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cAMP-Dependent Protein Kinase Enhances Inositol 1,4,5-Trisphosphate-Induced Ca^{2+} Release in AR4-2J Cells

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Abstract In non-excitable cells, the inositol 1,4,5-trisphosphate receptor (IP_3R), a ligand-gated Ca^{2+} channel, plays an important role in the control of intracellular Ca^{2+}. There are three subtypes of IP_3R that are differentially distributed among cell types. AR4-2J cells express almost exclusively the IP_3R-2 subtype. The purpose of this study was to investigate the effect of cAMP-dependent protein kinase (PKA) on the activity of IP_3R-2 in AR4-2J cells. We showed that immunoprecipitated IP_3R-2 is a good substrate for PKA. Using a back-phosphorylation approach, we showed that endogenous PKA phosphorylates IP_3R-2 in intact AR4-2J cells. Pretreatment with PKA enhanced IP_3-induced Ca^{2+} release in permeabilized AR4-2J cells. Pretreatment with the cAMP generating agent's forskolin and vasoactive intestinal peptide (VIP) enhanced carbachol (Cch)-induced and epidermal growth factor (EGF)-induced Ca^{2+} responses in intact AR4-2J cells. Our results are consistent with an enhancing effect of PKA on IP_3R-2 activity. This conclusion supports the emerging concept of crosstalk between Ca^{2+} signaling and cAMP pathways and thus provides another way by which Ca^{2+} signals are finely encoded within non-excitable cells. J. Cell. Biochem. 101: 609–618, 2007.

Key words: inositol 1,4,5-trisphosphate receptor; phosphorylation; protein kinase A; calcium

Stimulation of a wide variety of cells by hormones, neurotransmitters, or growth factors leads to the activation of phospholipase C (PLC) and triggers inositol 1,4,5-trisphosphate (IP_3)-mediated Ca^{2+} signaling via activation of IP_3 receptors (IP_3Rs) on the endoplasmic reticulum [Berridge, 1993; Joseph and Ryan, 1993; Yoshida and Imai, 1997]. Intracellular Ca^{2+} is a key second messenger involved in a vast array of cellular processes such as contraction, secretion, growth, and differentiation. Three specific isoforms of IP_3R, encoded by different genes, have been isolated, with each isoform exhibiting unique tissue-specific expression patterns [Furuichi et al., 1993; Maranto, 1994; Wojcikiewicz, 1995; Perez et al., 1997; Holtzclaw et al., 2002]. Co-immunoprecipitation studies have revealed that IP_3R isoforms can assemble as homotetramers (four identical isoforms) or as heterotetramers (two or three different isoforms within a tetramer) to constitute functional Ca^{2+} channels [Maeda et al., 1991; Monkawa et al., 1995]. Despite sharing considerable homology within their ligand-binding and channel domains, the amino acids sequence of each IP_3R isoform are least conserved in their regulatory domains. Ca^{2+} release through the IP_3R is markedly influenced by many factors such as Ca^{2+} itself, proteins, adenine nucleotides, and phosphorylation [Patterson et al., 2004]. These features are thought to be the major determinants of the rich diversity of Ca^{2+} signaling events observed in cells.

An emerging body of evidences, suggests that specific regulatory control over Ca^{2+} signaling pathways can be achieved by the simultaneous activation of additional signaling pathways, in particular those that elevate cAMP [Volpe and Alderson-Lang, 1990; Hajnoczky et al., 1993; Bruce et al., 2003]. Several studies have shown

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an important regulatory effect of cAMP-dependent protein kinase (PKA) on the activity of IP_3R-1 [DeSouza et al., 2002; Tang et al., 2003; Soulsby et al., 2004; Tu et al., 2004; Wagner et al., 2004]. Although these recent studies suggest that PKA increases the Ca^{2+} release sensitivity of IP_3R-1, little is known about the effect of PKA on IP_3R-2 and IP_3R-3.

The purpose of the present study was therefore to investigate the phosphoregulation of IP_3R-2 by PKA in AR4-2J cells, a pancreatic acinar cell line which expresses predominantly (86%) the IP_3R-2 isoform [Wojcikiewicz, 1995]. Our results demonstrate that IP_3R-2 is efficiently phosphorylated by exogenous and endogenous PKA. Pretreatment of AR4-2J cells with PKA or with cAMP generating agents enhanced IP_3-induced Ca^{2+} release and Cch- or EGF-induced Ca^{2+} responses. These results are consistent with a potentiating effect of PKA on IP_3R-2 activity.

MATERIALS AND METHODS

Materials

Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), and penicillin–streptomycin–glutamine were from Gibco Life Technologies (Gaithersburg, MD). [γ-^{32}P]ATP was from Perkin Elmer (Boston, MA). IP_3 was from Alexis Biochemicals (San Diego, CA). Anti-IP_3R-2 antibody was obtained by immunizing New Zealand rabbits with the C-terminus of IP_3R-2 coupled to keyhole limpet hemocyanin [Poitras et al., 2000]. Protein AG-agarose was from Santa Cruz Technology (Santa Cruz, CA). Vasoactive intestinal peptide (VIP), forskolin, ATP, bovine serum albumin (BSA), creatine phosphokinase, phosphocreatine, poly-L-lysine hydrobromide, saponin, and thapsigargine were from Sigma-Aldrich (Oakville, ON). The catalytic subunit of cAMP-dependent protein kinase was from Promega (Madison, WI). Carbachol (CCh), fura-2 (free acid), fura-2/AM, phosphatases inhbitors cocktail set II, and okadaic acid were from Calbiochem (San Diego, CA). Proteases inhibitors cocktail (Complete^{TM}) was from Roche Molecular Biochemicals (Laval, QC).

