Acetylcholine evokes an $InsP_3R1$-dependent transient Ca^{2+} signal in rat duodenum myocytes

Nicolas Fritz, Fabrice Dabertrand, Jean Mironneau, Nathalie Macrez, and Jean-Luc Morel

Abstract: In smooth muscle myocytes, agonist-activated release of calcium ions (Ca^{2+}) stored in the sarcoplasmic reticulum (SR) occurs via different but overlapping transduction pathways. Hence, to fully study how SR Ca^{2+} channels are activated, the simultaneous activation of different Ca^{2+} signals should be separated. In rat duodenum myocytes, we have previously characterized that acetylcholine (ACh) induces Ca^{2+} oscillations by binding to its M_2 muscarinic receptor and activating the ryanodine receptor subtype 2. Here, we show that ACh simultaneously evokes a Ca^{2+} signal dependent on activation of inositol 1,4,5-trisphosphate ($InsP_3$) receptor subtype 1. A pharmacologic approach, the use of antisense oligonucleotides directed against $InsP_3R1$, and the expression of a specific biosensor derived from green-fluorescent protein coupled to the pleckstrin homology domain of phospholipase C, suggested that the $InsP_3R1$-dependent Ca^{2+} signal is transient and due to a transient synthesis of $InsP_3$ via M_3 muscarinic receptor. Moreover, we suggest that both M_2 and M_3 signalling pathways are modulating phosphatidylinositol 4,5-bisphosphate and $InsP_3$ concentration, thus describing closely interacting pathways activated by ACh in duodenum myocytes.

Key words: duodenum, calcium signalling, $InsP_3$, acetylcholine, $InsP_3$ receptor.

Résumé : Sous l'action d'un agoniste, la libération des ions de calcium (Ca^{2+}) stockés dans le réticulum sarcoplasmique (SR) des myocytes des muscles lisses se fait par diverses voies de transduction enchevêtrées. Pour bien comprendre les mécanismes d'activation des canaux de calcium dans le SR, on doit isoler les unes des autres les activations simultanées de divers signaux calciques. Dans des travaux antérieurs, nous avons démontré que l'acétylcholine (ACh) entraîne des oscillations du Ca^{2+} en se liant au récepteur muscarinique M_2 et active le sous-type 2 du récepteur de la ryanodine dans les myocytes du duodénum du rat. Dans cette étude, nous démontrons que l'Ach active en même temps l'inositol 1,4,5-trisphosphate ($InsP_3$), sous-type 1 du récepteur, qui répond au signal donné par le Ca^{2+}. Par une approche pharmacologique, l'utilisation d'oligonucléotides antisens s'attaquant au $InsP_3R1$ et stoppant l'expression d'un biocapteur spécifique provenant d'une protéine à fluorescence verte associée au domaine d'homologie à la pleckstrine de la phospholipase C suggère que le signal issu de l'$InsP_3R1$ dépendant du calcium est transitoire et causé par la synthèse transitoire du $InsP_3$ par l'entremise du récepteur muscarinique M_3. De plus, selon nous, les voies de signalisation M_2 et M_3 ont un effet sur la concentration de phosphatidylinositol 4,5-bisphosphate et de $InsP_3$; il s'agit donc de voies de signalisation reliées entre elles et qui sont activées par l'Ach dans les myocytes du duodénum.

Mots-clés : duodénum, signaux calciques, $InsP_3$, acétylcholine, récepteur $InsP_3$.

[Traduit par la Rédaction]

Introduction

The specificity of the calcium (Ca^{2+}) signals activated by G protein-coupled receptors ultimately resides in the Ca^{2+} channels that are activated. In smooth muscle myocytes, agonists induce different Ca^{2+} signals involved in contraction by activating the release of the sarcoplasmic reticulum (SR)-stored Ca^{2+} ions through inositol 1,4,5-trisphosphate ($InsP_3$) receptors ($InsP_3Rs$) or ryanodine receptors (RYRs) (for review, Morel et al. 2007). $InsP_3Rs$ are coactivated by $InsP_3$ and Ca^{2+} (Bezprozvanny 2005). RYRs are activated by cyclic ADP-ribose (cADPR) (Fritz et al. 2005) or by $InsP_3Rs$-mediated Ca^{2+} release (Boittin et al. 1999) and Ca^{2+} influx from plasma membrane voltage-dependent Ca^{2+} channels through the Ca^{2+}-induced Ca^{2+} release (CICR) mechanism (Morel et al. 1996). Regeneration of Ca^{2+} waves produces Ca^{2+} oscillations, which have been measured in several types of myocytes (for review, Lee et al. 2005). Both RYRs and $InsP_3Rs$ have been implicated in the generation of Ca^{2+} oscillations. The RYRs encode Ca^{2+} oscillations via the CICR mechanism after agonist-activated Ca^{2+} influx or membrane potential oscillations (Morel et al. 1996; Sergeeva et al. 2003; Sneyd et al. 2004) and via activation of cADPR and the binding protein FKBP12/12.6 (Poirier et al. 2001; Fritz et al. 2005). Several mechanisms have been proposed to explain $InsP_3$-dependent Ca^{2+} oscilla-

