated at 30 °C for different time periods to verify the stability of the phosphorylation. (C) The *in vitro* phosphorylation of IP₃R-2 for 30 min at 30 °C with different recombinant PKC isozymes (3 U/ml). In each panel, the upper shows the autoradiogram and the lower shows the Coomassie blue staining of the same bands. These typical results are representative of at least three independent experiments.

Phosphorylation of IP₃R-2 in intact AR4-2J cells

AR4-2J cells were metabolically labelled with [³²P]P_i and stimulated for different time periods with PMA, a direct activator of PKC. Under basal conditions, IP₃R-2 was already phosphorylated (Fig. 3A, time 0), however, its level of phosphorylation was increased after stimulation of AR4-2J cells with PMA (Fig. 3A). Densitometric analysis of band intensities revealed that treatments with PMA for 10 or 15 min significantly increased the phosphorylation of IP₃R-2 (Fig. 3B). AR4-2J cells endogenously express different Gq-coupled receptors for the Ca²⁺ mobilizing agonists CCh, cerulein (Cer) and ATP. These receptors can activate phospholipase C that produces the PKC activator diacylglycerol. Fig. 3C shows that the phosphorylation of IP₃R-2 was increased upon stimulation of AR4-2J cells with Ca²⁺ mobilizing agonists. Densitometric analysis of these results (Fig. 3D) revealed that CCh and Cer significantly increased the phosphorylation of IP₃R-2, whereas the minor effect of ATP was not statistically significant (likely due to a low level of purinergic receptor expression in our cell line). Together, these results suggest that endogenous PKC can phosphorylate IP₃R-2 in AR4-2J cells.

Effect of PKC on IP₃-induced Ca²⁺ release in permeabilized AR4-2J cells

We used a direct IP₃-induced Ca²⁺ release assay that measures ambient Ca²⁺ concentrations by fura2 fluorescence to study the functional consequences of IP₃R phosphorylation by

PKC. Fig. 4A (upper panel) shows a typical experiment where 0.1 μM IP₃ released 4.0 nmol Ca²⁺ from permeabilized AR4-2J cells (the amount of Ca²⁺ released was calibrated by the exogenous addition of 4 nmol CaCl₂). In PKC-treated permeabilized cells (Fig. 4A, lower panel), 0.1 μM IP₃ released 2.5 nmol Ca²⁺, an amount which is smaller than in untreated cells. Histograms shown in Fig. 4B indicate that PKC-treated permeabilized AR4-2J cells released significantly less Ca²⁺ than untreated cells in response to 0.1 μM IP₃. These results show that PKC decreases IP₃-induced Ca²⁺ release in AR4-2J cells.

The effect of PKC on IP₃-induced Ca²⁺ release was also investigated using a slightly different protocol. Intact AR4-2J cells were pre-treated with PMA or vehicle and then were permeabilized with saponin and their IP₃-induced Ca²⁺ release activity was evaluated as described above. Under these conditions, the successive addition of increasing doses of IP₃ released increasing amounts of Ca²⁺ (Fig. 5A and B). The dose-response curve for IP₃-induced Ca²⁺ release from PMA-treated cells showed a rightward shift (EC₅₀ of 0.22 ± 0.03 μM, average and s.d. of three independent experiments, *P* < 0.05) compared with the dose-response curve for IP₃-induced Ca²⁺ release from control cells (EC₅₀ of 0.15 ± 0.02 μM; Fig. 5C). In paired experiments (similar to those shown in Fig. 4A), there was no significant difference in the amount of Ca²⁺ released by untreated and by PMA-treated permeabilized cells at a maximal dose of IP₃. It is also important to note that there was no significant difference in the amount of Ca²⁺ released by ionomycin between PMA-treated or control cells (Fig. 5A and B). These results suggest

Figure 3 *In cellulo* phosphorylation of IP₃R-2. AR4-2J cells were labelled for 5 h with [³²P]P_i and then stimulated for different time periods with 2 μM PMA (A) and (B) or for 3 min with different agonists: 100 μM carbachol (CCh), 10 nM cerulein (Cer) or 100 μM ATP (C) and (D). After solubilization in lysis buffer, IP₃R-2 (arrow) was immunoprecipitated, resolved SDS-PAGE (4%) and autoradiographed. (A) and (C) show the autoradiograms (B) and (D) the densitometric analysis (mean ± s.d. **P* < 0.05 compared with control) from three independent experiments.