Cell Culture

AR4-2J cells (American Type Culture Collection, Manassas, VA) were cultured at 37°C in a humidified atmosphere of 5% CO_2 in DMEM medium containing L-glutamine (2 mM), FBS (15%, v/v), penicillin (100 U/ml), and streptomycin (100 μg/ml).

In Vitro Phosphorylation of IP_3R-2

AR4-2J cells (2×10^7 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^{TM} (1×). 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-IP_3R-2 antibody and 50 μl of protein AG-agarose beads. The immunoprecipitated IP_3Rs were phosphorylated for different time periods at 30°C in phosphorylation buffer [40 mM Tris-HCl (pH 7.4), 20 mM $MgCl_2$] containing 10 μCi of [γ^{-32}P]ATP, 200 μM non-radioactive ATP, and the catalytic subunit of PKA (100–400 U/ml) in a final volume of 50 μl. The reaction was stopped by adding 1 ml of ice-cold phosphorylation buffer containing 1 mM ATP. Immune complexes were then precipitated by centrifugation for 1 min at 15,000*g*, washed twice with 1 ml of phosphorylation buffer containing 1 mM ATP, and resuspended in Laemmli buffer [Laemmli, 1970] for 10 min at 95°C. Samples were loaded onto a 4% SDS–PAGE gel that was run at 20 mA for 120 min. Gels were vacuum dried for 1 h at 80°C, and exposed for 24 h on a BioMax MR film with an intensifying screen. In some experiments, IP_3R subtypes were identified by immunoblotting with our anti-IP_3R-2 antibody or with the anti-IP_3R-1 antibody obtained from Affinity Bioreagents (Golden, CO). IP_3R subtypes were also identified by partial sequencing using a MALDI-TOF mass spectrometry approach after in-gel trypsin digestion. MALDI-MS mass spectra were recorded on a TofSpec 2E (Waters/Micromass Corporation, Manchester, England) using the delayed extraction and reflector mode. Acceleration and pulse voltages were set at 20,000 and 2,300 V, respectively, and the low-mass gate was 500. Peptide mass searches were performed with the Protein Prospector search engine (http://prospector.ucsf.edu) and the MASCOT algorithm (http://www.matrixscience.com). The latest versions of the NCBI and Swiss-Prot protein databases were used.

Back-Phosphorylation of IP_3R-2

AR4-2J cells were stimulated with 10 μM forskolin in HBSS buffer [15 mM HEPES (pH 7.4), 140 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 10 mM dextrose, 1.8 mM $CaCl_2$, 0.1% (w/v) BSA]. Cells were then solubilized in 1 ml of ice-cold lysis buffer supplemented with 100 nM okadaic acid, (1×) phosphatases inhibitors cocktail set II, and (1×) proteases inhibitors cocktail Complete™. IP_3R-2 was then immunoprecipitated and phosphorylated in vitro as described above. In the back-phosphorylation procedure, it must be understood that an important in vivo phosphorylation of IP_3R-2 in treated AR4-2J cells will markedly diminish the level of incorporation of ^{32}Pi in the in vitro phosphorylation assay.

Dynamic Video Imaging of Cytosolic Ca^{2+}

Fluorescence from fura 2-loaded cells was monitored as previously described [Auger-Messier et al., 2003]. Briefly, AR4-2J cells grown on glass coverslips were washed twice with HBSS buffer and loaded with 0.15 μM fura-2/AM in HBSS buffer for 20 min at room temperature in the dark. After a de-esterification step (incubation in fresh HBSS for 20 min at room temperature) the coverslips were inserted into a circular open-bottom chamber and mounted on the stage of a Zeiss Axiovert microscope fitted with an Attofluor Digital Imaging and Photometry System (Attofluor, Inc., Rockville, MD). The system allows simultaneous data acquisition from up to 99 user-defined variably sized regions of interest (or cells) per field of view. As shown in the manuscript, among these single cells, some may not respond, some may show Ca^{2+} oscillations, and some may produce a single Ca^{2+} transient. Very interestingly, the same Attofluor system can calculate the average response of all the cells on the field. The average response is the mean fluorescence value calculated from individual fluorescence values obtained for all the selected cells in the field, at any time point. Average responses usually show Ca^{2+} transients of different intensities because all the cells start their response at about the same time but later on, oscillating cells usually lose their synchrony. In our study we have used the single cell application and also the average response application of the Attofluor system. Fluorescence from isolated fura-2-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 are expressed as intracellular free Ca^{2+} concentration (nM) calculated from 334:380 fluorescence ratio according to Grynkiewicz et al. [1985].