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tions, including (*i*) rhythmic regulation of phospholipase C (PLC) inducing a cyclic $InsP_3$ production (Hirose et al. 1999) and (*ii*) regulation of $InsP_3R$ by $InsP_3$, Ca^{2+}, or ATP binding (Harootunian et al. 1991; for review, Bezprozvanny 2005), and $InsP_3R$ phosphorylation (Yule et al. 2003). In these latter cases, the expression pattern of $InsP_3R$ subtypes appears decisive. Indeed, the 3 $InsP_3R$ subtypes ($InsP_3R1$–3) have different molecular properties and can thus support different Ca^{2+} signals. The ability of each subtype to encode Ca^{2+} oscillations is still under debate; however, $InsP_3R2$ is suggested to carry a key role in this signal (Miyakawa et al. 1999; Morel et al. 2003; Dupont and Combettes 2006; Fritz et al. 2008).

The co-expression of muscarinic M_2 and M_3 receptors in most smooth muscle myocytes gives acetylcholine (ACh) the ability to simultaneously activate 2 different transduction pathways affecting the intracellular Ca^{2+} concentration. It is well known that the M_3-activated pathway induces an $InsP_3$-dependent Ca^{2+} signal in different types of myocytes (Thomas and Ehlert 1994; Morel et al. 1997), whereas we have shown that in rat duodenum myocytes, the M_2-activated pathway leads to an oscillating RYR-dependent Ca^{2+} signal (Fritz et al. 2005). Here, we show that in these myocytes, ACh induces a simultaneous transient $InsP_3$-dependent Ca^{2+} release by activation of the $M_3/InsP_3R1$ pathway in addition to the Ca^{2+} oscillations encoded by the M_2/cADPR/RYR2 pathway. Whether this transient Ca^{2+} signal is amplifying the RYR-dependent Ca^{2+} signal or in fact performs a more specific physiologic role remains to be determined.

Materials and methods

Cell preparation

The dissociation of duodenum myocytes was previously described in detail (Fritz et al. 2005). Briefly, rats were killed by cervical dislocation and the longitudinal layer of duodenal smooth muscle was stripped and cut into several pieces. Tissues were incubated with 0.8 mg/mL collagenase, 0.2 mg/mL pronase E (EC 3.4.24.31), and 1 mg/mL bovine serum albumin at 37 °C. Tissues were then placed in an enzyme-free solution and triturated to release cells. Cells were seeded on glass slides and maintained in short-term primary culture in medium M199 containing 10% fetal calf serum, 20 units/mL penicillin, and 20 μg/mL streptomycin. Cells were kept in an incubator gassed with 5% CO_2 at 37 °C and used within 30 h to 4 days. The investigation conformed to the European Community and French guiding principles in the care and use of animals. Authorization to perform animal experiments (A-33-063-003) was obtained from the préfecture de la Gironde, France.

Cytosolic Ca^{2+} measurements

Cells were loaded by incubation in physiologic solution containing 2 μmol/L fluo-4 acetoxymethyl ester (Fluo-4 AM) for 20 min at 37 °C, washed with fresh solution, and used after 10 min. Images were acquired using the image series mode of a Bio-Rad MRC1024ES (Carl Zeiss, Oberkochen, Germany) confocal microscope as previously described (Fritz et al. 2005; Dabertrand et al. 2006). Image series were composed of images of the same confocal section of the cells taken at 1.2 s intervals. To analyze variation of fluorescence, regions of interest (ROI) were drawn around each myocyte. The same ROI were used for each frame of the series. Total fluorescence values of the ROI in each frame (F) were divided by the total fluorescence of the ROI in the first frame and reported as F/F0 in time-course graphs. All pharmacologic agents were applied by pressure ejection from a glass pipette for the period indicated on the records. All experiments were carried out at 26 ± 1 °C.

Cell permeabilization

To gain rapid access to the SR Ca^{2+} channels with pharmacologic agents such as heparin or 8-bromo-cADP ribose (8-Br-cADPR), cell permeabilization was performed as previously described (Fritz et al. 2005). Briefly, the extracellular medium was replaced by a permeabilization solution containing 140 mmol/L KCl, 20 mmol/L HEPES, 0.5 mmol/L $MgCl_2$, 100 μmol/L ATP, and 10 μg/mL saponin, pH 7.4 with NaOH. We tested that both ACh- and caffeine-induced Ca^{2+} responses were similar in cells before and 15 min after permeabilization, suggesting that the Ca^{2+} stores were not emptied or destroyed (data not shown). Moreover, the levels of fluorescence observed before and after agonist application were not significantly modified, indicating no significant leakage of Fluo-4 out of the cells during this period.

