Chapter 5

Clustering of the type 3 inositol 1,4,5-trisphosphate receptor in rat vascular smooth muscle cells is abolished by long-term agonist treatment

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Abstract
Stimulation of PLC-coupled plasma membrane receptor leads to activation of the inositol 1,4,5-trisphosphate receptor (InsP₃R). Prolonged stimulation of rat A7r5 smooth muscle cells with the PLC activating agonist vasopressin led to down-regulation of the type 1 InsP₃ receptor (InsP₃R-1) and of the type 3 InsP₃ receptor (InsP₃R-3). Pre-treatment with the Ca²⁺ channel blocker verapamil induced InsP₃R-1 down-regulation without down-regulation of InsP₃R-3. The effects of InsP₃R down-regulation on Ca²⁺ responses were investigated in permeabilized A7r5 cells. Pre-treatment with vasopressin resulted in impaired Ca²⁺ release at submaximal InsP₃ concentrations. Pre-treatment with verapamil did not affect Ca²⁺ release. Since the differences in Ca²⁺ efflux in the vasopressin and verapamil pre-treated group seem to arise from differences in the regulation of InsP₃R-3, we further investigated the regulation of InsP₃R-3. Therefore, the intracellular distribution of InsP₃R-3 in A7r5 cells in response to long-term agonist treatment was determined using immuno-electron microscopy. We show that InsP₃R-3 labeling is strongly clustered on structures that appear to be regions of specialized endoplasmic reticulum, whereas most of the endoplasmic reticulum did not show InsP₃R-3 labeling. Pre-treatment with vasopressin resulted in a dramatic reduction of InsP₃R-3 staining and clustering, whereas exposure to verapamil resulted in dispersion of the InsP₃R-3 clusters. We conclude that down-regulation of both InsP₃R-1 and InsP₃R-3 in A7r5 cells by vasopressin results in impaired Ca²⁺ release. Further, dispersion of InsP₃R-3 clusters may involve a mechanism to compensate for down-regulation of InsP₃R-1, resulting in unchanged Ca²⁺ responses in verapamil pre-treated A7r5 cells.
Introduction:
Stimulation of PLC-coupled plasma membrane receptor leads to the formation of the second messenger inositol 1,4,5-trisphosphate (InsP$_3$). InsP$_3$ interacts with the intracellular inositol 1,4,5-trisphosphate receptor (InsP$_3$R), resulting in the release of Ca$^{2+}$ from intracellular stores (Berridge, 1993). Cloning studies showed that three types of InsP$_3$R (InsP$_3$R-1, InsP$_3$R-2 and InsP$_3$R-3) exist in rat tissue (Mignery et al., 1990). Most cell types express more than one InsP$_3$R type, but the ratio at which those are expressed differ considerably between cell types (Sugiyama et al., 1994; De Smedt et al., 1994a; Wojcikiewicz, 1995). The functional differences of InsP$_3$Rs are still not completely elucidated. However, InsP$_3$R-3 is thought to be situated near the plasma membrane and has been implicated in store operated Ca$^{2+}$ entry (Berridge, 1995b; DeLisle et al., 1996; Khan et al., 1996; Putney, 1997).

The objective of the present study was to further investigate the function and regulation of InsP$_3$Rs. We therefore aimed at obtaining an experimental model in which we could down-regulate specific InsP$_3$R types.

Previously, we demonstrated that rat A7r5 smooth muscle cells express predominately InsP$_3$R-1 and InsP$_3$R-3 and hardly any InsP$_3$R-2 (De Smedt et al., 1994b). Further, we demonstrated that InsP$_3$R-1 and InsP$_3$R-3 were differentially susceptible to down-regulation in A7r5 cells in response to long-term agonist pre-treatment (Sipma et al., 1998). Therefore, A7r5 cells seem to represent a good model for studying the function and regulation of InsP$_3$Rs.

In addition to down-regulation of InsP$_3$Rs, evidence has emerged that the function of InsP$_3$R-2 and InsP$_3$R-3 may also be regulated through clustering of these receptors. Clustering of InsP$_3$R-2 and InsP$_3$R-3 has been observed in several non-smooth muscle cell lines (Wilson et al., 1998), a mechanism that seems to function independently of InsP$_3$R down-regulation. However, the function of InsP$_3$R clustering is still unknown.

To further investigate the regulation and function of InsP$_3$Rs in smooth muscle cells, we investigated down-regulation of InsP$_3$Rs and InsP$_3$ mediated Ca$^{2+}$ release in A7r5 smooth muscle cells in response to long-term agonist treatment. To determine a possible relation with clustering of InsP$_3$R-3, we determined the intracellular distribution of InsP$_3$R-3 using immunoelectron microscopy.

