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Decavanadate displaces inositol 1,4,5-trisphosphate (IP_3) from its receptor and inhibits IP_3 induced Ca^{2+} release in permeabilized pancreatic acinar cells

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Abstract — Inositol 1,4,5-trisphosphate (IP_3) induced Ca^{2+} release in digitonin permeabilized rat pancreatic acinar cells is specifically inhibited by decavanadate. The Ca^{2+} release induced with 0.18 μM IP_3 is half maximally inhibited with approximately 5 μM decavanadate. Complete inhibition is achieved with around 20 μM decavanadate. Removal of decavanadate from the permeabilized cells fully restores sensitivity towards IP_3, indicating the reversibility of the inhibition. Oligovanadate, which inhibits ATP dependent Ca^{2+} uptake into intracellular stores, does not influence IP_3 induced Ca^{2+} release. In order to reveal the mechanism underlying the effects of the different vanadate species, binding of IP_3 to the same cellular preparations was investigated. We found that binding of IP_3 to a high affinity receptor site (K_d approx. 1.2 nM) could be abolished by decavanadate but not by oligovanadate. With 0.5 μM decavanadate, IP_3 binding was half maximally inhibited. A similar potency of decavanadate was also found with adrenal cortex microsomes which bind IP_3 with the same affinity (K_d approx. 1.4 nM) as permeabilized pancreatic acinar cells. Labelled IP_3 was displaced from these subcellular membranes with similar kinetics by unlabelled IP_3 and decavanadate. The data suggest that the inhibitory action of decavanadate on IP_3 induced Ca^{2+} release is a consequence of its effect on binding of IP_3 to its receptor.

Intracellular inositol 1,4,5-trisphosphate (IP_3), formed at the plasma membrane by the hydrolysis of phosphatidylinositol-4,5-bisphosphate, causes the release of Ca^{2+} from intracellular stores [1]. The latter was shown for the first time with permeabilized rat pancreatic acinar cells [2] and was subsequently confirmed by innumerable investigations in a variety of cells. Our knowledge concerning the mechanism of the IP_3 induced Ca^{2+} release is still limited although the primary structure of the IP_3 receptor has been determined [3, 4]. A useful tool to characterize the IP_3 receptor is heparin. It inhibits binding of IP_3 to its receptor [5–7] and IP_3 induced Ca^{2+} release [8–10]. The isolation of the IP_3 receptor on heparin agarose and

Abbreviations used : EGTA, ethylene-glycol-bis (2-aminoethylether)-N,N,N′,N′-tetraacetic acid; EDTA, ethylenediaminetetraacetic acid; PEG, polyethylene glycol; IP_3, inositol 1,4,5-trisphosphate; MOPS, morpholinopropane sulfonic acid; HEPES, N-(2-hydroxyethyl)-piperazine-N′-2-ethanesulfonic acid; Tris, tris(hydroxymethyl)-aminomethane

its incorporation into lipid vesicles led to the conclusion that the IP_3 receptor itself forms the Ca^{2+} channel [11].

In a previous report we introduced decavanadate as a specific inhibitor of the IP_3 induced Ca^{2+} release of rat insulinoma and rat pheochromocytoma cells [12]. In the present study we focused our interest on rat pancreatic acinar cells, which are similar to permeabilized chromaffin cells [13], but in contrast to the above mentioned tumor cells require GTP during the repetitive IP_3 induced Ca^{2+} release [14]. The investigation of decavanadate effects on Ca^{2+} release in these different cell types is of interest, since heparin reportedly only inhibits the IP_3 induced Ca^{2+} release, whereas the GTP-activated Ca^{2+} translocation is unaffected [9]. Here we show that decavanadate completely inhibits IP_3 induced and GTP modulated Ca^{2+} release from permeabilized pancreatic acinar cells, indicating that decavanadate influences a step in the Ca^{2+} release mechanism common to several different cell types. Furthermore, we found that decavanadate inhibits binding of IP_3 to its receptor in permeabilized pancreatic acinar cells as well as in isolated adrenal cortex microsomes. By contrast, the related compound oligovanadate, which inhibits ATP dependent Ca^{2+} uptake into intracellular stores, has no effect on IP_3 induced Ca^{2+} release or binding of IP_3 to its receptor protein. Thus, oligovanadate and decavanadate are highly selective substances which specifically inhibit either Ca^{2+} uptake or Ca^{2+} release by the IP_3 sensitive intracellular Ca^{2+} store.

Materials and Methods

Chemicals

Collagenase was purchased from Serva (Heidelberg, Germany). Soybean trypsin inhibitor and IP_3 were from Boehringer (Mannheim, Germany). [^{3}H]-IP_3 (specific activity 17 Ci/mmol) was from New England Nuclear (UK). Ca^{2+} selective membranes containing the neutral carrier ETH 129 were prepared as described [15]. KCl suprapur was from Merck (Darmstadt, Germany), and Azur A was from Fluka (Neu-Ulm, Germany). All other chemicals were of analytical grade.