RT-PCR

Total RNA was extracted from duodenum myocytes using the RNA preparation kit following the supplier instructions (Epicentre, Tebu-Bio, Le Perray en Yveline, France). The reverse transcription reaction was performed using RT-transcriptor kit (Roche, Meylan, France). PCR conditions and primer design were previously detailed (Morel et al. 2003). PCR were performed with a Mastercycler thermal cycler (Eppendorf, Le Pecq, France). Amplification products were separated by electrophoresis (2% agarose gel) and visualized by ethidium bromide staining. Gels were photographed with EDAS120 and analyzed with KDS1D 2.0 software (Kodak Digital Science, Rochester, N.Y.).

$InsP_3R$ immunostaining

Immunostaining was performed as previously described (Morel et al. 2003). Briefly, myocytes were washed with PBS, fixed with 4% (*v*/*v*) formaldehyde and 0.05% (*v*/*v*) glutaraldehyde (10 min, room temperature) and permeabilized in PBS containing 3% fetal calf serum and 1 mg/mL saponin (20 min, room temperature). Images of the stained cells were obtained with a confocal microscope, and fluorescence was estimated by grey-level analysis using IDL software (RSI) in 0.5 μm confocal sections. Cells were compared by keeping acquisition parameters (grey scale, exposure time, iris aperture, gain, and laser power) constant. Nonspecific fluorescence was determined when each specific anti-$InsP_3R$ antibody was preincubated with its antigenic peptide 1 h before performing immunostaining. When the cell fluorescence was significantly higher than nonspecific fluorescence, the cell was considered positive and fluorescence-specific. Primary and secondary antibodies were used at the concentrations of 1:500 and 1:250, respectively.

Microinjection of oligonucleotides

Phosphorothioate antisense oligonucleotides (denoted with the prefix "as") used here were previously described (Morel et al. 2003). Oligonucleotides were injected into the nucleus of myocytes that were in a square imprinted on a glass slide. Injections were realized with a manual injection system with femtotips II (Eppendorf). The myocytes were then cultured for 2 to 4 days in culture medium and the glass slides were transferred into the perfusion chamber for physiologic experiments.

Transfection of $InsP_3$ biosensor

The $InsP_3$ biosensor is the plasmid encoding the protein containing the PH domain of PLCδ1 fused to enhanced green fluorescent protein (PLCδ1-PH-EGFP). The use of the $InsP_3$ biosensor in smooth muscle cells in primary culture was extensively described and validated previously by our group (Fritz et al. 2008). Briefly, after 20 h in culture, myocytes were electroporated (0.4 μg/mL in PBS, square wave 100 V/cm, 10 ms) using a 35 mm petri dish electrode (T 820 electroporator, BTX, Holliston, Mass.). After electroporation, cells were placed in new culture medium for 6–10 h, then the expression of the fused protein was observed by confocal microscopy. Plasma membrane and cytosolic fluorescence were measured with a specific application in IDL software developed by J.L. Lavie. An ROI was drawn around the myocyte membrane perimeter on the first image to separate the membrane and the cytosolic compartments. A threshold of fluorescence on the first image was determined to provide a mask image used as a reference to distinguish the plasma membrane from the cytosol in the following image frames of the image series. Fluorescence in the ROI was then measured and implemented in an IDL algorithm to produce the time-course analysis of $InsP_3$ biosensor localization. Values of fluorescence were graphed in time-course manner and reported as a relative fluorescence to the baseline (obtained by the mean of the first 5 images). Any cells showing an ACh-induced contraction were excluded from the analysis.

Chemicals and drugs

Collagenase (EC 3.4.24.3) was obtained from Worthington (Lakewood, N.J.). Anti-goat Fluoprobe 488 secondary antibody, Fluo-4, and fluo-4 acetoxymethyl ester were from Interchim (Montluçon, France). Caffeine and (2-aminoethoxy)-diphenylborane (2APB) were from Calbiochem-Merck (Nottingham, UK). Medium M199, streptomycin, and penicillin were from Invitrogen (Cergy-Pontoise, France). Anti-$InsP_3R$ subtype antibodies were from Santa Cruz Biotechnology (Santa Cruz, Calif.). All primers and phosphorothioate antisense oligonucleotides were synthesized and purchased from Eurogentec (Angers, France). All other chemicals were from Sigma (Lyon, France). PLCδ1-PH-EGFP construct was a generous gift from Tobias Meyer (Stanford University, Calif.).

Data analysis

Data were expressed as means ± SE; n represents the number of tested cells. Significance was tested by means of Student's test, and p values < 0.05 were considered significant.