IP3-Induced Ca^{2+} Release

AR4-2J cells (5×10^7 cells) were permeabilized in 2 ml of cytosol-like buffer [20 mM Tris-HCl (pH 7.4), 10 mM NaCl, 110 mM KCl, 5 mM KH_2PO_4, and 2 mM $MgCl_2$] supplemented with 50 μg/ml saponin, 0.5 μM fura-2 acid, 20 Units creatine kinase, and 20 mM phosphocreatine. Fura-2 fluorescence was monitored at 37°C with a Hitachi F-2000 spectrofluorometer (Hitachi Scientific Instruments, Inc., Hialeah, FL) with alternative excitation wavelengths of 340 and 380 nm and with emission wavelength of 510 nm. The amount of Ca^{2+} released by IP_3 was calculated according to the fluorescent signal obtained with an exogenously added Ca^{2+} standard (4 nmol $CaCl_2$). At the end of each recording, maximal fluorescence ratio (R_{max}) and minimal fluorescence ratio (R_{min}) were determined by adding successively 2 mM Ca^{2+} and 10 mM EGTA to the cells suspension. When needed, free Ca^{2+} concentration was calculated according to Grynkiewicz et al. [1985].

Data Analysis

Results are presented as the mean ± standard deviation. When needed, data were analyzed by a Student's *t*-test. *P* values of <0.05 were considered statistically significant (*).

RESULTS

Phosphorylation of IP_3R-2

Previous studies have shown that AR4-2J cells express predominantly (86%) the IP_3R-2, making them a convenient model to study the pharmacological and functional properties of this specific IP_3R subtype [Wojcikiewicz, 1995]. IP_3Rs from AR4-2J cells were solubilized in lysis buffer, immunoprecipitated with anti-IP_3R-2 antibody, and phosphorylated in vitro as described in Materials and Methods section. Figure 1A shows that within a period of 30 min, efficient phosphorylation of IP_3R-2 was obtained with a relatively high concentration of PKA (400 U/ml). The bands migrating with

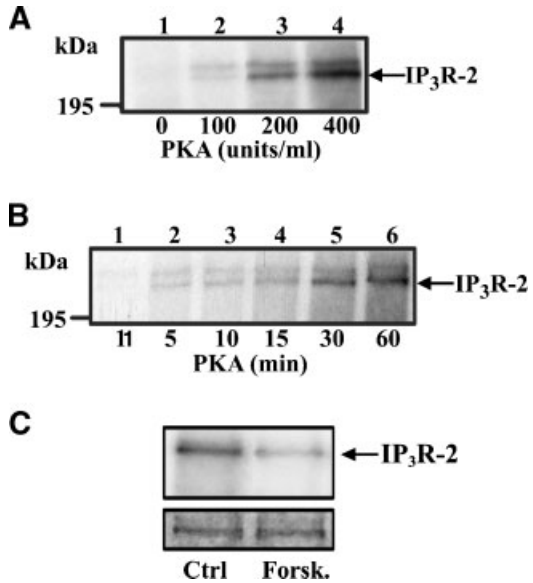

Fig. 1. Phosphorylation of IP_3R-2. IP_3R-2 from 2×10^7 AR4-2J cells was solubilized in lysis buffer, immunoprecipitated with anti-IP_3R-2 antibody, phosphorylated in vitro with the catalytic subunit of PKA, and analyzed by 4% SDS–PAGE and autoradiography, as described in Materials and Methods section. In **panel A**, IP_3R-2 was phosphorylated in vitro for 30 min with increasing amounts of PKA. In **panel B**, IP_3R-2 was phosphorylated in vitro with exogenous PKA (400 U/ml), for different time periods. These results are representative of three independent experiments. In **panel C**, AR4-2J cells were pre-treated with 10 μM forskolin before solubilization in lysis buffer. IP_3R-2 was then immunoprecipitated and phosphorylated in vitro. Proteins were then resolved on a 4% SDS–PAGE gel and autoradiographed (**upper panel**). The **lower panel** shows the Coomassie blue staining of the same bands. The results shown in panel C are representative of four independent experiments.

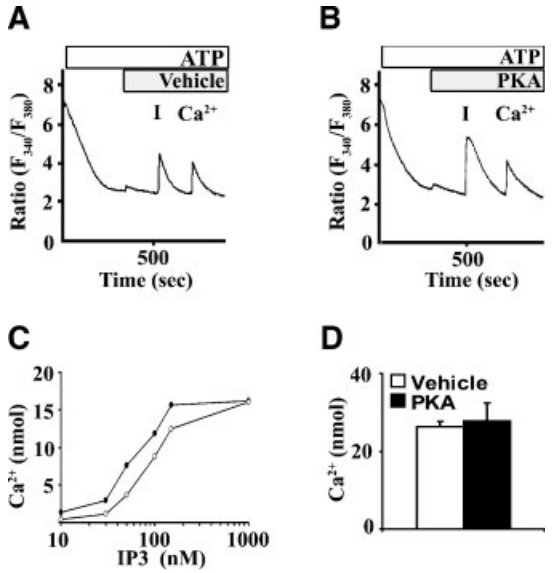

Fig. 2. PKA increases IP_3-induced Ca^{2+} release in permeabilized AR4-2J cells. AR4-2J cells (5×10^7) were permeabilized with 100 μg/ml saponin and pre-treated for 2 min with vehicle (**panel A**) or 400 U/ml PKA (**panel B**) in a cytosol-like medium containing 0.5 μM fura-2 acid. Ca^{2+} was then partially released with 0.1 μM IP_3 (I). The amount of Ca^{2+} released by IP_3 was calibrated by adding 4 nmoles of exogenous Ca^{2+} (Ca^{2+}). These typical traces are representative of at least three independent experiments. **Panel C** shows the dose-response curves (under the same experimental conditions) for IP_3-induced Ca^{2+} release from permeabilized untreated (open circle) or PKA-treated (filled circle) AR4-2J cells. **Panel D** shows the total content of the Ca^{2+} pool released with 2 μM ionomycin from permeabilized AR4-2J cells pre-treated with vehicle (empty bar) or with PKA (filled bar). The results shown in panels C and D are representative of five independent experiments.