Figure 4 PKC decreases IP₃-induced Ca^{2+} release. Permeabilized AR4-2J cells (5×10^7 cells/assay) were permeabilized in a cytosol-like medium containing $0.5 \mu\text{M}$ fura2 acid and $100 \mu\text{g/ml}$ saponin. Cells were treated for 5 min with vehicle or with PKC (0.5 IU/ml). IP₃-induced Ca^{2+} release was measured as described in Materials and Methods. (A) Typical traces where a dose of $0.1 \mu\text{M}$ IP₃ partially releases stored Ca^{2+} from vehicle-treated (upper) or PKC-treated cells (lower). The amount of Ca^{2+} released was calibrated by addition of 4 nmol exogenous Ca^{2+} (C). (B) Average Ca^{2+} released by $0.1 \mu\text{M}$ IP₃ means $\pm$ s.d. of eight independent experiments; * $P < 0.05$), for cells pre-treated with vehicle (solid bar) or with PKC (open bar).

that PKC attenuates IP₃-induced Ca^{2+} release by decreasing the apparent affinity of IP₃R-2.

Effect of PKC on Ca^{2+} movements within AR4-2J cells

To determine the functional consequence of PKC-mediated phosphorylation, we also evaluated the cytosolic Ca^{2+} concentration in intact AR4-2J cells loaded with fura2/AM. Fig. 6A shows that when cells were incubated in a medium prepared without Ca^{2+} (nominally Ca^{2+} -free), a low dose of the Ca^{2+} -mobilizing agonist CCh (CCh, $3 \mu\text{M}$) produced a transient Ca^{2+} increase with a peak amplitude of $135 \pm 25 \text{ nM}$ Ca^{2+} . After a 5-min pre-treatment with $2 \mu\text{M}$ PMA, the effect of $3 \mu\text{M}$ CCh was very importantly reduced to a small transient with peak amplitude of $48 \pm 19 \text{ nM}$ Ca^{2+} . The effect of PMA on CCh-induced intracellular Ca^{2+} mobilization was completely abolished after a pre-treatment with the PKC inhibitor GF1 (Fig. 6C). AR4-2J cells are sensitive to epidermal growth factor (EGF), which activates a receptor-tyrosine kinase that in turn activates phospholipase C γ and produces IP₃. Fig. 6B shows that, in a nominally Ca^{2+} -free medium, a high dose of EGF (100 ng/ml) caused a relatively weak Ca^{2+} increase with a peak amplitude of $30 \pm 8 \text{ nM}$ Ca^{2+} . After a 5-min pre-treatment with PMA, 100 ng/ml EGF produced a barely detectable Ca^{2+} transient with a peak amplitude of $7 \pm 5 \text{ nM}$ Ca^{2+} (Fig. 6B). Because this series of experiments was done in the absence of extracellular Ca^{2+} , it is likely that the Ca^{2+} transients produced by CCh and EGF were due to IP₃-induced Ca^{2+} release from intracellular stores.

Our dynamic video imaging system allows single cell responses to be recorded. When the Ca^{2+} movements were investigated at the single cell level, different types of responses were observed. In upgrading intensity, cells either did not respond (NR) at all to $3 \mu\text{M}$ CCh, or responded with rapid Ca^{2+} oscillations that lasted for about 2 min (as illustrated in Fig. 7A) or responded with a single Ca^{2+} transient of high amplitude (as illustrated in Fig. 7B). After a 5-min pre-treatment with PMA, the proportion of cells that did NR to $3 \mu\text{M}$ CCh went from 1.3 ± 2.3 to $43.6 \pm 10.3\%$, whereas the proportion of cells that showed an oscillatory pattern went from 27.0 ± 3.8 to $14.5 \pm 4.1\%$ and the proportion of cells that showed a single transient went from 71.6 ± 2.8 to $41.9 \pm 13.6\%$ (Fig. 7C). These results show that PMA decreases the response intensity of single cells stimulated with $3 \mu\text{M}$ CCh. A 15-min pre-treatment with GF1 did not modify the responses of cells pre-treated with vehicle but completely abolished the effect of PMA (Fig. 7C). Again, these results were obtained with intact cells bathed in an extracellular medium that did not contain extracellular Ca^{2+} , therefore it is likely that the single cell Ca^{2+} responses elicited by CCh were due to IP₃-induced Ca^{2+} release from intracellular stores.