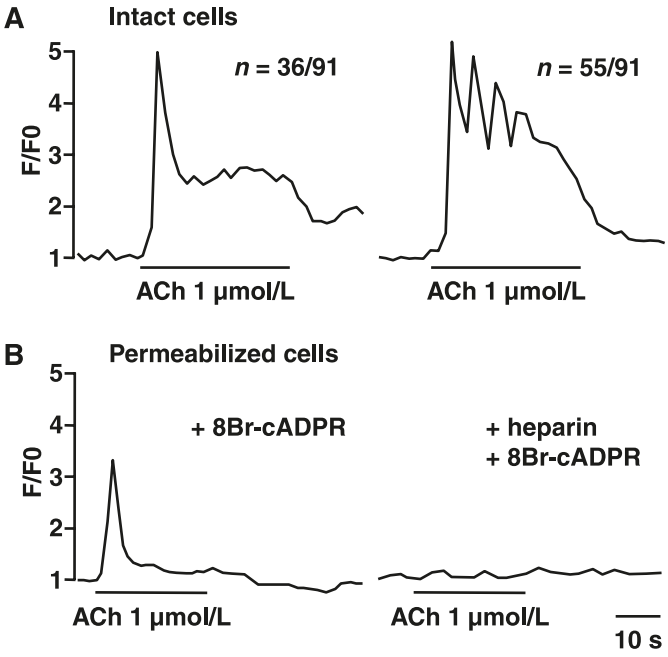


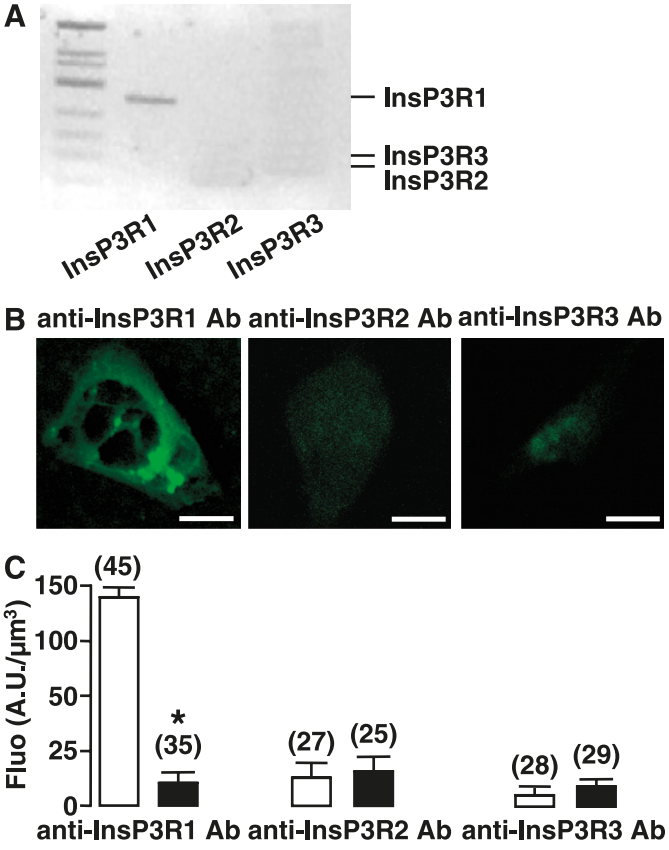
Fig. 1. Time course of typical Ca^{2+} responses evoked by 1 μmol/L ACh in rat duodenum myocytes. (A) Typical ACh-induced Ca^{2+} transient (in 40% of tested cells, left) and Ca^{2+} oscillations (in 60% of tested cells, right). (B) Representative traces in permeabilized cells show the effect on ACh-induced Ca^{2+} response of (left) 20 μmol/L 8Br-cADPR (n = 18) and (right) the cocktail of 8Br-cADPR (20 μmol/L) plus heparin (1 mg/mL). Myocytes were loaded with 2 μmol/L Fluo-4 AM. Total fluorescence values of the ROI in each frame (F) were divided by the total fluorescence of the ROI in the first frame and reported as F/F0. Scale bar indicates 10 seconds. Ach, acetylcholine; 8Br-cADPR, 8-bromo-cADP ribose; ROI, region of interest.

Results

Characterization of an $InsP_3$-dependent Ca^{2+} signal evoked by acetylcholine

In rat duodenum myocytes, ACh (1 μmol/L, 30 s) induced either a Ca^{2+} peak followed by regenerative oscillations (60% of the cells, n = 55/91) or a single Ca^{2+} transient followed by a sustained phase (40% of the cells, n = 36/91) (Fig. 1A). The mean amplitude of the Ca^{2+} transient peak was 5.3 ± 0.2 ratio units (n = 36) and the first peak of the oscillating Ca^{2+} response was 5.4 ± 0.3 ratio units (n = 55). Oscillations were characterized by their frequency (5.3 ± 0.3 oscillations/min, n = 55) because this parameter was more reproducible than amplitude of each oscillation. Previously, we demonstrated that permeabilizing myocytes did not significantly modify peak amplitude and percentage of oscillating cells, indicating that these Ca^{2+} signals are dependent on Ca^{2+} release from the SR (Fritz et al. 2005). Here, specific pharmacologic agents modulating SR Ca^{2+} channels were used to fully characterize the ACh-mediated Ca^{2+} response. In permeabilized cells, 8Br-cADPR (20 μmol/L, 5 min), a cADPR antagonist, totally abolished Ca^{2+} oscillations (0% oscillating cells, n = 0/18), but a single Ca^{2+} transient remained in all tested cells. Amplitude of this remaining Ca^{2+} transient was significantly reduced (3.2 ± 0.4 ratio units, n = 18, in presence of 8Br-cADPR versus 5.2 ± 0.4 ratio units,

