Micromolar calcium decreases affinity of inositol trisphosphate receptor in vascular smooth muscle

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The mechanism by which Ca^{2+} inhibits $InsP_3$-induced Ca^{2+} release from sarcoplasmic reticulum of vascular smooth muscle was investigated. $InsP_3$ binding to sarcoplasmic-reticulum vesicles from dog aortic smooth muscle was inhibited by $51 \pm 6\%$ by 2 μM Ca^{2+} in the presence of 10 nM [^{3}H]$InsP_3$. Scatchard analysis indicated the presence of two $InsP_3$-binding sites in the absence of Ca^{2+} ($K_d = 2.5 \pm 0.9$ and 49 ± 8 nM $InsP_3$), though the low-affinity site was more prevalent (representing $92 \pm 3\%$ of the total number of binding sites). Ca^{2+} (2 μM) did not alter $InsP_3$ binding to the high-affinity site ($P > 0.05$), but increased the K_d of the low-affinity site 3-fold ($K_d = 155 \pm 4$ nM $InsP_3$; $P < 0.001$). The possibility that the apparent decrease in $InsP_3$ affinity was caused by Ca^{2+}-dependent activation of an endogenous phospholipase C could be excluded, because the Ca^{2+}-dependent inhibition of $InsP_3$ binding was completely reversible and insensitive to an inhibitor of phospholipase C. Moreover, Ca^{2+} did not inhibit $InsP_3$ binding to $InsP_3$ receptor partially purified by heparin–Sepharose chromatography, though another fraction (devoid of $InsP_3$ receptor) restored Ca^{2+}-sensitivity of the partially purified $InsP_3$ receptor. Thus Ca^{2+} binding to a Ca^{2+}-sensitizing factor associated with the $InsP_3$ receptor decreases the affinity of the receptor complex for $InsP_3$. This Ca^{2+}-sensitizing factor may provide a negative-feedback mechanism for regulating the rise in cytosolic Ca^{2+} concentration in vascular smooth muscle after hormone activation of the phosphoinositide cascade.

INTRODUCTION

Inositol 1,4,5-trisphosphate ($InsP_3$) represents an important second messenger for a variety of Ca^{2+}-mobilizing receptors in vascular smooth muscle [1,2]. In this phosphoinositide cascade, agonist-induced activation of phospholipase C leads to the hydrolysis of the sarcolemmal phospholipid phosphatidylinositol 4,5-bisphosphate [PtdIns(4,5)P_2] to produce $InsP_3$ that, when bound to a Ca^{2+} channel in the sarcoplasmic reticulum, releases Ca^{2+} from this intracellular store. The resulting increase in intracellular Ca^{2+} initiates contraction of the vascular smooth muscle.

Recent studies have suggested that the $InsP_3$-gated Ca^{2+} channel may be regulated by Ca^{2+}. In cerebellar membranes, Ca^{2+} concentrations in excess of 0.2 μM decreased the open probability of the $InsP_3$-gated Ca^{2+} channel [3]. Similar results were also reported for reticular membranes isolated from rat forebrain [4]. The mechanism underlying this inhibitory effect, however, is unclear. Worley et al. [5] have shown that Ca^{2+} inhibits $InsP_3$ binding, with half-maximal inhibition at 0.3 μM Ca^{2+}. Mignery et al. [6], on the other hand, suggested that this Ca^{2+}-dependent inhibition of $InsP_3$ binding was an artifact resulting from Ca^{2+}-dependent stimulation of an endogenous phospholipase C.

Vascular and intestinal smooth muscle also show a Ca^{2+}-dependent inhibition of $InsP_3$-induced Ca^{2+} release [7,8]. The mechanism underlying this inhibition, however, has not been investigated. In the present study, we report that, as in the cerebellum, micromolar Ca^{2+} inhibits $InsP_3$ binding. In view of the report by Mignery et al. [6], particular attention has been paid to the possibility of Ca^{2+}-dependent activation of an endogenous phospholipase C. Our results indicate that micromolar Ca^{2+} inhibits the $InsP_3$ binding by decreasing the affinity of the receptor for $InsP_3$. Morover, this Ca^{2+}-dependent change in $InsP_3$ affinity is not due to a direct effect of Ca^{2+} on the $InsP_3$ receptor, but instead is mediated by a Ca^{2+}-sensitizing factor that is associated with the $InsP_3$ receptor. An abstract describing these findings has been published [9].

MATERIALS AND METHODS

Reagents

[^{3}H]$InsP_3$ and $InsP_3$ were obtained from NEN and Calbiochem, respectively. Heparin–Sepharose was from Pharmacia. Triton X-100 was from either Sigma or Bio-Rad. Other reagents were commercial preparations of analytical grade. Milli-Q water was used in all studies.