In this study, we demonstrate down-regulation of InsP$_3$R-1 and InsP$_3$R-3 in A7r5 smooth muscle cells in response to long-term pre-treatment with vasopressin which was accompanied by an impaired InsP$_3$-induced release of Ca$^{2+}$ in permeabilized cells. Pre-treatment with verapamil, a blocker of L-type Ca$^{2+}$ channels, resulted in down-regulation of only InsP$_3$R-1 without an effect
on Ca\textsuperscript{2+} release. Clustering of InsP\textsubscript{3}R-3 is abolished by pre-treatment with vasopressin and verapamil, implicating that redistribution of InsP\textsubscript{3}R-3 is independent on down-regulation.

**Material and Methods:**

**Cell culture**

A7r5 cells were grown in Dulbecco’s modified Eagle Medium (Life technologies, USA) and were cultured at 37\(^\circ\)C in 5% CO\textsubscript{2} in air. Medium was supplemented with 10% fetal calf serum, 100 IU/ml penicillin (Life technologies, USA) and 100 IU/ml streptomycin (Life technologies, USA). For preparation of microsomes, A7r5 cells were cultured in 180 cm\textsuperscript{2} culture flasks (Costar, USA). For immunoelectron microscopy, cells were cultured in 24 well clusters (1.9 cm\textsuperscript{2}, Costar, USA). For \textsuperscript{45}Ca\textsuperscript{2+} fluxes, A7r5 cells were cultured in 12 well dishes (4 cm\textsuperscript{2}, Costar, USA). Medium was replaced every 2-3 days. Cells were confluent at the time of experiments.

**Antibodies**

InsP\textsubscript{3}R-1 was detected with a specific polyclonal antibody raised against the unique C-terminus of InsP\textsubscript{3}R-1 (Parys et al., 1995). InsP\textsubscript{3}R-3 was detected with a specific monoclonal antibody, which was obtained from Transduction Laboratories (Lexington, KY, USA). SERCA 2b Ca\textsuperscript{2+} ATPase was detected with an isoform-specific polyclonal antibody (Wuytack et al., 1989).

**Western blotting**

Total microsomes from A7r5 cells were prepared according to Parys et al. (Parys et al., 1995). Protein content was determined, using bovine serum albumin as standard (Lowry et al., 1951). Microsomal proteins were analyzed by SDS-PAGE (Laemmli, 1970) on a 3-12 % linear gradient and transferred to Immobilon-P (Millipore, USA). Blots were blocked for 1 h in a buffer containing KH\textsubscript{2}PO\textsubscript{4}, 10 mM; NaHPO\textsubscript{4}, 30 mM (pH = 7.5); NaCl, 153 mM; Tween-20, 0.1 %; milkpowder 5% and incubated with primary antibodies for 1 h in the same buffer without milkpowder. Alkaline phosphatase-coupled anti-rabbit or anti-mouse antibodies were used as secondary antibodies. The immunoreactivity was visualized by conversion of the substrate into a fluorescent probe (Vistra, ECF Western Blotting kit, USA) and quantified with the Storm 840 FluorImager, equipped with the Imagequant NT4.2 software (Molecular Dynamics, USA). Linearity of the fluorescent signal was verified.
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by blotting different amounts of the same protein batch, as described previously (Vanlingen et al., 1997).

\textit{\textsuperscript{45}Ca\textsuperscript{2+} -flux experiments}

\textsuperscript{45}Ca\textsuperscript{2+} fluxes on permeabilized cells were done on a thermostatically controlled plate at 25 °C as described previously (Sipma et al., 1998) Briefly, the culture medium was replaced by 1 ml permeabilization medium containing 120 mM KCl, 30 mM imidazole/HCl (pH 6.8), 2 mM MgCl\textsubscript{2}, 1 mM ATP, 1 mM EGTA and 20 µg/ml saponin. The saponin-containing solution was removed after 10 min and the cells were washed with a similar saponin-free solution. \textsuperscript{45}Ca\textsuperscript{2+} uptake into the non-mitochondrial Ca\textsuperscript{2+} stores was accomplished by incubation for 60 min in 2 ml of loading medium containing 120 mM KCl, 30 mM imidazole/HCl (pH 6.8), 5 mM MgCl\textsubscript{2}, 5 mM ATP, 0.44 mM EGTA, 10 mM NaN\textsubscript{3}, and 100 nM free \textsuperscript{45}Ca\textsuperscript{2+}. Thereafter, cells were incubated in 1 ml of efflux medium containing 120 mM KCl, 30 mM imidazole/HCl (pH 6.8), 1 mM ATP, 1 mM EGTA and 2 µM thapsigargin. The efflux solutions supplemented with increasing concentrations of InsP\textsubscript{3} were replaced every 6 sec until 8.5 min had elapsed. At the end of the experiment the \textsuperscript{45}Ca\textsuperscript{2+} remaining in the stores was released by incubation with 1 ml of a 2% sodium dodecyl sulfate solution for 30 min.