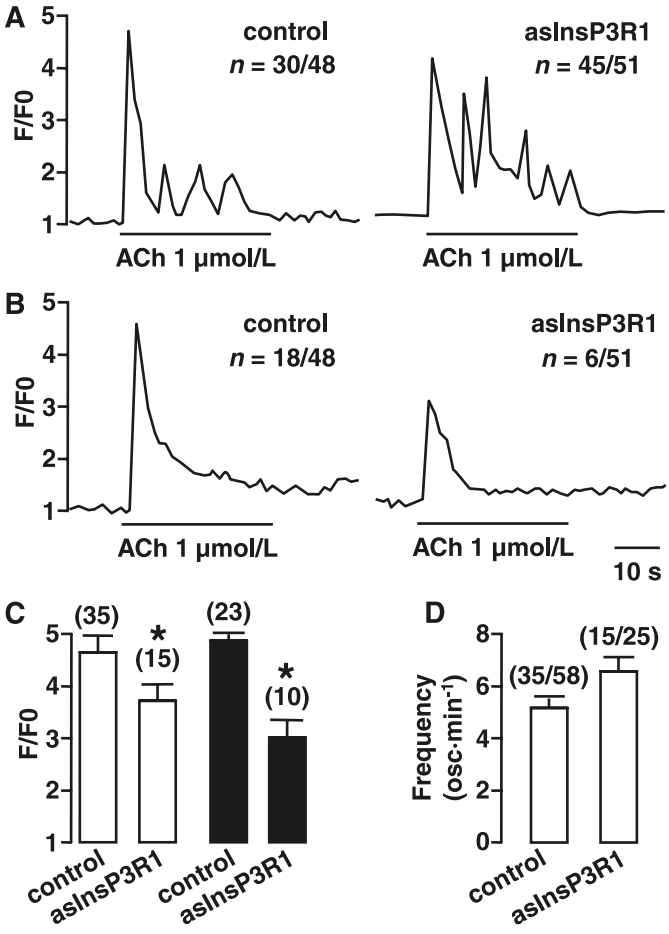
Fig. 2. Expression of $InsP_3R$ subtypes in rat duodenum myocytes. (A) cDNA fragments of $InsP_3R$ subtypes were amplified from duodenum myocytes and separated on a 2% agarose gel to be visualized by staining with ethidium bromide. Molecular size standard is ϕ174-HinfI scale. (B) Typical confocal sections show immunostaining with anti-$InsP_3R1$ (1:500), anti-$InsP_3R2$ (1:500), and anti-$InsP_3R3$ (1:500) antibodies. Scale bar represents 10 µm. **(C)** Compiled data of quantification of specific (white bars) and nonspecific (black bars) fluorescence. Data are means ± SE; number of tested cells indicated in parentheses. Asterisk (*) indicates significant difference ($p < 0.05$) from specific fluorescence. AU, arbitrary units; $InsP_3R$, inositol 1,4,5-trisphosphate receptor.



$n = 21$, in control conditions, $p < 0.05$). Addition of heparin ($InsP_3R$ antagonist, 1 mg/mL) together with 8Br-cADPR in permeabilized cells totally inhibited the 8Br-cADPR-resistant ACh-induced Ca^{2+} response (Fig. 1B). This suggests that the 8Br-cADPR-resistant Ca^{2+} signal is dependent on the $InsP_3$ pathway.

To more precisely investigate the function of $InsP_3R$ subtypes in the ACh-induced Ca^{2+} response, the expression of these 3 receptors was examined by RT-PCR and immunostainings. RT-PCR revealed that only $InsP_3R1$ was potentially expressed (Fig. 2A). Detection of $InsP_3R$ subtype proteins by immunocytochemistry with specific anti-$InsP_3R1$ (1:500), anti-$InsP_3R2$ (1:500), and anti-$InsP_3R3$ (1:500) antibodies confirmed that only $InsP_3R1$ is expressed in duodenum myocytes (Figs. 2B and 2C). The specificity and non-crossreactivity of the anti-$InsP_3R$ subtype antibodies were previously described (Morel et al. 2003). The same anti-goat Fluoprobe 488 secondary antibody (1:250) was used to reveal each primary antibody.