Membrane preparation

Sarcoplasmic-reticulum vesicles were isolated from dog aortic smooth muscle by differential centrifugation, as previously described [10,11]. Briefly, tissue was minced with a Waring blender and then homogenized by Polytron in 20 mM Hepes (pH 7.1)/100 μM EGTA/5 mM NaN_3. All solutions contained leupeptin and pepstatin A (1 μg/ml each). The homogenate was centrifuged at 4200 *g* for 20 min, then the supernatant was centrifuged at 72000 *g* for 30 min. The pellet from the latter spin was resuspended in 0.6 M KCl/20 mM sodium pyrophosphate/5 mM NaN_3/10 mM Hepes (pH 7.2) and then centrifuged at 4200 *g* (20 min). The supernatant was centrifuged at 72000 *g* (30 min). The pellet from this final spin was resuspended in 10% sucrose/20 mM Mops (pH 6.8) (final protein concn. ~ 8 mg/ml), frozen in liquid N_2, and stored at −80 °C.

Abbreviations used: DOC, deoxycholate; DTT, DL-dithiothreitol; HEDTA, *N*-hydroxyethylethylenediaminetriacetic acid.

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$InsP_3$ binding

Aortic sarcoplasmic-reticulum membranes (100 μl) were diluted into 1 ml of buffer A [2 mM *N*-hydroxyethylethylenediaminetriacetic acid (HEDTA)/1 mM EGTA/1 mM dithiothreitol (DTT), with 50 mM Tris or Hepes, pH 8.3, 2 °C], and centrifuged for 15 min at 16000 *g*. The pellet (containing 90% of the initial membrane protein) was resuspended in buffer A (final concn. ~ 4 mg/ml), and used directly in the $InsP_3$-binding assay. The resuspended aortic sarcoplasmic-reticulum membranes (10 μl) were incubated on ice for 10 min in buffer A, containing 0.1 M NaCl, 0.8–20 nM [^{3}H]$InsP_3$, 0–1200 nM $InsP_3$, and the specified $CaCl_2$ concentration (40 μl final volume). Binding reactions were terminated by centrifugation (16000 *g*, 15 min, 4 °C). The supernatant was aspirated, and the pellet was rinsed quickly with 0.25 ml of buffer A. Identical results were obtained when the rinse was omitted. Pellets were solubilized with 0.1 ml of Packard Soluene 350 (55 °C, 30 min), then the radioactivity was measured by liquid-scintillation counting (by using ICN Cytoscint). Non-specific binding was determined under identical conditions, in the presence of 40 μM $InsP_3$. Similar results were obtained when non-specific binding was performed in the presence of 250 μM $InsP_3$. Iterative curve-fitting routines (SigmaPlot) were used to calculate the $InsP_3$ affinities and maximal binding levels.

$InsP_3$ binding to the solubilized receptor and to fractions eluted from the heparin–Sepharose column was measured by a poly(ethylene glycol) precipitation technique [12], as described below.

Phospholipase C activity

Phospholipase C activity was measured as the production of [^{3}H]$InsP_3$ from phosphatidyl[2-^{3}H]inositol 4,5-bisphosphate ([^{3}H]Ptd$InsP_2$) [13]. Assay media contained buffer A, with 0.1 M NaCl, 20 μM [^{3}H]Ptd$InsP_2$, and specified concentrations of $CaCl_2$, $BaCl_2$ and deoxycholate (DOC).

Heparin–Sepharose chromatography

Aortic sarcoplasmic-reticulum membranes (2 mg/ml) were solubilized in 1.5% Triton X-100 in 1 mM EDTA/1 mM DTT/50 mM Tris (pH 8.3, 2 °C, 30 min). Samples were centrifuged (16000 *g*, 20 min, 4 °C), then the supernatant was applied to a Pharmacia heparin–Sepharose column (attached to a Pharmacia f.p.l.c. system). The column was washed with the same buffer containing 0.25 M NaCl and 0.1% Triton (0.5 ml/min). The $InsP_3$ receptor was eluted by increasing NaCl concentration to 0.5 M. Fractions were assayed for protein concentration with the Bio-Rad Protein Assay kit, with BSA as a standard. Peak fractions were used for $InsP_3$ binding in 1 mM EDTA/1 mM DTT/50 mM Tris (pH 8.3) as described above. Salt concentration was adjusted to 0.125 M for all binding reactions. Reactions were terminated by addition of 0.25 vol. of 12 mg/ml rabbit γ-globulin plus 1 vol. of 30% poly(ethylene glycol) in 50 mM Tris, pH 8.3, followed by centrifugation (16000 *g*, 15 min, 4 °C). Pellets were rinsed with buffer A, and then assayed for radioactivity (as described above). Identical results were obtained if the rinsing was omitted.