\textit{Immunocytochemistry and electron microscopy}

Cells were fixed in the culture wells by gently removing the medium and adding a mixture of freshly prepared 2% paraformaldehyde and 1% glutaraldehyde in 0.1M sodium cacodylate buffer, pH 7.4, at room temperature. After 5-10 min. the wells were rinsed with tris buffered saline, quenched in 1% sodium borohydride, rinsed five times in TBS and preincubated in 5 % normal goat serum (Nordic, Netherlands) for 30 min., washed and incubated for two hours at room temperature in InsP\textsubscript{3}:R-3 antibodies, diluted 1:2000 in TBS and supplemented with 12.5mM Triton X100. Subsequently the wells were thoroughly rinsed with TBS and the cells were incubated according the ABC method (Vectastain Elite ABC Kit, Vector laboratories, USA). Cells were reacted with DAB according to standard procedures. After the DAB procedure a gold substituted silver peroxidase enhancement reaction was performed to improve the visibility of the reaction product (van den Pol and Gorcs, 1986). In a control series the primary antibody has been omitted from the incubation procedure, endogenous peroxidase reactivity was suppressed by washing cells in 1% hydrogen peroxide in methanol.
After the incubation procedure the cells were osmicated and dehydrated in graded series of ethanol and propylene oxide and embedded in epon, in the culture wells. After polymerization the wells were broken and the epon blocks were trimmed to fit in the ultramicrotome. Ultrathin sections were contrasted with uranyl acetate and lead citrate.

**Chemicals**

Carbachol, [Arg8]vasopressin and verapamil were from Sigma (USA). All chemicals used were of analytical grade.

**Statistics**

Data are represented as means ± s.e.mean. Data were considered significantly different when P < 0.05 by use of Student's unpaired t-test.

**Results:**

In order to obtain an experimental model in which specific InsP3R types are down-regulated, A7r5 cells were exposed to several PLC-activating agonists. The expression of InsP3R-1 and InsP3R-3 in response to long-term agonist treatment was quantified by Western blotting. Treatment of A7r5 cells with vasopressin (3 µM) induced an about 50% down-regulation of both InsP3R-1 and InsP3R-3. Down-regulation started after one hour and was maximal after 4-6 hours (Fig. 1A, B and Fig.2). Down-regulation of both InsP3R-1 and InsP3R-3 was still apparent after 20 hours of vasopressin treatment. Other PLC-activating agonists in A7r5 cells, including ATP (1 mM, 4 h), bradykinin (10 µM, 4 h), carbachol (1 mM, 4 h) or histamine (100 µM, 4 h) did not result in down-regulation of InsP3R-1 or InsP3R-3 in A7r5 cells (data not shown). A7r5 cells exhibit spontaneous Ca2+ oscillations, which are mediated by L-type voltage-gated Ca2+ channels (Byron and Taylor, 1993) and are blocked by a high dose of vasopressin (Missiaen et al., 1994; Byron, 1996). Because inhibition of Ca2+ spiking might be involved in the mechanism of InsP3R down-regulation by vasopressin, we investigated whether the Ca2+ channel blocker verapamil induced a similar down-regulation of InsP3Rs. Exposure of A7r5 cells to verapamil (2 µM, 4 h) resulted in down-regulation of InsP3R-1 similar to the down-regulation of InsP3R-1 by vasopressin (Fig. 2). In contrast to vasopressin, exposure to verapamil did not result in down-regulation of InsP3R-3 (Fig. 2).
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Fig. 1 The effect of vasopressin on the expression levels of InsP₃R-1 and InsP₃R-3 in A7r5 cells. A) InsP₃R-1 and InsP₃R-3 immunoreactivity in A7r5 cells after exposure to vasopressin (3 µM) for 0 h (C), 1 h (1), 2 h (2), 3 h (3), 4 h (4), 6 h (6) (typical experiment). B) The vasopressin-induced decrease in InsP₃R-1 and InsP₃R-3 immunoreactivity, expressed as percentage of reactivity in non-pretreated cells. From 3 h onwards, InsP₃R-1 and InsP₃R-3 immunoreactivity was significantly different (P < 0.05) from reactivity in non-treated cells, n = 4.