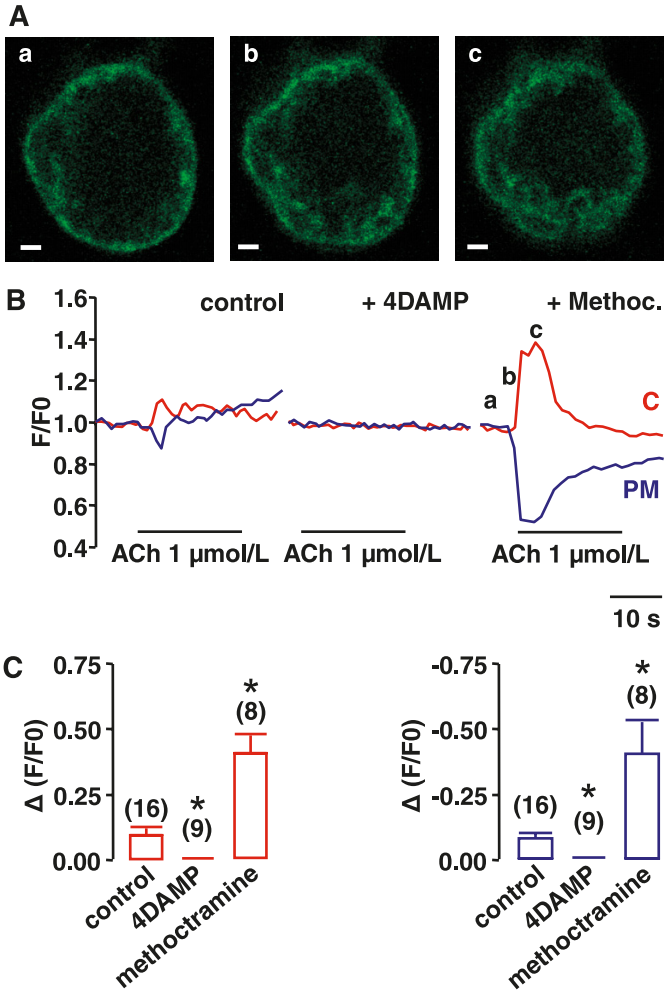
Fig. 3. Effect of as$InsP_3R1$ antisense oligonucleotide on Ca^{2+} signals evoked by 1 µmol/L ACh in rat duodenum myocytes. (A) Time course of typical Ca^{2+} oscillations induced by ACh in control cells (left) and in as$InsP_3R1$-injected cells (right). (B) Typical Ca^{2+} transient induced by ACh in control cells (left) and in as$InsP_3R1$-injected cells (right). Scale bar indicates 10 seconds; n = proportion of tested cells exhibiting the response. (C) Compiled data show the effect of as$InsP_3R1$ on the first peak of the ACh-induced oscillating response (white bars) and on the ACh-induced Ca^{2+} transient (black bars). ACh-induced Ca^{2+} responses were observed 2 days after injection of 10 µmol/L as$InsP_3R1$ antisense oligonucleotide. (D) Compiled data of the effects of as$InsP_3R1$ on the frequency of ACh-induced Ca^{2+} oscillations. Data are means ± SE; number of cells tested indicated in parentheses. Asterisk (*) indicates significant difference ($p < 0.05$) from corresponding control.



Role of $InsP_3R1$ in ACh-induced Ca^{2+} responses

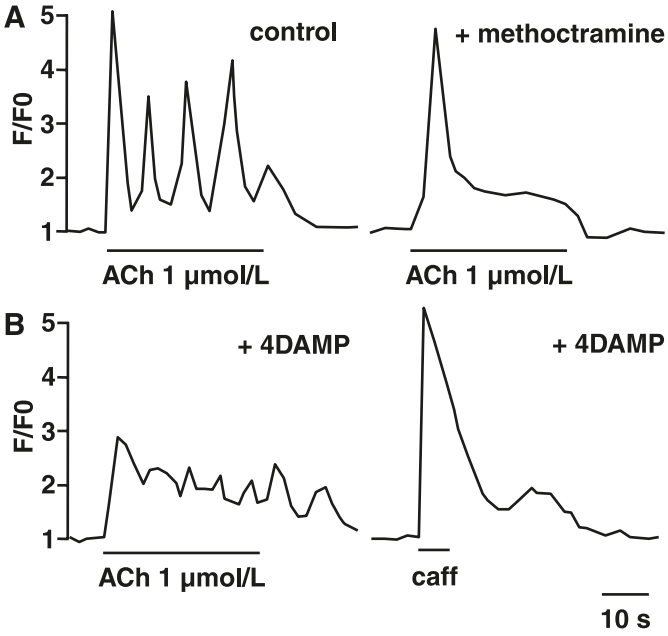
The role of $InsP_3R1$ subtype in ACh-induced Ca^{2+} response was studied using an antisense oligonucleotide strategy. The time-course efficiency of the designed as$InsP_3R1$ was previously described (Morel et al. 2003). We verified here by immunostaining with the anti-$InsP_3R1$ antibody that the expression of $InsP_3R1$ was maximally decreased 2–3 days after nuclear injection of 10 µmol/L as$InsP_3R1$ in myocytes located in a marked area of glass slides (data not shown). Noninjected cells outside this marked area stably expressed $InsP_3R1$ and were subsequently used as control cells. Injection of 10 µmol/L