Free cation concentrations

Calculations of the total Ca^{2+} or Ba^{2+} concentrations needed to obtain a given free concentration were based on previously published association constants [14,15]. The apparent association constants (M^{-1}) used for these calculations were 8.25×10^8 for Ca–EGTA, 2.94×10^6 for Ca-HEDTA, 1.83×10^6 for Ba-EGTA and 3.78×10^4 for Ba-HEDTA.

RESULTS

Ca^{2+}-dependent inhibition of $InsP_3$ binding

$InsP_3$ binding to aortic sarcoplasmic-reticulum vesicles were inhibited by 51±6% ($P < 0.05$) when free Ca^{2+} concentration was increased from 30 nM to 2 μM. Increasing the free Ca^{2+} concentration to 140 μM had no further inhibitory effect (56±7% inhibition; $P > 0.05$). Similar results (62% inhibition) were obtained when the free Ca^{2+} concentration was raised to 593 μM (results not shown). Thus maximal inhibition of $InsP_3$ binding amounts to only 50–60% in the presence of 10 nM $InsP_3$. Half-maximal inhibition occurred at 0.3 μM free Ca^{2+} (Figure 1).

Possible influence of an endogenous phospholipase C

To determine if activation of Ca^{2+}-dependent phospholipase C could account for the apparent Ca^{2+}-dependent decrease in $InsP_3$ binding [6], the reversibility of the Ca^{2+} effect was examined. As shown in Table 1, addition of EGTA to the binding assay after

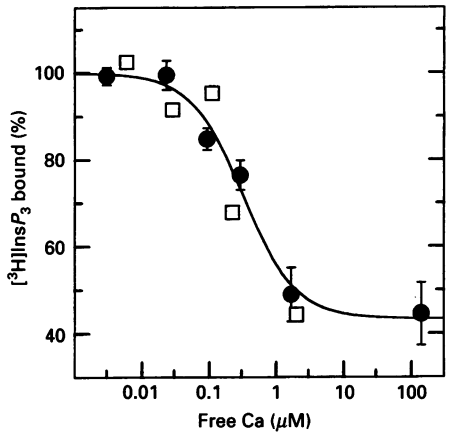

Figure 1 Ca^{2+}-dependent inhibition of $InsP_3$ binding to sarcoplasmic reticulum from dog aortic smooth muscle

Circles represent data obtained in the presence of 1 mM EDTA, 2 mM HEDTA, with 0–3.125 mM $CaCl_2$ (as described in the Materials and methods section). Similar results were obtained in the presence of 2 mM HEDTA, 2.7 mM $CaCl_2$ and 1–10 mM EGTA (squares). The continuous line represents a computer fit of the data (circles; Hill coefficient = 1, $K_{0.5}$ = 0.3 μM Ca^{2+}). The 100% value is 0.98±0.15 pmol/mg. Non-specific binding was 25±0.8% of the total, and was subtracted from values shown in this Figure.

Table 1 Reversibility of Ca^{2+}-dependent inhibition of $InsP_3$ binding under different preincubation conditions

Samples were preincubated at specified temperature (with or without DOC or Ba^{2+}, as indicated) in the presence of 2 μM Ca^{2+} for 10 min. Ca^{2+}-dependent inhibition was then measured at 0 °C, in the presence of 10 nM $InsP_3$ (2 μM free Ca). Reversibility of this inhibition was measured by adding 2.5 mM EGTA (4 nM free Ca^{2+}) to the binding assay. Data (means±S.E.M.) represent relative binding (%) to control conditions when both preincubation and incubation were done in the presence of EGTA (4 nM Ca^{2+}): *statistically different from corresponding numbers obtained at 0 °C ($P < 0.05$).

	Incubation conditions	
Preincubation conditions	2 μM Ca^{2+}	4 nM Ca^{2+}
0 °C + 2 μM Ca^{2+}	39±3 ($n = 8$)	95±2 ($n = 8$)
37 °C + 0.05% DOC + 2 μM Ca^{2+}	24±3* ($n = 3$)	42±3* ($n = 4$)
37 °C + 0.05% DOC + 2 μM Ca^{2+} + 100 μM Ba^{2+}	48±7 ($n = 3$)	92±3 ($n = 4$)

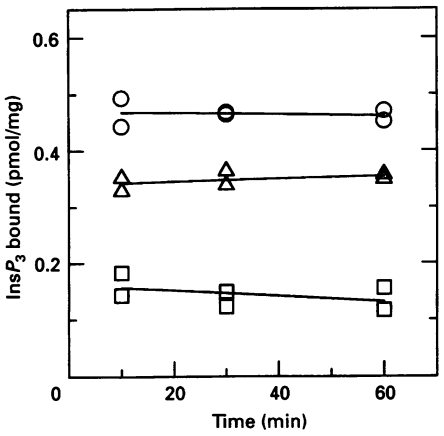

Figure 2 Ca^{2+}-dependent inhibition of InsP_3 binding is stable for up to 60 min

Aortic sarcoplasmic reticulum was incubated in the presence of 0.4 μM Ca^{2+} (triangles) or 2 μM Ca^{2+} (squares) for various times before termination of the binding reaction by centrifugation (as described in the Materials and methods section). Circles represent data obtained in the absence of added Ca^{2+} (in the presence of 1 mM EGTA, 2 mM HEDTA). Data shown are from two experiments.