Fig. 2 The effect of vasopressin and verapamil on the expression level of InsP₃R-1 and InsP₃R-3 in A7r5 cells. InsP₃R-1 (left panel) and InsP₃R-3 (right panel) immunoreactivity is shown after treatment with vasopressin (AVP, 3 µM, 4 h) and verapamil (vera, 2 µM, 4 h). * Significantly different from immunoreactivity in non-pretreated cells, P < 0.05, n = 4.
The down-regulation of InsP₃Rs by vasopressin and verapamil might result from a non-specific process that affects several different endoplasmic reticulum proteins. However, treatment of A7r5 cells with vasopressin and verapamil did not significantly change the expression of the type 2b isoform of endoplasmic reticulum Ca²⁺-ATPase (SERCA 2b, 82 ± 10 % and 78 ± 10 % of control expression for vasopressin and verapamil, respectively, n=3) as measured by Western blotting using an antibody raised against SERCA 2b (Wuytack et al., 1989).

Prolonged exposure to vasopressin and verapamil may provide a good experimental model to determine the functional differences of InsP₃R-1 and InsP₃R-3, since exposure to vasopressin resulted in down-regulation of both InsP₃R-1 and InsP₃R-3, and exposure to verapamil resulted in down-regulation of only InsP₃R-1. To investigate the effects of down-regulation of InsP₃Rs on Ca²⁺ responses in A7r5 cells, ⁴⁵Ca²⁺ efflux from preloaded stores was determined in permeabilized A7r5 cells. Pre-treatment with vasopressin resulted in impaired ⁴⁵Ca²⁺ release at submaximal InsP₃ concentrations (Fig.3). In contrast, pre-treatment with verapamil did not result in significant changes in ⁴⁵Ca²⁺ release in permeabilized A7r5 cells (Fig. 3). Exposure to vasopressin and verapamil did not affect basal leak from Ca²⁺ stores or total loading of the stores or the relative amount of Ca²⁺ that could be released by a maximal dose of InsP₃ (20 µM) (data not shown).

**Fig. 3** ⁴⁵Ca²⁺ release from permeabilized A7r5 cells after pre-treatment with vasopressin and verapamil. The non-mitochondrial stores of control cells, vasopressin-pretreated cells and verapamil treated cells were loaded to steady state with ⁴⁵Ca²⁺ and then incubated in efflux medium containing progressively increasing [InsP₃]. The [InsP₃] was increased logarithmically in steps each lasting 6 s. The extent of Ca²⁺ release was normalized to that induced by 5 µM InsP₃. These tracings are typical for 3 separate experiments. * P<0.05, n=3.
Since the differences in Ca\(^{2+}\) efflux in the vasopressin and verapamil pre-treated group seem to arise from differences in the regulation of InsP\(_3\)R-3, we further investigated the regulation of InsP\(_3\)R-3. We therefore determined the intracellular distribution of InsP\(_3\)R-3 in A7r5 cells in response to long-term agonist treatment using immunoelectron microscopy.

In untreated A7r5 cells, InsP\(_3\)R-3 labeling appeared strongly clustered on structures that seem to be regions of specialized endoplasmic reticulum (Fig. 4A, 4B), while the remainder of the endoplasmic reticulum showed little or no labeling.

![Fig. 4](image)

**Fig. 4** The intracellular distribution of InsP\(_3\)R-3 in untreated A7r5 smooth muscle cells. A) InsP\(_3\)R-3 labeling is strongly clustered in untreated A7r5 cells (arrows indicate InsP\(_3\)R-3 clusters). Diffuse labeling of InsP\(_3\)R-3 is present throughout the cytoplasm and on the dense areas of chromatin. B) Detail of a InsP\(_3\)R-3 cluster. Clusters of InsP\(_3\)R-3 are always associated with smooth endoplasmic reticulum. AF: actin filaments, ER: endoplasmic reticulum, Mt: mitochondria.

The clusters of InsP\(_3\)R-3 labeling were predominately present in the perinuclear region. From this area, there was a gradient of decreasing clustering. In addition, diffuse InsP\(_3\)R-3 labeling was observed throughout the cytoplasm. Mitochondria and actin filaments were mostly free of label. Although most cells showed InsP\(_3\)R-3 immunoreactivity, some cells were free of labeling (<10%). These cells differed in their ultrastructural characteristics: they were spherical and more electron-dense. On the whole, untreated A7r5 cells did not display any abnormal morphological characteristics.

To determine a possible relation between agonist-mediated down-regulation of InsP\(_3\)R-3 and clustering of InsP\(_3\)R-3, we determined the intracellular distribution of InsP\(_3\)R-3 in response to prolonged treatment with PLC activating agonists and verapamil.

Treatment of A7r5 cells with vasopressin (3\(\mu\)M, 12 h) resulted in the almost complete abolition of InsP\(_3\)R-3 clustering (Fig. 5A). In addition, diffuse...
cytoplasmicInsP₃R-3 labeling was reduced when compared to untreated cells. The endoplasmic reticulum was dilated. No other morphological changes were observed.