Mrs 260 and 230 kDa were identified as IP_3R-1 and IP_3R-2, respectively, by partial sequencing using a MALDI-TOF mass spectrometry approach and by immunoblotting (data not shown). Figure 1B shows that in vitro phosphorylation of IP_3R-2 (with 400 U/ml exogenous PKA) was detectable within 5 min and reached a high level by 60 min. These results clearly indicate that exogenous PKA can phosphorylate IP_3R-2. Next, we used a back-phosphorylation procedure to determine whether endogenous PKA can phosphorylate IP_3R-2. Intact AR4-2J cells were first stimulated for 1 min with forskolin, an indirect activator of PKA. IP_3R-2 was then solubilized, immunoprecipitated, and phosphorylated in vitro with exogenous PKA and [γ-^{32}P] ATP. Figure 1C shows a robust PKA-mediated back-phosphorylation of IP_3R-2 that had been immunoprecipitated from untreated AR4-2J cells, indicating that very little phosphorylation occurs under basal conditions (Fig. 2C, control). After 1 min pre-treatment with forskolin (Fig. 2C, Forsk.), back-phosphorylation with PKA decreased significantly, suggesting that forskolin increases IP_3R-2 phosphorylation by PKA in intact AR4-2J cells.

PKA Enhances IP_3-Induced Ca^{2+} Release in AR4-2J Cells

We used a direct IP_3-induced Ca^{2+} release assay that measures ambient Ca^{2+} concentrations by fura-2 fluorescence to study the functional consequences of PKA phosphorylation. Figure 2A shows a typical experiment where 0.1 μM IP_3 released 4.5 nmol of Ca^{2+} from permeabilized AR4-2J cells. The amount of Ca^{2+} released was calibrated by the exogenous addition of 4 nmol $CaCl_2$. In PKA-treated permeabilized cells (Fig. 2B), 0.1 μM IP_3 released 6.8 nmol of Ca^{2+}, an amount which is

larger than in untreated cells. These results indicate that PKA enhances IP_3-induced Ca^{2+} release in AR4-2J cells. The dose-response curve for IP_3-induced Ca^{2+} release from PKA-treated cells showed a leftward shift (EC_{50} of 58 ± 6 nM, average and standard deviation of three experiments) compared to the dose-response curve for IP_3-induced Ca^{2+} release from control cells (EC_{50} of 92 ± 10 nM; Fig. 2C). In paired experiments (similar to those shown in panels 2A and B), there was no significant difference in the amount of Ca^{2+} released by untreated and PKA-treated permeabilized cells at a maximal dose of IP_3 (Fig. 2C). Treatment with 2 μM ionomycin (Fig. 2D) or with 2 μM thapsigargin (data not shown) revealed that the IP_3-releasable Ca^{2+} pool of PKA-treated cells was similar to that of vehicle-treated cells. These results suggest that PKA enhances IP_3-induced Ca^{2+} release by increasing the apparent affinity of IP_3Rs.

Forskolin Enhances CCH-Induced Ca^{2+} Responses in Intact AR4-2J Cells

To determine the functional consequence of PKA phosphorylation, we also evaluated the cytosolic Ca^{2+} concentration in intact AR4-2J cells loaded with fura-2/AM. When cells were incubated in a medium prepared without Ca^{2+} (0.5 mM EGTA), a low dose of the Ca^{2+}-mobilizing agonist Cch (1 μM) produced a Ca^{2+} increase with a peak amplitude of 95 ± 13 nM (Fig. 3A). After a 3 min pre-treatment with forskolin, 1 μM Cch produced a much stronger Ca^{2+} increase with a peak amplitude of 135 ± 11 nM (Fig. 3B). Because these experiments were done in the absence of extracellular Ca^{2+}, it is likely that the Ca^{2+} responses produced by Cch were due to IP_3-induced Ca^{2+} release from intracellular stores. These results, which were obtained with intact cells, are consistent with a potentiating effect of PKA on IP_3-induced Ca^{2+} release.

Our dynamic video imaging system allows single cell responses to be recorded. When the Ca^{2+} movements were investigated at the single cell level, two types of responses were observed. At a low dose of Cch (1 μM) $23 \pm 6\%$ of cells did not show any change in their Ca^{2+} concentration whereas $27 \pm 7\%$ of cells responded with a single Ca^{2+} transient of high amplitude (Fig. 4A) and $50 \pm 6\%$ of cells began rapid Ca^{2+} oscillations that lasted for about 3 min (Fig. 4B). At higher doses of Cch, the proportion of cells