10 min preincubation with 2 μM free Ca^{2+} completely restored InsP_3 binding, demonstrating the complete reversibility of the Ca^{2+}-dependent inhibition of InsP_3 binding. If Ca^{2+} had activated an endogenous phospholipase C that produced InsP_3, chelation of Ca^{2+} by EGTA would not have restored InsP_3 binding.

Under certain conditions (such as preincubation of the aortic SR membranes with 2 μM Ca^{2+} at 37 °C in the presence of 0.05% DOC), the Ca^{2+}-dependent inhibition of InsP_3 binding was only partially reversible (Table 1). We hypothesized that this partial reversibility was due to an activation of an endogenous Ca^{2+}-dependent phospholipase C. With [^{3}H]PtdInsP_2, Ca^{2+}-dependent phospholipase C activity could be observed in the membrane preparation when assayed at 37 °C in the presence of 0.05% deoxycholate. The commercially available inhibitor U231166 (100 μM [16]) did not block this phospholipase activity, whereas 100 μM Ba^{2+} decreased the Ca^{2+}-activated phospholipase C activity by 84±2% (n = 4). Consequently, the reversibility of Ca^{2+}-dependent inhibition of InsP_3 binding was re-examined in the presence of Ba^{2+}. As shown in Table 1, inclusion of 100 μM Ba^{2+} in the preincubation (containing 0.05% DOC; 37 °C) restored reversibility of the Ca^{2+}-dependent inhibition of InsP_3 binding (92% versus 42%). Thus, under some conditions (such as high temperature and in the presence of detergents), Ca^{2+}-dependent activation of an endogenous phospholipase C may contribute to the apparent Ca^{2+}-dependent inhibition of InsP_3 binding. Under normal binding conditions (0 °C), or when phospholipase C is inhibited by Ba^{2+}, micromolar Ca^{2+} inhibits InsP_3 binding independently of phospholipase C.

Further evidence against the involvement of phospholipase C in the Ca^{2+}-dependent inhibition of InsP_3 binding to aortic sarcoplasmic-reticulum membranes is provided by Figure 2, which shows the time course of InsP_3 binding at three calcium concentrations. Had Ca^{2+}-dependent phospholipase C been activated in this assay, one would expect InsP_3 binding to decrease with time, particularly at sub-maximal Ca^{2+} concentrations. As shown in Figure 2, however, the inhibition of InsP_3 binding at 0.4 μM free Ca^{2+} remained constant over 60 min.

Effect of Ca^{2+} on InsP_3 affinity

InsP_3 binding to aortic membranes was measured over a broad range of InsP_3 concentrations (0.8–1220 nM), but, as shown in Figure 3, Scatchard plots of these data were non-linear. These InsP_3-binding data could be fitted by assuming the presence of two InsP_3-binding sites. In the absence of Ca^{2+} (Figure 3a), the calculated dissociation constants (K_d) were 4 nM and 47 nM, with 84% of the binding sites being low affinity. In the presence of 2 μM Ca^{2+}, there was dramatic increase in the K_d of this low-affinity InsP_3-binding site (K_d = 315 nM), but no increase in the K_d of the high-affinity InsP_3-binding site (K_d = 2 nM). In three separate experiments, the K_d of the low-affinity site increased

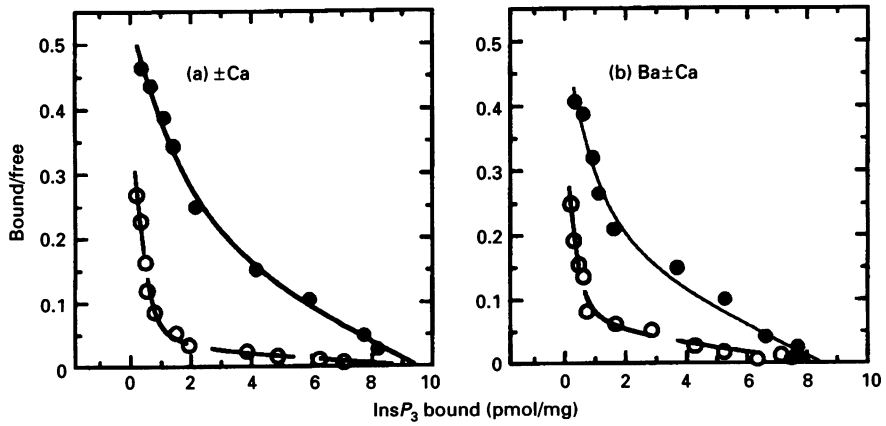

Figure 3 Ca^{2+} decreases the affinity of InsP_3 binding to aortic sarcoplasmic reticulum