Although treatment of A7r5 cells with verapamil (2µM, 12 h) did not result in down-regulation of InsP₃R-3 immunoreactivity as measured by Western blotting, verapamil treatment resulted also in an almost complete abolishment of the InsP₃R-3 clusters (Fig. 5B). However, diffuse InsP₃R-3 labeling was strongly increased when compared to control cells (Fig. 5C). Morphological inspection of verapamil-treated A7r5 cells revealed a poor cellular ultrastructure. The cytoplasm was fragmented and highly granular. In addition, dilated membranes were observed.

![Fig. 5](image)

**Fig. 5** The effect of carbachol, vasopressin and verapamil on the distribution of InsP₃R-3 in A7r5 cells. A) Exposure to vasopressin (3µM, 12 h) also resulted in an almost complete abolishment of the InsP₃R-3 clusters. B) Cells treated with verapamil (2µM, 12 h) have a poor cellular ultrastructure. InsP₃R-3 clustering has almost completely disappeared. C) Detail of a cell treated with verapamil. Strong diffuse labeling can be observed. D) Exposure to carbachol (1mM, 12 h) did not result in a strong reduction of InsP₃R-3 clusters. Nu: nucleus.

Treatment of A7r5 cells with carbachol (1mM, 12 h) resulted in similar InsP₃R-3 labeling patterns as for control cells (Fig. 5D). Again, InsP₃R-3 labeling was strongly clustered on structures that appeared to be regions of specialized endoplasmic reticulum. In addition, diffuse labeling was found in the
cytoplasm. Besides a slightly dilated endoplasmic reticulum, no morphological changes were observed. It has been suggested that InsP_3 R-3 situated at or near the plasma membrane, might play a role in (store operated) Ca^{2+} entry (Berridge, 1995b; DeLisle et al., 1996; Khan et al., 1996; Putney, 1997). However, no aggregation of InsP_3 R-3 was observed near the plasma membrane in any of the observed A7r5 cells. In a control series the primary InsP_3 R-3 antibody was omitted from the incubation procedure. Some labeling was observed on the chromatin-dense regions within the nucleus (data not shown), indicating that labeling of the chromatin-dense regions arose from non-specific labeling. However, no non-specific labeling was observed either on clusters or diffusely in the cytoplasm (data not shown), indicating the specificity of the InsP_3 R-3 labeling reaction.

**Discussion:**
In this study, we demonstrate down-regulation of InsP_3 R-1 and InsP_3 R-3 in A7r5 smooth muscle cells in response to long-term exposure to vasopressin, whereas exposure to verapamil resulted in down-regulation of only InsP_3 R-1. Vasopressin pre-treatment resulted in impaired Ca^{2+} release from InsP_3 sensitive stores whereas verapamil pre-treatment did not affect Ca^{2+} release. Further, we show that InsP_3 R-3 labeling was strongly clustered on structures that appeared to be regions of specialized endoplasmic reticulum. Surprisingly, most of the endoplasmic reticulum did not show InsP_3 R-3 labeling. Pre-treatment with vasopressin and verapamil resulted in a dramatic reduction of InsP_3 R-3 clusters, suggesting that redistribution of InsP_3 R-3 is independent on InsP_3 R-3 down-regulation.

**Down-regulation of specific InsP_3 Rs types**
In the present study, we investigated the function and regulation of InsP_3 R-1 and InsP_3 R-3. We therefore aimed at obtaining an experimental model in which specific InsP_3 R types could be down-regulated. Long-term exposure of A7r5 cells to vasopressin resulted in down-regulation of both InsP_3 R-1 and InsP_3 R-3. Exposure to verapamil resulted in down-regulation of only InsP_3 R-1. The extent of down-regulation of InsP_3 R-1 was similar for vasopressin and verapamil pre-treatment. From this, we conclude that A7r5 cells may provide a good model for studying the regulation and function of specific InsP_3 R types. Down-regulation of InsP_3 Rs was not the result of a non-specific process that affected several different proteins, as the expression of SERCA 2b did not differ statistically from controls in vasopressin or verapamil treated cells.
Despite the lack of statistic significance, SERCA 2b expression seemed slightly decreased in both vasopressin and verapamil treated cells. However, SERCA 2b expression levels were identical for vasopressin and verapamil treated cells, indicating that down-regulation of InsP₃R-1 in vasopressin and verapamil treated cells is comparable.

Down-regulation of InsP₃R-1 and InsP₃R-3 in A7r5 cells was observed by long-term treatment with vasopressin in a time-dependent manner. Treatment with the PLC-activating agonists ATP, bradykinin, carbachol or histamine did not result in down-regulation of InsP₃Rs. Therefore, the down-regulation of InsP₃R-1 and InsP₃R-3 by PLC-activating agonists seems to be agonist specific.