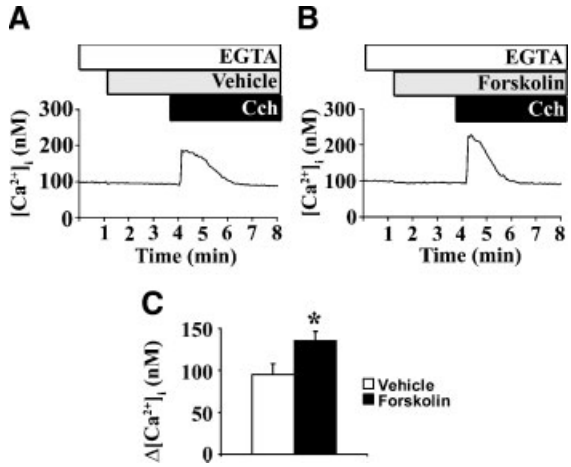

Fig. 3. Forskolin increases Cch-induced Ca^{2+} responses in intact AR4-2J cells. AR4-2J cells were loaded with fura-2/AM and bathed in a Ca^{2+}-free extracellular medium. Cells were imaged using a Zeiss Axiovert microscope (40× oil immersion objective) coupled to an Attofluor imaging system. Representative recordings of intracellular Ca^{2+} levels before and after the addition of 1 μM Cch to cells pre-treated with vehicle (**panel A**, average response of 84 cells), or with forskolin (**panel B**, average response of 61 cells). **Panel C** shows the average Ca^{2+} responses (means ± SD, $*P < 0.05$) obtained from three independent experiments, for cells pretreated with vehicle (empty bar, average response of 244 cells) or with forskolin (filled bar, average response of 195 cells) and stimulated with 1 μM Cch.

that did not respond decreased and the proportion of cells that responded with a single transient increased (Fig. 4C). These results illustrate the graded responses obtained with stimuli of increasing intensity, going from no significant response to an oscillatory pattern and to a single transient of high amplitude. A similarly enhanced response to 1 μM Cch was observed after a pretreatment of AR4-2J cells with 10 μM forskolin. As demonstrated by the average response of a cell population stimulated with Cch (Fig. 3), analysis of single cell responses revealed that forskolin enhances Cch-induced Ca^{2+} responses (Fig. 4D). Indeed, after a pretreatment with forskolin, only $8 \pm 5\%$ of cells did not show any change in their Ca^{2+} concentration whereas $36 \pm 3\%$ of cells began rapid Ca^{2+} oscillations that lasted for about 3 min, and $56 \pm 4\%$ of cells responded with a single Ca^{2+} transient of high amplitude. These results are consistent with a potentiating effect of PKA on IP_3-induced Ca^{2+} release.

Forskolin Enhances EGF-Induced Ca^{2+} Responses In AR4-2J Cells

Epidermal growth factor (EGF) activates a receptor-tyrosine kinase that in turn activates

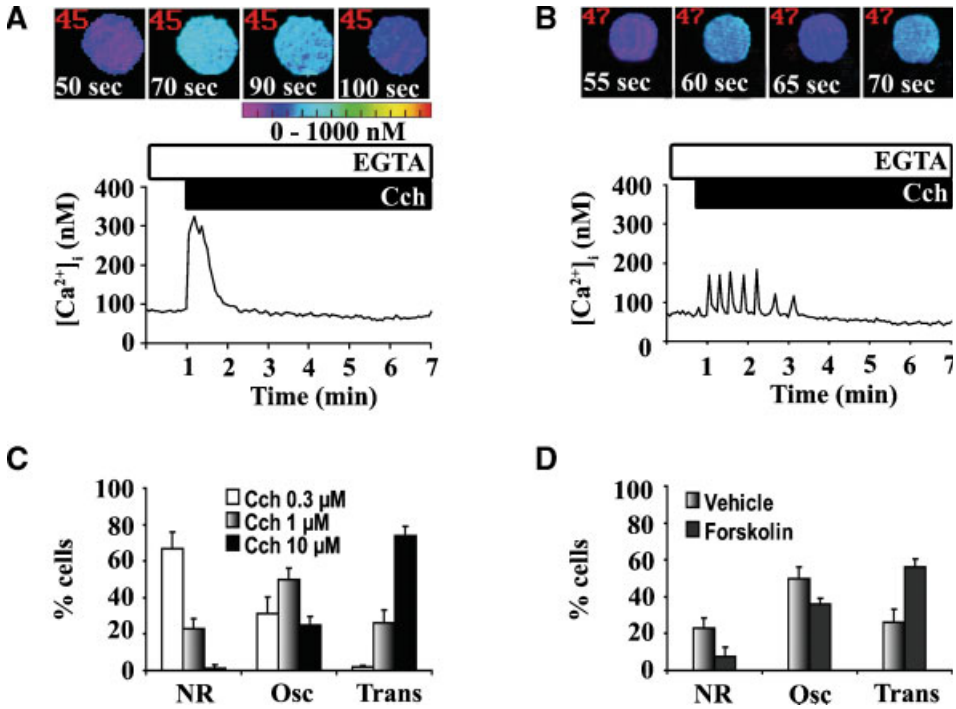

Fig. 4. Cch-induced Ca^{2+} signaling in single AR4-2J cells loaded with fura-2/AM, in the absence of extracellular Ca^{2+}. **A**: It shows serial images depicting a typical Ca^{2+} transient (according to the pseudocolor scale) within a cell stimulated with 1 μM Cch. The trace below shows the full time Ca^{2+} response of the same cell. **B**: It shows serial images depicting typical Ca^{2+} oscillations within a cell stimulated with 1 μM Cch. The trace below shows the full time Ca^{2+} response of the same cell. **C**: It shows the proportions of cells responding with Ca^{2+} oscillations (Osc), Ca^{2+} transient (Trans) or not responding (NR) to increasing doses of Cch. **D**: It shows the proportions of cells responding with Ca^{2+} oscillations (Osc), Ca^{2+} transient (Trans), or not responding (NR) to 1 μM Cch after a 3 min pre-treatment with vehicle (empty bars) or with forskolin (filled bars). These results are representative of at least three independent experiments.