InsP_3 binding was measured as described in the Materials and methods section (0 °C) in the absence of Ca^{2+} (●) or in the presence of 2 μM free Ca^{2+} (○). In panel (**b**), the incubation medium was supplemented with 100 μM free Ba^{2+} (to inhibit any endogenous phospholipase C activity). The lines represent predicted InsP_3 binding assuming the presence of both high-affinity and low-affinity InsP_3-binding sites (see the text and Table 2 for affinities and number of binding sites for panel **b**). Curve-fitting was done by using SigmaPlot for Windows. Incubation media included 1 mM EGTA, 2 mM HEDTA, with 0–2.7 mM $CaCl_2$ (for **a**) or 0.915–2.68 mM $BaCl_2$ plus 0–1.99 mM $CaCl_2$ (for **b**).

Table 2 Micromolar Ca^{2+} increases the K_d of the low-affinity $InsP_3$ binding site

$InsP_3$ binding was measured as in Figure 3(b), in the presence of 100 μM free Ba^{2+} to inhibit any possible endogenous phospholipase C. Similar values were obtained in the absence of Ba^{2+} (cf. Figure 3a and the text). An iterative curve-fitting routine (Sigma Plot) was used to calculate the dissociation constants (K_{d1} and K_{d2}) and numbers of binding sites. $B_{max.}$ is the total number of $InsP_3$-binding sites (high-affinity plus low-affinity sites). $\%B_2$ is the relative amount of low-affinity sites. Values represent means ± S.E.M. for three preparations: *statistically different from the values obtained in the absence of Ca^{2+}.

0 Ca^{2+}	2 μM Ca^{2+}
K_{d1} 2.5 ± 0.9 nM $InsP_3$	2.2 ± 0.6 nM $InsP_3$
K_{d2} 49.3 ± 8 nM $InsP_3$	155 ± 4.6 nM $InsP_3$ ($P < 0.001$)*
$B_{max.}$ 7.7 ± 0.41 pmol/mg	7.6 ± 0.5 pmol/mg
$\%B_2$ 91.8 ± 3.1	94.6 ± 0.7

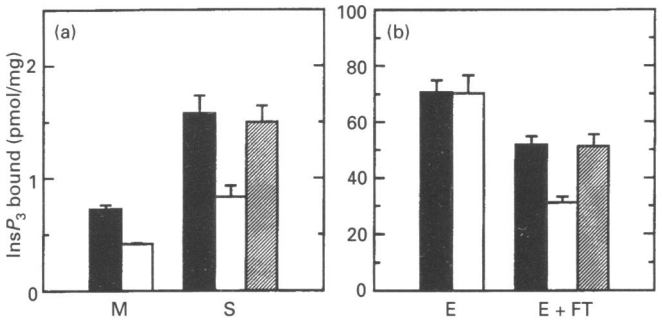

Figure 4 Ca^{2+}-sensitizing factor can be separated from the $InsP_3$ receptor

$InsP_3$ binding was measured in the presence of 0.6 nM free Ca^{2+} (black bars) or 50 μM free Ca^{2+} (white bars) (as described in the Materials and methods section). Reversibility of the Ca^{2+}-dependent inhibition was measured by preincubating samples in 50 μM free Ca^{2+}, then lowering Ca^{2+} concentration to 0.6 nM by addition of EGTA (striped bars). Abbreviations: M, aortic sarcoplasmic-reticulum membranes; S, solubilized membranes; E, eluate from heparin–Sepharose column; E + FT, mixture of eluate plus flow-through from heparin–Sepharose column. Data represent means ± S.E.M. ($n = 3$).

by an average of 3.9-fold in the presence of 2 μM free Ca^{2+} ($P < 0.05$).

As indicated above, this Ca^{2+}-dependent increase in the K_d is unlikely to be the result of a Ca^{2+}-dependent activation of an endogenous phospholipase C. Under certain conditions, however, an endogenous Ca^{2+}-dependent phospholipase C in the membrane preparation can be activated, though this can be inhibited by Ba^{2+} (100 μM). $InsP_3$ binding was therefore repeated in the presence of 100 μM free Ba^{2+}, with or without 2 μM free Ca^{2+}.