Discussion

Despite their relatively high sequence homology, the three IP₃R subtypes differ in many points including their affinity for IP₃, their intracellular location, their tissue distribution and possibly their regulatory mechanisms and their specific physiological roles (Patel *et al.* 1999, Patterson *et al.* 2004).

Figure 5 PKC decreases the apparent affinity of IP₃R in permeabilized AR4-2J cells. Intact AR4-2J cells were pre-treated for 10 min with vehicle (A) or with PMA (B), permeabilized with saponin in a cytosol-like medium containing 0.5 μ M fura2 acid and then stimulated with increasing doses of IP₃. The amount of Ca^{2+} released was calibrated by adding a known amount (6 nmol) of exogenous Ca^{2+} (C) and the total content of the Ca^{2+} pool was released with 2 μ M Ionomycin (I₀). (C) The dose-response curves for IP₃-induced Ca^{2+} released from permeabilized AR4-2J cells pre-treated with PMA (solid symbols) or with vehicle (open symbols). These typical results are representative of results obtained in at least three independent experiments.

For example, neurons express almost exclusively the IP₃R-1 which plays crucial roles in neuronal plasticity, brain development and motor coordination (Itoh *et al.* 2001, Ogura *et al.* 2001), whereas the digestive system expresses predominantly the IP₃R-2 and IP₃R-3 (Futatsugi *et al.* 2005). It is therefore important to characterize the specific pharmacological properties and the regulatory mechanisms of each IP₃R subtype. In the study presented here, we used AR4-2J cells that express mostly the IP₃R-2 and evaluated the regulatory effect of PKC on this specific IP₃R subtype. Using a direct *in vitro* phosphorylation assay, we demonstrated that IP₃R-2 is a good substrate for conventional PKC isozymes. We

also demonstrated that endogenous PKC phosphorylates IP₃R-2 in intact AR4-2J cells. Previous studies demonstrated that IP₃R is a substrate for PKC but these studies were done with cells or tissues expressing exclusively the IP₃R-1 subtype (Cameron *et al.* 1995), or a mixture of all three IP₃R subtypes (Matter *et al.* 1993, Poirier *et al.* 2001). A recent study reported that full-length mouse IP₃R-1 and full-length rat IP₃R-3 expressed in Sf9 insect cells are good substrates for PKC (Vermassen *et al.* 2004). To our knowledge, we are the first to report that IP₃R-2 is a substrate for PKC.

Using two different protocols, we directly evaluated the functional consequence of IP₃R-2 phosphorylation by

Figure 6 PKC decreases carbachol-induced Ca^{2+} response in intact AR4-2J cells. AR4-2J cells were loaded with fura2/AM (0.15 μ M) and bathed in a nominally Ca^{2+} -free HBSS. Cells were imaged using a Zeiss Axiovert microscope (Prior Scientific Inc., Rockland, MA, USA; 40 $\times$ oil immersion objective) coupled to an Attolfluor imaging system (Attolfluor Inc.). (A) and (B) show representative recordings of intracellular Ca^{2+} levels before and after the addition of 3 μ M CCh (A) or of 100 ng/ml EGF (B) to cells pre-treated for 5 min with vehicle (grey trace) or with 2 μ M PMA (black trace). Each trace shows the average response of 60–80 cells per microscope field. (C) The average Ca^{2+} responses to 3 μ M CCh (means $\pm$ s.d. * $P < 0.05$) obtained from three independent experiments, for cells pre-treated for 5 min with vehicle, or with 2 μ M PMA, and/or for 15 min with 100 nM GF1.