phospholipase Cβ and produces small amounts of IP_3. In a Ca^{2+}-free medium (0.5 mM EGTA), 200 ng/ml EGF caused a slow, weak Ca^{2+} increase with a peak amplitude of 45 ± 9 nM (Fig. 5A). After a 2 min pre-treatment with 10 μM forskolin, EGF produced a stronger Ca^{2+} increase with a peak amplitude of 65 ± 6 nM (Fig. 5B). These results demonstrate that the potentiating effect of forskolin is due to the regulation of a component common to the distinct Ca^{2+} signaling pathways used by Cch and EGF. These results are consistent with a potentiating effect of PKA on IP_3-induced Ca^{2+} release.

Vip Enhances CCH-Induced Ca^{2+} Responses in AR4-2J Cells

On AR4-2J cells, VIP activates a G-protein-coupled receptor that couples to Gs, elevates cAMP levels, and activates PKA. After a 3 min pretreatment with 1 μM VIP, the Ca^{2+} response produced by 1 μM Cch (Fig. 6A, filled circle) showed a peak amplitude of 106 ± 10 nM (Fig. 6B) a value significantly higher than that observed with untreated cells (Fig. 6A, empty circle) 67 ± 13 nM (Fig. 6B). When these results were analyzed at the single cell level, VIP clearly enhanced Cch-induced Ca^{2+} responses. The proportion of cells that did not respond to Cch (1 μM) went from $33 \pm 7\%$ for untreated cells to $9 \pm 3\%$ for VIP-pretreated cells, the proportion of cells that responded with oscillations went from $49 \pm 7\%$ for untreated cells to $37 \pm 2\%$ for VIP-pretreated cells and the proportion of cells that showed a single transient went from $18 \pm 4\%$ for untreated cells to $55 \pm 4\%$ for VIP-pretreated cells. These results demonstrate that the potentiating effect of PKA activation could also be obtained by the activation G-protein-coupled receptor that couples to Gs. These results are consistent with a potentiating effect of PKA on IP_3-induced Ca^{2+} release.

VIP Enhances Overall CCH-Induced Ca^{2+} Responses in AR4-2J Cells

We also evaluated the functional impact of a treatment with VIP on the overall Ca^{2+}

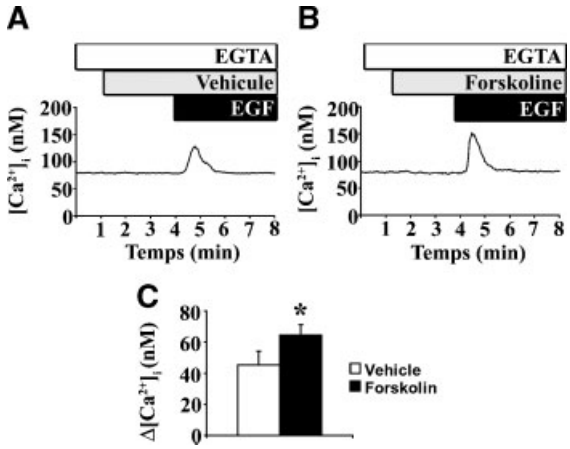

Fig. 5. Forskolin increases EGF-induced Ca^{2+} response in intact AR4-2J cells. AR4-2J cells were loaded with fura-2/AM, bathed in a Ca^{2+}-free extracellular medium, and imaged with the Attofluor system. Representative average Ca^{2+} response, before and after the addition of 200 ng/ml EGF to cells pre-treated with vehicle (**panel A**, average response of 82 cells), or with forskolin (**panel B**, average response of 77 cells). **Panel C** shows data (means ± SD, $*P < 0.01$) obtained from three independent experiments, for cells pretreated for 3 min with vehicle (empty bar, average response of 315 cells) or with 10 μM forskolin (filled bar, average response of 354 cells) and stimulated with 200 ng/ml EGF.