As shown in Figure 3(b), 100 μM free Ba^{2+} did not influence $InsP_3$ binding. Thus, in the absence of Ca^{2+}, the data could be fitted by assuming the presence of two $InsP_3$-binding sites ($K_d = 2.5 \pm 0.9$ nM and 49.3 ± 8 nM). In the presence of 2 μM free Ca^{2+}, however, there was a 3-fold increase in the K_d of the low-affinity site, with no change in the K_d of the high-affinity site (Table 2). Thus the low-affinity $InsP_3$-binding site appears to be Ca^{2+}-sensitive, whereas the high-affinity site is Ca^{2+}-insensitive.

In this two-site model for $InsP_3$ binding, the low-affinity site constitutes 92 ± 3% of the maximal $InsP_3$ binding level, and therefore is the more prevalent form of the receptor (Table 2). Ca^{2+} (2 μM) did not alter this distribution (95 ± 0.7%; $P > 0.05$), nor did it alter the calculated maximal level of $InsP_3$ binding ($P > 0.05$). Thus micromolar Ca^{2+} does not appear to change the maximal $InsP_3$ binding, but rather increases the K_d of the low-affinity $InsP_3$-binding site.

Role of Ca^{2+}-sensitizing factor

To determine whether the inhibitory effect of Ca^{2+} was due to a direct effect on the $InsP_3$ receptor, the latter was solubilized in 1.5% Triton X-100. After incubation for 30 min in the Triton-containing solution, insoluble material was separated by centrifugation, and the clear supernatant was assayed for $InsP_3$ binding. The supernatant contained 85% of the total $InsP_3$ binding, but contained only 40% of the total protein. Consequently, the specific $InsP_3$-binding level of the supernatant was approximately twice that of the original membranes (1.8 versus 0.8 pmol/mg) (Figure 4a). As in the intact membranes, 50 μM Ca^{2+} markedly inhibited $InsP_3$ binding to the solubilized receptor (Figure 4a), and this Ca^{2+}-dependent inhibition was completely reversed upon addition of EGTA (Figure 4a).

When the solubilized material was applied to a heparin–Sepharose column in the presence of 0.25 M NaCl, the $InsP_3$ receptor bound to the column. The $InsP_3$ receptor was eluted with 0.5 M NaCl, similarly to the cerebellar $InsP_3$ receptor [12,17]. The eluate (which was enriched 50-fold in $InsP_3$ receptor) was no longer sensitive to Ca^{2+} (Figure 4b). The flow-through (which contained no detectable $InsP_3$ binding), however, restored Ca^{2+}-sensitivity to the eluate (Figure 4b). Furthermore, as in the intact membranes, this Ca^{2+} inhibition was completely reversible upon addition of EGTA (Figure 4b).

DISCUSSION

The present study shows that micromolar Ca^{2+} inhibits $InsP_3$ binding to sarcoplasmic-reticulum vesicles isolated from vascular smooth muscle by decreasing the affinity of the $InsP_3$ receptor. The inhibition of $InsP_3$ binding occurred at physiologically relevant Ca^{2+} concentrations, with half-maximal inhibition at 0.3 μM Ca^{2+}. This Ca^{2+}-dependent inhibition of $InsP_3$ binding could explain the Ca^{2+}-dependent inhibition of $InsP_3$-induced Ca^{2+} release seen in vascular smooth muscle [8,18], and possibly intestinal smooth muscle [7], where half-maximal inhibition of Ca^{2+} release was reported to occur at 0.3–0.6 μM Ca^{2+}. Kinetic analyses have indicated that Ca^{2+}-dependent inhibition of $InsP_3$-induced Ca^{2+} release in vascular smooth muscle is rapid [8], and thus may provide immediate negative feedback that would promote an abbreviated, perhaps locally confined, Ca^{2+} release within the cell [8].

The inhibitory effect of Ca^{2+} on $InsP_3$ binding in aortic smooth muscle is similar in many respects to the effect in cerebellum. Half-maximal inhibition of $InsP_3$ binding to rat cerebellar membranes, for example, occurred at 0.3 μM Ca^{2+} [5], similar to results obtained with aortic smooth muscle (IC_{50} = 0.3 μM Ca^{2+}). Moreover, 1 μM Ca^{2+} has been reported to alter the $InsP_3$ affinity of rat cerebellar membranes from 64 nM to 220 nM [19], consistent with the results shown in the present study for dog aortic smooth-muscle sarcoplasmic reticulum. There are, however, some inconsistencies in the literature concerning the extent of inhibition by Ca^{2+} (with values ranging from 40 to 90% inhibition of $InsP_3$ binding to rat cerebellar membranes in the presence of 1–2 μM Ca^{2+}, 2.5–15 nM $InsP_3$) [5,19]. Under conditions identical with those for aortic sarcoplasmic reticulum, $InsP_3$ binding to rat cerebellar membranes was decreased by 80%

in the presence of 2 μM Ca^{2+} and 10 nM $Ins P_3$ (results not shown), and this inhibition was completely reversible upon addition of EGTA. Thus 2 μM Ca^{2+} only partially inhibits $Ins P_3$ binding to both aortic and cerebellar membranes, consistent with a Ca^{2+}-dependent decrease in affinity for $Ins P_3$.