Fig. 4. Hydrolysis of phosphatidylinositol 4,5-bisphosphate (PtdIns(4,5)P_2) induced by 1 µmol/L ACh in rat duodenum myocytes transfected with $InsP_3$ biosensor. (A) Typical localization of $InsP_3$ biosensor before (*a*) and during (*b*, *c*) application of 1 µmol/L ACh. Panels *a*, *b*, and *c* refer to the time points indicated in (B). Scale bar represents 2 µm. (B) Time-course measurement of fluorescence in plasma membrane (blue) and in cytoplasmic compartment (red). 1 µmol/L ACh was applied in control conditions and in the presence of 10 nmol/L 4DAMP and 10 µmol/L methoctramine. (C) Compiled data of maximal fluorescence increase in cytoplasmic compartment (left) and maximal fluorescence decrease in plasma membrane (right) in control conditions and in the presence of 10 nmol/L 4DAMP or 10 µmol/L methoctramine. Data are means ± SE; number of cells tested indicated in parentheses. Asterisk (*) indicates significant difference ($p < 0.05$) from control.



asInsP$_3$R1 had no significant effect on Ca^{2+} oscillation frequency (Figs. 3A and 3D) but inhibited the amplitude of the ACh-evoked first Ca^{2+} peak in both oscillating and non-oscillating cells (Figs. 3B and 3C). The percentage of cells presenting Ca^{2+} oscillations was increased from 60% to 88%. These results suggest that while Ca^{2+} oscillations are not encoded by InsP$_3$R1, a significant part of the first Ca^{2+} peak is due to activation of InsP$_3$R1.

Fig. 5. Typical ACh-induced Ca^{2+} signals evoked in presence of muscarinic antagonists. (A) Typical 1 µmol/L ACh-induced Ca^{2+} oscillations in control cells (left) and in presence of 1 µmol/L methoctramine. (B) Typical Ca^{2+} responses induced by 1 µmol/L ACh (left) and by caffeine (right) in presence of 1 nmol/L 4DAMP.



Effects of muscarinic antagonists on PtdIns(4,5)P_2 hydrolysis

To reveal the relevance of the transduction pathway that induces the InsP$_3$-dependent Ca^{2+} signal, we measured $InsP_3$ production dynamics in duodenum myocytes. To monitor dynamics of phosphatidylinositol 4,5-bisphosphate (PtdIns(4,5)P_2) and $InsP_3$ in duodenum myocytes, the $InsP_3$ biosensor was expressed as previously described (Fritz et al. 2008). Expression of the $InsP_3$ biosensor did not interfere with caffeine-induced contractions (data not shown). Panel *a* in Fig. 4A illustrates that in resting myocytes, the $InsP_3$ biosensor is accumulated in the plasma membrane whereas cytosolic fluorescence is very low. Upon ACh application in control conditions, the $InsP_3$ biosensor is transferred from the membrane to the cytosol (Fig. 4A, panels *b* and *c*, and Fig. 4B). The kinetics of these fluorescence changes are compatible with kinetics of ACh-induced Ca^{2+} responses. In the presence of 10 nmol/L 4DAMP, a M_3 muscarinic receptor inhibitor in nanomolar range, no changes in plasma membrane or cytosolic fluorescence were observed (Figs. 4B and 4C). In contrast, 10 µmol/L methoctramine, which at this concentration specifically inhibits M_2 without effect on M_3, significantly accentuated the fluorescence transfer from the plasma membrane to the cytosolic compartment (Figs. 4B and 4C). To validate this protocol, transfected cells were patch-clamped with an intracellular solution containing 1 µmol/L 3F-$InsP_3$, a poorly hydrolysable analog of $InsP_3$. A decrease of plasma membrane fluorescence ($\Delta F/F0 = -0.23 \pm 0.02$, $n = 7$) accompanied by an increase in cytosolic fluorescence ($\Delta F/F0 = 0.21 \pm 0.03$, $n = 7$) was observed, confirming that $InsP_3$ is able to transfer the $InsP_3$ biosensor from plasma membrane to cytosol (not shown).

We have previously demonstrated that 10 μmol/L methoctramine inhibited cADPR production (Fritz et al. 2005). Here, 1 μmol/L methoctramine inhibited ACh-evoked Ca^{2+} oscillations, whereas the Ca^{2+} transient was not modified (Fig. 5A). As illustrated in Fig. 5B, application of 4DAMP (1 nmol/L) significantly reduced the first Ca^{2+} transient (3.4 ± 0.5 ratio units, $n = 21$, in presence of 4DAMP, versus 5.0 ± 0.4 ratio units, $n = 31$, in control conditions, $p < 0.05$), but did not modify the frequency of Ca^{2+} oscillations (6.1 ± 0.6 oscillations/min in presence of 4DAMP versus 5.3 ± 0.8 oscillations/min in control conditions). The decrease in the amplitude of the first Ca^{2+} transient was not due to the decrease of SR Ca^{2+} content because the caffeine-induced Ca^{2+} response was not modified by the presence of 4DAMP (Fig. 5B).