Down-regulation of InsP₃Rs by specific PLC-activating agonists has also been described previously. Chronic treatment of WB rat liver epithelial cell with AII resulted in down-regulation of InsP₃R-1 and InsP₃R-3, whereas vasopressin, bradykinin or 12-O-tetradecanoylphorbal-13-acetate was without effect (Bokkala and Joseph, 1997). Further, down-regulation of InsP₃Rs by chronic carbachol treatment has been described for SH-SY5Y human neuroblastoma cells (Wojcikiewicz et al., 1994). Therefore, the down-regulation of InsP₃R-1 and InsP₃R-3 by PLC-activating agonists seems to be agonist specific as well as cell type specific.

Exposure of A7r5 cells to verapamil resulted in down-regulation of InsP₃R-1 to a similar extent as in vasopressin treated cells. In contrast, pre-treatment with verapamil did not result in down-regulation of InsP₃R-3. Therefore, inhibition of spontaneous Ca²⁺ spiking by verapamil only seems to affect InsP₃R-1 expression in A7r5 cells.

**Functional consequences of specific InsP₃R down-regulation**

As InsP₃R-1 and InsP₃R-3 can be differentially down-regulated in A7r5 cells, functional differences between InsP₃R-1 and InsP₃R-3 may be determined in vasopressin and verapamil pre-treated A7r5 cells. InsP₃ mediated Ca²⁺ release was impaired in vasopressin pre-treated A7r5 cells, whereas InsP₃ mediated Ca²⁺ release in verapamil pre-treated cells was unchanged. Therefore, the differences in InsP₃ mediated Ca²⁺ release seem to arise from differences in the regulation of InsP₃R-3.

Pre-treatment with vasopressin resulting in a down-regulation of both InsP₃R-1 and InsP₃R-3 was accompanied by a reduction of Ca²⁺ release at submaximal InsP₃ concentrations without changes in total loading and the relative amount of Ca²⁺ that could be released by InsP₃. These results indicate that the impaired Ca²⁺ responses in vasopressin treated cells were caused by down-regulation of...
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InsP$_3$Rs and not by changes in the size of the InsP$_3$-releasable Ca$^{2+}$ pool. As all Ca$^{2+}$ flux experiments were performed in the presence of the SERCA blocker thapsigargin, impaired Ca$^{2+}$ responses were not caused by differences in SERCA 2b levels.

As impaired Ca$^{2+}$ release was accompanied by down-regulation of InsP$_3$R-1 and InsP$_3$R-3 whereas downregulation of only InsP$_3$R-1 was without affect, one might speculate that the impaired Ca$^{2+}$ release is caused primarily by down-regulation of InsP$_3$R-3. However, the role of InsP$_3$R-1 in Ca$^{2+}$ release from intracellular stores is well established. In addition, as InsP$_3$R-1 is expressed most abundantly in A7r5 cells (De Smedt et al., 1994a), it seems unlikely that down-regulation of InsP$_3$R-1 does not affect Ca$^{2+}$ release from InsP$_3$ sensitive stores.

Possibly, InsP$_3$R-3 may compensate for down-regulation of InsP$_3$R-1 in verapamil pre-treated A7r5 cells, resulting in unchanged Ca$^{2+}$ responses. Down-regulation of both InsP$_3$R-1 and InsP$_3$R-3 in vasopressin pre-treated cells may therefore no longer enable compensatory mechanisms, resulting in impaired Ca$^{2+}$ release. Such a compensatory mechanism has been demonstrated in B-cells. There it was demonstrated that B-cells in which a single type of InsP$_3$R had been deleted still mobilized calcium from internal stores (Sugawara et al., 1997). Calcium release was only abrogated in B-cells lacking all three types of InsP$_3$Rs, suggesting that InsP$_3$R types are functionally redundant.

**Intracellular distribution of InsP$_3$R-3**

To our knowledge this is the first time that the intracellular location and clustering of InsP$_3$R-3 is described in vascular smooth muscle cells using immunoelectron microscopy.

Although the impaired Ca$^{2+}$ release may be the consequence of down-regulation of both InsP$_3$R-1 and InsP$_3$R-3, the differences in InsP$_3$ mediated Ca$^{2+}$ release in vasopressin and verapamil treated cells seem to arise from differences in the regulation of InsP$_3$R-3. We therefore determined the intracellular distribution of InsP$_3$R-3 in A7r5 cells in response to long-term agonist treatment using immunoelectron microscopy.