Increasing the Ca^{2+} concentration to 50 μM resulted in more complete inhibition of $Ins P_3$ binding to rat cerebellar membranes (95% inhibition; results not shown), though this was only partially reversible upon addition of EGTA (55% reversibility; results not shown). The incomplete reversibility at this high Ca^{2+} concentration may reflect Ca^{2+} activation of an endogenous phospholipase C in the cerebellar preparation, as suggested previously [6]. A similar trend has recently been reported for myeloid cells, where low Ca^{2+} concentrations (0.5 μM) inhibited $Ins P_3$ binding reversibly [20]. At higher Ca^{2+} concentrations (1.3–100 μM), however, the reversibility appeared to be incomplete in these myeloid cells (Figure 1c in [20]). This contrasts with the present study, where Ca^{2+}-dependent inhibition of $Ins P_3$ binding to aortic sarcoplasmic reticulum was completely reversible at Ca^{2+} concentrations as high as 50 μM (Figure 4), which may reflect differences in the phospholipase C contamination in the membrane preparations from different cell types.

As indicated above, Ca^{2+}-stimulated phospholipase C could give rise to a spurious inhibitory effect of Ca^{2+} on $Ins P_3$ binding in cerebellum by endogenous generation of a significant amount of $Ins P_3$ in the assay. In the present study, we paid particular attention to this potential artifact, and concluded that under our conditions the Ca^{2+}-dependent inhibition of $Ins P_3$ binding was not due to such phospholipase C activity. We have, under certain conditions (i.e., at 37 °C in the presence of 0.05% DOC), been able to activate an endogenous phospholipase C in this aortic preparation, which led to a Ca^{2+}-dependent inhibition that was only slightly reversible (Table 1). Addition of 100 μM Ba^{2+} to the solution (which inhibits phospholipase C activity by 84%) restored the reversibility of the Ca^{2+}-dependent inhibition of $Ins P_3$ binding (92±3% reversibility; Table 1). Thus, although we could reproduce the result reported by Mignery et al. [6], the effect of Ca^{2+} on aortic sarcoplasmic reticulum under ordinary conditions appeared to be a true inhibition of $Ins P_3$ binding due to a decrease in $Ins P_3$ affinity.

Our data also argue against the involvement of phosphorylation/dephosphorylation in this Ca^{2+}-dependent inhibition of $Ins P_3$ binding. Firstly, the media lacked ATP and Mg^{2+}, which are needed for phosphorylation. Moreover, the Ca^{2+}-dependent inhibition was reversible (Table 1), which is difficult to reconcile with a phosphorylation/dephosphorylation pathway in the absence of MgATP. The time course of the Ca^{2+}-dependent inhibition at sub-maximal Ca^{2+} concentrations (Figure 2) also argues against involvement of an enzyme (kinase/phosphatase)-catalysed reaction.

It should be noted that in the present study, which examined $Ins P_3$ binding over a broad range of $Ins P_3$ concentrations (0.8–1220 nM), the Scatchard plots were non-linear. These data could be fitted by assuming the presence of both high-affinity and low-affinity $Ins P_3$-binding sites. The K_d of the high-affinity site (2.5 nM) is consistent with a previous report on aortic smooth muscle [21], though, as indicated in the Results section, the high-affinity $Ins P_3$-binding site was Ca^{2+}-insensitive. The low-affinity $Ins P_3$-binding site, on the other hand, was much more abundant than the high-affinity form, and represented approx. 92% of the maximal $Ins P_3$ binding. Moreover, the K_d of this low-affinity $Ins P_3$-binding site increased 3-fold in the presence of 2 μM Ca^{2+}. Thus the low-affinity form of the $Ins P_3$ receptor was Ca^{2+}-sensitive. This contrasts with the situation in hepatocytes, where Ca^{2+} was shown to decrease the K_d of the $Ins P_3$ receptor [22].