PKC. In the first protocol, saponin-permeabilized AR4-2J cells were treated with exogenous PKC and their IP₃-induced Ca^{2+} release activity was measured. In the second protocol, intact AR4-2J cells were pre-treated with PMA and then permeabilized and their IP₃-induced Ca^{2+} release activity was measured. Both protocols revealed that PKC decreases IP₃-induced Ca^{2+} release. Because PKC did not affect the ionomycin-sensitive Ca^{2+} pool, the diminished Ca^{2+} release must be attributed to a diminution of IP₃R-2 activity. IP₃ dose-response curves suggested that PKC decreases the apparent affinity of IP₃R-2 in AR4-2J cells. Using intact AR4-2J cells incubated in a Ca^{2+} -free medium, we showed that PKC reduces the global Ca^{2+} response induced by CCh and by EGF, two agonists that produce IP₃ through different pathways. A thorough review of the literature reveals conflicting results regarding the regulatory effect of PKC on intracellular Ca^{2+} release. On the one hand, a few studies concluded that PKC increases IP₃-induced Ca^{2+} release. In nuclei isolated from rat liver (unknown composition of IP₃R/channel), PKC enhanced

IP₃-induced Ca^{2+} release (Matter *et al.* 1993). In bovine adrenal glomerulosa cells, which express all three subtypes of IP₃R (but a large majority of IP₃R-1), PKC enhanced IP₃-induced Ca^{2+} release (Poirier *et al.* 2001). In rat brain microsomes, a tissue containing exclusively the IP₃R-1 subtype, PKC also increased IP₃-induced Ca^{2+} release (Cameron *et al.* 1995). On the other hand, in permeabilized rabbit pancreatic acinar cells (unknown composition of IP₃R/channel), PKC decreased IP₃-induced Ca^{2+} release (Willems *et al.* 1989). In a recent study, we showed that PKC decreases IP₃-induced Ca^{2+} release in RINm5F cells that express almost exclusively the IP₃R-3 subtype (Caron *et al.* 2007). These results suggest that the regulatory effect of PKC is qualitatively different depending on the specific subtype of IP₃R phosphorylated. To our knowledge, we are the first to clearly show that PKC decreases IP₃-induced Ca^{2+} release in a tissue expressing almost exclusively the IP₃R-2 subtype.

Using intact AR4-2J cells bathed in a Ca^{2+} -free medium, we consistently observed that the effect of PKC

Figure 7 PKC modifies carbachol-induced Ca^{2+} responses in single AR4-2J cells. AR4-2J cells were loaded with fura2/AM (0.15 μ M), bathed in a nominally Ca^{2+} -free HBSS and then stimulated with 3 μ M CCh. (A) A typical example of CCh-induced Ca^{2+} oscillations. (B) A typical example of CCh-induced Ca^{2+} transient. (C) The proportion of cells that did not respond (NR) or that responded to 3 μ M CCh with Ca^{2+} oscillations or with a Ca^{2+} transient, after a 5-min pre-treatment with vehicle or with PMA (2 μ M), and/or after a 15-min pre-treatment with GF1 (100 nM; means $\pm$ s.d. * $P < 0.05$). These results were obtained with at least 200 single cells, in three independent experiments.

on CCh- and EGF-induced Ca^{2+} responses was more important than its effect on IP₃-induced Ca^{2+} release from permeabilized cells. These results suggest that PKC may down-regulate more than one component of the intracellular Ca^{2+} signalling cascade. Indeed, several studies have shown that PKC is responsible for the desensitization of some Gq-coupled receptors (Lynch *et al.* 1985, Biden *et al.* 1988), whereas other studies showed that PKC may disrupt the interaction between the G protein and phospholipase C (Orellana *et al.* 1987, Smith *et al.* 1987). In the same way, PKC has been shown to inhibit the tyrosine kinase activity of EGF receptor, thus down-regulating the IP₃/PLC γ pathway (Davis 1988, Lund *et al.* 1990). A recent study also showed that PKC negatively regulates the activity of TRPC3, TRPC4 and TRPC5, which are Ca^{2+} channels responsible for the capacitative Ca^{2+} entry into cells (Venkatachalam *et al.* 2003). Interestingly, PKC is exerting an inhibitory effect on all these components of the Ca^{2+} signalling cascade (Gq-coupled receptors, Gq coupling, tyrosine kinase receptors, Ca^{2+} entry channels). Therefore, by acting at different levels of the Ca^{2+} signalling cascade,

PKC is providing diverse negative feedback loops that may produce additive inhibitory effects on intracellular Ca^{2+} increase. These effects of PKC are likely required to protect the cells against deleterious effects of sustained elevation of Ca^{2+} .

In conclusion, we have shown that PKC phosphorylates IP₃R-2, thus diminishing its sensitivity to IP₃. This regulatory effect of PKC is a further negative feedback mechanism through which PKC controls intracellular Ca^{2+} elevation.

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