Although immunoelectron microscopy only allows for a semi-quantitative evaluation of InsP$_3$R-3 labeling, the results obtained with immunoelectron microscopy were identical to those obtained with Western-blotting. Similar amounts of InsP$_3$R-3 labeling were observed in control cells, carbachol treated cells and verapamil treated cells, whereas InsP$_3$R-3 labeling was strongly reduced in vasopressin pre-treated cells. Using Western-blotting, we
demonstrated that InsP$_3$R-3 is maximally down-regulated by about 64% in vasopressin treated cells. Immunoelectron microscopy demonstrated an almost complete abolishment of InsP$_3$R-3 labeling in A7r5 cells. However, because of the semi-quantitative nature of immunolabeling, the reduction in InsP$_3$R-3 labeling may reflect the 64% down-regulation as observed by Western-blotting. InsP$_3$R-3 labeling was strongly clustered on structures that appeared to be regions of specialized endoplasmic reticulum. We concluded this from the fact that the clusters of InsP$_3$R-3 labeling were always associated with the endoplasmic reticulum. Further, several studies have demonstrated that InsP$_3$Rs are situated on endoplasmic reticulum membranes (Ross et al., 1989), although some studies suggest that InsP$_3$Rs are also present on the plasma (Fujimoto et al., 1992) and nuclear membrane (Humbert et al., 1996; Stehno-Bittel et al., 1995; Malviya, 1994).

Exposure to vasopressin resulted in a dramatic reduction of InsP$_3$R-3 clusters. As carbachol treatment was without effect, the reduction of InsP$_3$R-3 clustering in A7r5 cells is not only controlled by an increase of InsP$_3$ and Ca$^{2+}$ release. Pre-treatment with the Ca$^{2+}$ channel blocker verapamil also resulted in an almost complete abolishment of clustering. As high dosages of vasopressin and verapamil result in the inhibition of spontaneous Ca$^{2+}$ spiking through L-type calcium channels (Byron and Taylor, 1993; Sipma et al., 1998), the redistribution of InsP$_3$R-3 in A7r5 cells seems to depend on Ca$^{2+}$ entry through L-type Ca$^{2+}$ channels. Previously, in experiments performed on RBL-2H3 cells in absence of extracellular calcium, it has been demonstrated that redistribution of InsP$_3$R-2 is also dependent on Ca$^{2+}$ entry (Wilson et al., 1998). In A7r5 cells, Ca$^{2+}$ entry evoked by low concentrations of vasopressin is mediated largely by arachidonic acid (Broad et al., 1999). Therefore, the redistribution of InsP$_3$R-3 might be regulated through the PLC/ diacylglycerol lipase/ arachidonic acid pathway rather than the PLC/ InsP$_3$/ Ca$^{2+}$ pathway.

Exposure of A7r5 cells to verapamil resulted in an almost complete abolishment of the InsP$_3$R-3 clusters. The abolishment of InsP$_3$R-3 clusters was accompanied by a strong increase of diffuse staining. However, exposure of A7r5 cells to verapamil did not result in a decrease of InsP$_3$R-3 as measured by Western-blotting. Since InsP$_3$R-3 was quantified by Western-blotting on proteins from a microsomal fraction, the diffusely labeled InsP$_3$R-3 should still be bound to endoplasmic reticulum membrane. However, the cytoplasmic granulation and dilation of membranes in verapamil treated A7r5 cells, strongly hampered the visualization of intracellular membranes. Thus, we conclude that exposure to verapamil results in dispersion of the InsP$_3$R-3 clusters, implicating
that redistribution of InsP$_3$R-3 in verapamil-treated cells is independent on down-regulation. On the light-microscopic level, redistribution of InsP$_3$R-3 without down-regulation has also been suggested for InsP$_3$R-3 in hamster lung fibroblast E36(M3R) cells and for InsP$_3$R-2 in rat basophilic leukemia (RBL-2H3) cells, indicating that this process is not cell or species specific (Wilson et al., 1998).

In the present study, we observed clustering of the InsP$_3$R-3 using immunoelectron microscopy. At the light microscopic level, clustering of InsP$_3$R-3 has also been described in hamster lung fibroblast E36(M3R) cells (Wilson et al., 1998), whereas InsP$_3$R-2 clustering has been observed in rat basophilic leukemia (RBL-2H3) cells and rat pancreatoma (AR4-2J) cells (Wilson et al., 1998). Although the results obtained at the light microscopic level suggested that the InsP$_3$R clusters were associated with the endoplasmic reticulum, this is the first study demonstrating that InsP$_3$R-3 clusters are attached to the endoplasmic reticulum. In E36(M3R) cells, increased InsP$_3$R-3 clustering was observed after exposure to carbachol whereas in A7r5 smooth muscle cells, exposure to vasopressin and verapamil resulted in a decrease of InsP$_3$R-3 clustering. However, the comparison of redistribution of InsP$_3$R-3 in E36(M3R) and A7r5 cells is hampered by the differences in the techniques used. E.g. small clusters might not be detected by immunofluorescence microscopy since the resolution of this technique is lower than for immunoelectron microscopy. Besides technical differences, differences in InsP$_3$R clustering may have arisen from the fact that E36(M3R) cells were only stimulated with carbachol for 10-60 minutes whereas A7r5 cells were exposed to vasopressin and verapamil for 12 hours. In addition, E36(M3R) do not express muscarinic receptors and as hamster E36(M3R) cells were transfected with the human m3-muscarinic receptor, InsP$_3$R-3 clustering in E36(M3R) cells may not reflect normal physiological conditions. Further, we can not exclude that the regulation of redistribution of InsP$_3$R-3 is cell type specific.