The mechanism by which Ca^{2+} decreases the affinity of the $Ins P_3$ binding was not due to Ca^{2+} binding to the $Ins P_3$ receptor itself, as Ca^{2+} had no effect on binding to partially purified $Ins P_3$ receptor (Figure 4). Ca^{2+}-sensitivity of the partially purified receptor could, however, be restored by addition of a fraction devoid of $Ins P_3$ receptor. It is possible that this Ca^{2+}-sensitizing factor is analogous to calmedin, a monomer of 15 kDa reported to convey Ca^{2+}-sensitivity to the $Ins P_3$ receptor in cerebellum [23,24]. Calmedin, however, has not been observed in peripheral tissues [23]. Studies are currently underway to identify this Ca^{2+}-sensitizing factor in aortic sarcoplasmic reticulum.

It is noteworthy that, even after incubating the flow-through fraction from the heparin–Sepharose column for 8 min at 90 °C, that fraction continued to produce a Ca^{2+}-dependent inhibition of $Ins P_3$ binding to the partially purified $Ins P_3$ receptor. Approx. 70% of the Ca^{2+}-dependent inhibition was preserved after this heat treatment. This contrasts with the effect of 10% trichloroacetic acid. Precipitation of all detectable protein in the flow-through by 10% trichloroacetic acid (followed by centrifugation and extraction of the trichloroacetic acid from the supernatant by ether) eliminated this Ca^{2+}-dependent inhibition. The relative heat-stability, coupled with the trichloroacetic acid lability, raises the possibility that the Ca^{2+}-sensitizing factor may be a low-molecular-mass protein. Although calmedin has been reported to have a molecular mass of 15 kDa [24], its temperature-sensitivity has not been published.

It is important to point out that Ca^{2+}-inhibition of $Ins P_3$ binding is not present in all cell types. Membranes from uterine smooth muscle [25] and from myeloid cells [20] exhibit a Ca^{2+}-sensitivity similar to that reported here for aortic sarcoplasmic reticulum, whereas membranes from vas deferens smooth muscle were insensitive to Ca^{2+} concentrations as high as 500 μM [26]. In the presence of cerebellar calmedin, however, $Ins P_3$ binding to the vas deferens $Ins P_3$ receptor was Ca^{2+}-sensitive [26]. Thus calmedin can convey Ca^{2+}-sensitivity to $Ins P_3$ binding in a peripheral tissue. The presence of such a Ca^{2+}-sensitizing factor could explain the immediate negative feedback of Ca^{2+} on $Ins P_3$-induced Ca^{2+} release in vascular smooth muscle reported by Iino and Endo [8], and thereby regulate the rise in cytosolic Ca^{2+} during agonist-induced production of $Ins P_3$. Another consequence of such a negative-feedback mechanism would be the generation of Ca^{2+} oscillations, as suggested previously [19,24]. It remains to be determined, however, if the Ca^{2+}-sensitizing factor is 'calmedin'. Different isoforms of the $Ins P_3$ receptor have been identified in neuronal and peripheral tissues [27,28], raising the possibility that isoforms of Ca^{2+}-sensitizing factor(s) may also exist. Alternatively, the Ca^{2+}-sensitizing factor may be a novel protein.

It was obvious from the reconstitution experiments shown in Figure 4 that the flow-through from the heparin–Sepharose column caused a slight (~ 25%) decrease in $Ins P_3$ binding to the eluate at a low (0.6 nM) Ca^{2+} concentration. The mechanism for this Ca^{2+}-independent inhibition is not clear, but efforts are underway to assess if this 'inhibitor' can be separated from the Ca^{2+}-sensitizing factor. Like the Ca^{2+}-sensitizing factor, preliminary data indicate that this 'inhibitor' is trichloroacetic acid-labile and heat-stable.

In summary, $Ins P_3$ binding to sarcoplasmic reticulum isolated from dog aortic smooth muscle is consistent with the presence of both high-affinity (K_d = 2.5 nM) and low-affinity (K_d = 49 nM) binding sites. The calculated density of the high-affinity sites is small (representing only 5–7% of the total maximal $Ins P_3$ binding). Moreover, the calculated K_d of these high-affinity sites did not change in the presence of Ca^{2+}. This contrasts with the low-affinity $Ins P_3$-binding sites, which were prevalent and Ca^{2+}-

sensitive. Ca^{2+} (2 μM) increased the calculated K_d of the low-affinity $Ins P_3$-binding site 3-fold. This Ca^{2+}-dependent inhibition of $Ins P_3$ binding requires the presence of a Ca^{2+}-sensitizing factor that can be separated from the $Ins P_3$ receptor, analogous to cerebellum [23] and myeloid cells [20]. The inhibitory mechanism provided by this Ca^{2+}-sensitizing factor could participate in the immediate negative feedback of Ca^{2+} on the $Ins P_3$-gated Ca^{2+} channel in vascular smooth-muscle sarcoplasmic reticulum, and thereby contribute to the regulation of vascular tone following hormonal activation of the phosphoinositide cascade.

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