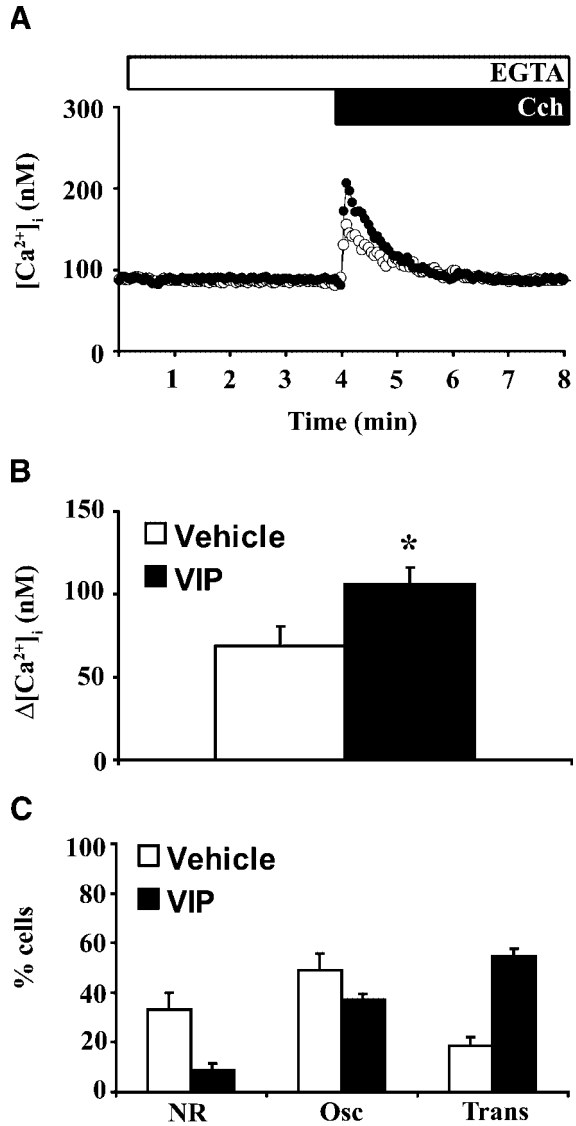

Fig. 6. VIP increases Cch-induced Ca^{2+} response in intact AR4-2J cells AR4-2J cells were loaded with fura-2/AM, bathed in a Ca^{2+}-free extracellular medium, and imaged with the Attofluor system. Representative recordings of intracellular Ca^{2+} levels before and after the addition of 1 μM Cch to cells pre-treated with vehicle (**panel A**, empty circle, average response of 49 cells), or with 1 μM VIP (panel A, filled circle, average response of 44 cells). **Panel B** shows data (means ± SD, $*P < 0.05$) obtained from three independent experiments, for cells pretreated for 3 min with vehicle (empty bar, average response of 138 cells) or with 1 μM VIP (filled bar, average response of 151 cells) and stimulated with 1 μM Cch. **Panel C** shows the proportions of cells responding with Ca^{2+} oscillations (Osc), Ca^{2+} transient (Trans), or not responding (NR) to 1 μM Cch after a 3 min pre-treatment with vehicle (empty bars) or with 1 μM VIP (filled bars). The results presented in panel C are representative of three independent experiments.

response of intact AR4-2J cells incubated in a medium containing a normal (1.8 mM) Ca^{2+} concentration. Under these conditions, agonist-induced intracellular Ca^{2+} movements are due to Ca^{2+} release from the intracellular stores and also to Ca^{2+} entry from the extracellular medium. Figure 7A shows a typical trace where, in the presence of extracellular Ca^{2+}, cells responded to 1 μM Cch by barely detectable baseline Ca^{2+} oscillations of low amplitude. The addition of 1 μM VIP to these cells induced strong oscillations of high amplitude. Under the same conditions few cells responded to 1 μM Cch by producing oscillations of high amplitude (Fig. 7B). The addition of 1 μM VIP to these cells accelerated the oscillatory pattern to generate a large single Ca^{2+} transient. It is interesting to note that the rapidity of the response to VIP (less than 1 min) is consistent with the rapidity of IP_3R-2 phosphorylation within intact AR4-2J cells (Fig. 1C). All together these results demonstrated that the activation of a Gs-coupled receptor enhances Cch-induced Ca^{2+} response in AR4-2J cells. This effect could occur, at least in part, through a potentiating effect of PKA on IP_3-induced Ca^{2+} release.

DISCUSSION

The regulatory domain of IP_3R-2 contains a putative phosphorylation site for PKA (R/K)(R/

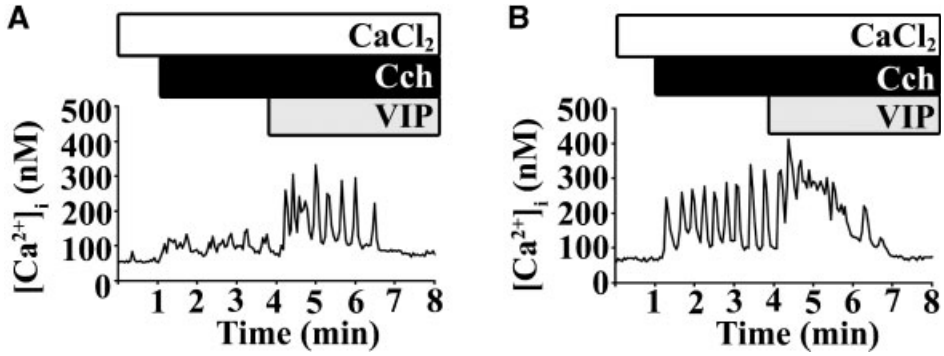

Fig. 7. VIP potentiates the overall Cch-induced Ca^{2+} response of AR4-2J cells. AR4-2J cells were loaded with fura-2/AM, bathed in a normal Ca^{2+} medium (1.8 mM), and imaged with the Attofluor system. Representative recordings of intracellular Ca^{2+} levels before and after the addition of 1 μM Cch followed by 1 μM VIP. The typical trace shown in **panel A** illustrates the response of a single cell that, upon VIP addition, goes from a barely detectable Ca^{2+} elevation to a strong Ca^{2+} oscillatory pattern. The typical trace shown in **panel B** illustrates the response of another single cell that, upon VIP addition goes from a Ca^{2+} oscillatory pattern to a single Ca^{2+} transient of high amplitude. These results are representative of responses obtained with more than 100 cells, in three independent experiments.