The functional properties of InsP$_3$R-3 clustering are unknown. In the present study we suggest that InsP$_3$R-3 may compensate for down-regulation of InsP$_3$R-1. Possibly, the diffuse redistribution of InsP$_3$R-3 in verapamil treated cells may reflect the compensatory function of InsP$_3$R-3. Alternatively, for pancreatic acinar cells, it has been suggested that plasma membrane receptors are coupled to their own specific internal Ca$^{2+}$ stores. Possibly the clusters of InsP$_3$R-3 may reflect such receptor-specific Ca$^{2+}$ stores in A7r5 cells. Although InsP$_3$R-3 clusters might be operated by specific plasma membrane receptors, the regulation of InsP$_3$R-3 clustering appears to be similar for all
clusters, as vasopressin treatment results in a nearly complete abolishment of all InsP₃R-3 clusters. Further, if the abolishment of clustering is the result of InsP₃-mediated Ca²⁺ release, this would imply that vasopressin receptors are coupled to all clusters.

In addition to InsP₃R-3 clustering on the endoplasmic reticulum, we found diffuse InsP₃R-3 labeling throughout the cytoplasm in untreated, carbachol treated and vasopressin treated A7r5 cells. The diffuse cytoplasmic InsP₃R-3 labeling was not associated with any membrane or organelle. Therefore, these receptors do not appear to be functional Ca²⁺ release channels. Possibly, the diffuse cytoplasmic labeling may reflect InsP₃R-3 degradation products. It has been demonstrated that cytosolic InsP₃R-I fragments can be stably expressed in NG108-15 neuroblastoma cells (Miyawaki et al., 1991) and in 3T3 fibroblasts (Fischer et al., 1994). Surprisingly, these fragments could still bind InsP₃ and had a marked effect on cell-growth and transformation. Possibly, InsP₃R degradation products may compete for InsP₃ binding or act as specific signaling peptides.

It has been suggested that InsP₃R-3 situated at or near the plasma membrane, might play a role in store-operated Ca²⁺ entry (Berridge, 1995b; DeLisle et al., 1996; Khan et al., 1996; Putney, 1997). However, no aggregation of InsP₃R-3 could be observed near the plasma membrane in any of the studied groups. In addition, we previously demonstrated that down-regulation of InsP₃R-1 and InsP₃R-3 in A7r5 cells did not affect store-operated Ca²⁺ entry (Sipma et al., 1998). Further, store operated Ca²⁺ entry remained intact in B cells lacking all three types of InsP₃R (Sugawara et al., 1997). From this, we conclude that InsP₃R-3 is unlikely to be involved in store-operated Ca²⁺ entry in A7r5 cells in general.

Although most cells showed InsP₃R-3 immunoreactivity, some cells were virtually free of labeling. These cells differed in their ultrastructural characteristics: they were spherical and more electron-dense. From these morphological features it appears that these cells were engaged in mitosis. This observation could implicate that InsP₃R-3 is involved in mitosis. Although, InsP₃Rs are involved in cell proliferation (for review see (Berridge, 1995a)), it is unknown whether InsP₃R-3 is down-regulated in mitotic cells.

In conclusion, we demonstrated down-regulation of InsP₃R-1 and InsP₃R-3 in A7r5 smooth muscle cells in response to long-term exposure to vasopressin, whereas exposure to verapamil resulted in down-regulation of only InsP₃R-1. Vasopressin pre-treatment resulted in impaired Ca²⁺ release from InsP₃ sensitive stores whereas verapamil pre-treatment did not affect Ca²⁺ release.
Further, we show that InsP₃R-3 labeling is strongly clustered on structures that appear to be regions of specialized endoplasmic reticulum, whereas most of the endoplasmic reticulum did not show InsP₃R-3 labeling. Pre-treatment with vasopressin and verapamil resulted in a dramatic reduction of InsP₃R-3 clusters, implicating that redistribution of InsP₃R-3 is independent on InsP₃R-3 down-regulation.
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