Discussion

Upon binding to its muscarinic receptors, ACh activated 2 different signaling pathways involved in Ca^{2+} release. Binding to M_3 muscarinic receptors stimulated a transient $InsP_3$ production that activated $InsP_3R1$-dependent release of stored Ca^{2+}, whereas binding to M_2 receptors activated the cADPR/FKBP/RYR2 pathway and Ca^{2+} oscillations, as we previously described (Fritz et al. 2005). Rat duodenum myocytes expressed only $InsP_3R1$, as revealed by RT-PCR and immunostaining using antibodies the specificities of which were previously demonstrated (Morel et al. 2003). We thus used this cell type to investigate the relevance of $InsP_3R1$ in ACh-induced Ca^{2+} responses. The persistence of ACh-induced Ca^{2+} oscillations in cells treated with 4DAMP and in cells injected with as$InsP_3R1$ indicated that M_3 and $InsP_3R1$ are not required for Ca^{2+} oscillations. The inhibition of ACh-induced Ca^{2+} oscillations by methoctramine and 8Br-cADPR confirmed that they were dependent on the M_2/cADPR pathway, as previously described (Fritz et al. 2005). On the other hand, the remaining transient Ca^{2+} signal activated by ACh in the presence of 8Br-cADPR was inhibited by heparin, confirming that in rat duodenum myocytes, as described in other species (Morel et al. 1997) or other tissues (Boittin et al. 2000), $InsP_3R1$ is activated by ACh. The $InsP_3R1$-dependent Ca^{2+} signal appears to be a single transient Ca^{2+} signal, as described in vascular and ureteric myocytes (Morel et al. 2003) or in DT40 B cells engineered to express only $InsP_3R1$ (Miyakawa et al. 1999). Overall, we demonstrate here that the first Ca^{2+} peak of the ACh-evoked Ca^{2+} response is a mixed signal composed of the $InsP_3R1$-dependent Ca^{2+} transient and the RYR-dependent first Ca^{2+} oscillation.

In MDCK (Madin–Darby canine kidney) and CHO (Chinese hamster ovary) cells, $InsP_3$ production oscillations visualized with the $InsP_3$ biosensor and Ca^{2+} oscillations had similar frequencies (Hirose et al. 1999; Nash et al. 2001 and Nash et al. 2002). In duodenum myocytes, we here show that the kinetics of the $InsP_3R1$-encoded transient Ca^{2+} signal evoked by ACh are in accordance with the kinetics of $InsP_3$ production. ACh-activated $InsP_3$ production measured with the $InsP_3$ biosensor is indeed transient and the kinetic parameters of $InsP_3$ production and Ca^{2+} signals are very similar. Moreover, biochemical studies using radiolabelled inositol have demonstrated that the linear $InsP_3$ accumulation depends on M_3 muscarinic activation (Prestwich and Bolton 1995). Our results showed that (*i*) M_3 muscarinic receptors are responsible for the $InsP_3$ production, and (*ii*) inhibition of M_2 muscarinic receptor amplifies the $InsP_3$ biosensor signal. As the $InsP_3$ biosensor is also an indicator of the presence of $PtdIns(4,5)P_2$ in the plasma membrane (Stauffer et al. 1998), this indicates that activation of the M_2 receptor could modulate the M_3-activated pathway, either by inhibiting $PtdIns(4,5)P_2$ availability or by decreasing the $InsP_3$ concentration. In smooth muscle myocytes, M_2 activation induced the production of $PtdIns(3,4,5)P_3$ and was implicated in cADPR production (Wang et al. 1999; Callaghan et al. 2004; Kim et al. 2008). $PtdIns(3,4,5)P_3$ is a $PtdIns(4,5)P_2$ derivative that stays in the plasma membrane and, as other phosphoinositides, has a very low affinity for the $InsP_3$ biosensor (Varnai and Balla 1998).

In conclusion, we demonstrate here that 2 different Ca^{2+} signals are induced simultaneously by ACh in rat duodenum myocytes. The resulting mixed Ca^{2+} signal is composed of an $InsP_3R1$-dependent Ca^{2+} transient due to a transient $InsP_3$ production activated via M_3 receptor added to the Ca^{2+} oscillations activated by the M_2/cADPR pathway that we have previously described (Fritz et al. 2005). Whether the first Ca^{2+} oscillation activated by the M_2 pathway is spatially merged with the transient Ca^{2+} peak activated by the M_3 pathway remains to be determined and opens relevant physiologic questions regarding their respective roles. Finally, our work also indicated that both M_2 and M_3 pathways modulate the $PtdIns(4,5)P_2$ membrane concentration and (or) availability.

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