K)X(S/T) at serine 1,687, suggesting that it could be a substrate for PKA. In the present study, we used a direct in vitro phosphorylation assay to demonstrate that PKA dose-dependently and time-dependently phosphorylates IP_3R-2. These results support and extend those of a previous study reporting that IP_3R-2 was a relatively poor substrate for PKA [Wojcikiewicz and Luo, 1998]. Using an indirect back-phosphorylation approach, we further showed that IP_3R-2 is phosphorylated by endogenous PKA in AR4-2J cells. Several studies already demonstrated that IP_3Rs are substrates for PKA but these studies were done with tissues expressing almost exclusively the IP_3R-1 subtype or various proportions of the different subtypes of IP_3Rs [Ferris et al., 1991; Hajnoczky et al., 1993; Giovannucci et al., 2000; Wagner et al., 2003; Soulsby and Wojcikiewicz, 2005]. Our results clearly demonstrated that IP_3R-2 is a good substrate for PKA.

There is still no clear consensus on the functional consequences of IP_3R subtype specific phosphorylation by PKA. Several studies concluded that PKA decreases IP_3-induced Ca^{2+} release. In rat brain microsomes, a tissue containing almost exclusively IP_3R-1, PKA decreased IP_3-induced Ca^{2+} release [Supattapone et al., 1988]. In canine tracheal smooth muscle cells (undefined composition of IP_3R-channel), cAMP elevating agents diminished Cch-induced intracellular Ca^{2+} mobilization [Yang et al., 1996]. In mouse pancreatic acinar cells containing a mixture of IP_3R-1, IP_3R-2, and IP_3R-3, PKA reduced the amplitude and frequency of Cch-induced intracellular Ca^{2+} oscillations [Giovannucci et al., 2000] and markedly slowed the kinetics of cholecystokinin-evoked Ca^{2+} oscillations [Straub et al., 2002].

On the contrary, several studies concluded that PKA increases IP_3-induced Ca^{2+} release. In rat hepatocytes (undefined IP_3R/channel composition), $[^3H]IP_3$-binding affinity and IP_3-induced Ca^{2+} mobilization were enhanced by PKA [Hajnoczky et al., 1993; Joseph and Ryan, 1993]. With purified IP_3R-1 reconstituted into lipid vesicles, both the rate and extent of $^{45}Ca^{2+}$ influx into proteoliposomes increased by 20% after incubation with the catalytic subunit of PKA [Nakade et al., 1994]. In DT-40 cells expressing exclusively the IP_3R-1 subtype, it was unambiguously shown that PKA markedly enhances IP_3-induced Ca^{2+} release [Wagner et al., 2003, 2004]. The sensitivity of recombinant IP_3R-1 reconstituted into planar lipid bilayers was increased by fourfold after phosphorylation with PKA [Tang et al., 2003]. In permeabilized SH-SY5Y cells (which contain almost exclusively IP_3R-1), PKA produced a biphasic effect (stimulatory at low concentrations and inhibitory at high concentrations) on IP_3-induced Ca^{2+} release [Dyer et al., 2003]. For many reasons, including cell type-specific expression of IP_3R subtypes and of accessory proteins or factors that can influence the Ca^{2+}-handling machinery, it has been difficult to functionally assess the effect of PKA phosphorylation of IP_3R. Although the most recent studies show that PKA increases the Ca^{2+} release sensitivity of IP_3R-1, little is known about the effect of PKA on IP_3R-2 and IP_3R-3.

Here we showed that PKA enhances IP_3-induced Ca^{2+} release in permeabilized AR4-2J cells. This is a straightforward assay that evaluates the direct effect of IP_3 on its receptor/channel. Because PKA did not modify the content of the thapsigargin- and ionomycin-sensitive Ca^{2+} pool, the enhanced Ca^{2+} release must be due to the enhanced activity of IP_3R. Dose-response curves for IP_3-induced Ca^{2+} release revealed that PKA increases the apparent affinity of IP_3R in AR4-2J cells. It must be noted that AR4-2J cells express a very low level of IP_3R-1 that is also a substrate for PKA (as shown in Fig. 1). Recent evidences suggest that the activity of IP_3R-1 is potentiated by PKA [Nakade et al., 1994; Tang et al., 2003; Wagner et al., 2003, 2004]. However, AR4-2J cells express predominantly the IP_3R-2, and we assume that the Ca^{2+} responses within these cells are mostly due to the activity of IP_3R-2. Therefore, our results suggest that PKA enhances the activity of IP_3R-2.

It was previously shown that VIP and forskolin activate the adenylyl cyclase and PKA in AR4-2J cells [Simeone et al., 1995; Wojcikiewicz and Luo, 1998]. Using intact AR4-2J cells incubated in a Ca^{2+}-free medium, we showed that a pretreatment with forskolin (an indirect activator of PKA) or with VIP (a cAMP elevating agonist) enhanced Cch- and EGF-induced Ca^{2+} elevations. Experiments performed at the single cell level also showed that forskolin and VIP enhance Cch-induced Ca^{2+} responses in AR4-2J cells. Lastly, using intact AR4-2J cells bathing in a normal Ca^{2+}-containing medium, we showed that VIP unequivocally boosted the Ca^{2+} response induced by Cch. Notwithstanding the fact that within intact cells, PKA may have numerous other substrates, these results are consistent with an enhancing effect of PKA on IP_3R-2 activity. The weight of evidences presented here suggests that PKA phosphorylates IP_3R-2 and potentiates Cch-induced Ca^{2+} signaling in AR4-2J cells. These results further support the emerging concept of crosstalk between Ca^{2+} signaling and cAMP pathways and thus provide another way by which Ca^{2+} signals are finely encoded within cells.

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