Isolation of exocrine pancreatic acinar cells

Pancreatic acinar cells were prepared as described previously [16] with modifications according to [17]. Briefly, pancreatic tissue from four male Wistar rats (200–250 g) fasted overnight was chopped with scissors and incubated in a standard medium containing (in mM): 145 NaCl, 4.7 KCl, 1.2 KH_2PO_4, 1.2 $MgCl_2$, 2 $CaCl_2$, 10 HEPES (pH 7.4), 15 glucose, 0.2 % BSA, 0.01 % soybean trypsin inhibitor plus collagenase (150 U/ml) for 15 min at 37°C. In order to get single cells a washing step in the above Ca^{2+} free medium ($CaCl_2$ replaced by 1 mM EDTA, without collagenase) was interposed, followed by a further digestion by collagenase (210 U/ml) for 50–60 min at 37°C. During cell isolation the medium was continuously gassed with oxygen in a water bath at 37°C. Acinar cells were then mechanically dissociated by sequential passages through polypropylene pipettes of decreasing tip diameter (about 5, 3 and 2 mm) after 40 min of the second collagenase digestion step. The cell suspension was diluted with standard medium, and centrifuged for 5 min at 85 *g*. The pellet was resuspended in standard buffer and filtered through a double layer of medical gauze, placed over 30 ml of standard medium containing 8% albumin. The suspension was centrifuged again. Finally, the pellet was washed three times in the standard medium as described above.

Permeabilization of pancreatic acinar cells with digitonin

The resultant cell suspension was washed three times by centrifugation (80 g_{av}, 3 min) in medium A containing in mM: 150 KCl, 5 NaN_3, 1 EGTA and 20 MOPS (pH 7.2). Permeabilization was carried out on ice with 10 μM digitonin which was added to medium A from a stock where it was dissolved in dimethyl sulfoxide (DMSO). The final concentration of DMSO was less than 0.1 %. Permeabilization was controlled by counting the Azur A (0.25 % in medium A) stained cells. Following permeabilization, the cells were washed three times in medium B (medium A without EGTA) by centrifugation (400 g_{av}, 3 min).

Measurement of free Ca^{2+} concentration

Ca^{2+} specific mini-electrodes were prepared as described [15]. Ca^{2+} specific electrodes were calibrated using the same ionic background for calibration as used for Ca^{2+} measurements. The poly (vinyl chloride) membrane of the electrode contained the recently developed neutral carrier ETH 129, which allows reliable measurements of Ca^{2+} down to the nanomolar range [18, 19]. Experiments were carried out at room temperature and pH 7.2 in 400 µl of medium B containing 3–6 x 10^6 cells. 10^7 cells correspond to 1.5 mg of cell protein. During Ca^{2+} uptake experiments, medium B contained 1 mM Mg^{2+} and 20 µM ATP, and during Ca^{2+} release experiments, medium B contained 2 mM Mg^{2+} and 2 mM ATP [14]. The absolute amount of Ca^{2+} fluxes was calibrated in each experiment by the addition of suitable dilutions of a neutral Ca^{2+} standard (Orion, Lorch, Germany). Guanine nucleotides were present during Ca^{2+} measurements as indicated in the figure captions.

Preparation of bovine adrenal cortex microsomes

Bovine adrenal glands were obtained from the local slaughter house. Cortex microsomes were essentially prepared as previously described [20]. Briefly, the isolated cortex of adrenal glands was homogenized in 20 mM $NaHCO_3$, 1 mM DTT, pH 8.0 in a Teflon-to-glass homogenizer with 5 strokes at 800 revs/min. The homogenate was centrifuged at 5000 *g*, 4°C for 15 min and rehomogenized. From the pooled supernatants, the microsomes were spun down at 100 000 *g*, 4°C for 30 min and the pellet was washed again. The final pellet was resuspended in the homogenisation buffer described above at a protein concentration of 5 mg/ml and stored at –20°C.

Determination of IP_3 binding

IP_3 binding assays were carried out according to the method of Challis [20]. Briefly, digitonin permeabilized cells were washed three times by centrifugation (450 g_{av}, 3 min) in a medium containing in mM: 100 Tris, 20 MOPS, 240 sucrose, 2 EDTA, 2 EGTA, 2% PEG and a final pH of 8.0. Each assay tube contained 0.9 nM [^{3}H]-IP_3 (6000 cpm/assay) and unlabelled IP_3 as indicated in the figure captions. The assays were carried out at 4°C with 0.5 mg protein of permeabilized cells or 0.4 mg protein of adrenal cortex microsomes in a final volume of 400 µl. During the incubation (15 min), samples were vortex-mixed every 3 min. Thereafter, bound and free IP_3 were separated by centrifugation (10 000 *g*, 30 s) in an Ole Dich microcentrifuge 154. The supernatant was aspirated and the pellet dissolved in 5 ml scintillant. Nonspecific binding (NSB) was defined as binding of [^{3}H]-IP_3 in the presence of 2 µM IP_3 (final).

Preparation of defined vanadate species

Oligovanadate and decavanadate were prepared as described [12] with the following modifications. In order to avoid high salt concentrations (due to dissolving of vanadate in 1 M KOH), crystals of orthovanadate were dissolved in medium B as a stock of 50 mM. For the preparation of decavanadate, the pH was adjusted to 3.75 and for the preparation of oligovanadate the pH was adjusted to 7.2 with HCl. Finally the preparations were boiled for 5 min. Oligovanadate concentrations refer to the monomeric form.

Results and Discussion

Decavanadate inhibits IP_3 induced Ca^{2+} release

In the presence of 5 mM sodium azide (NaN_3) and 2 mM Mg^{2+}/ATP, permeabilized exocrine pancreatic acinar cells sequester Ca^{2+} mainly within non-mitochondrial intracellular Ca^{2+} stores [14]. The application of submicromolar concentrations of inositol 1,4,5-trisphosphate results in a transient Ca^{2+} release which is followed by the immediate reuptake of released Ca^{2+}. In the presence of 20 µM GTP, a constant Ca^{2+} release occurs during the repeated application of the same amount of IP_3 from permeabilized pancreatic acinar cells (Fig. 1; *see* also [14]). Streptolysin O permeabilized chromaffin cells exhibit the same behaviour [13]. We found that increasing concentrations of decavanadate dose dependently inhibited the Ca^{2+} transients induced by

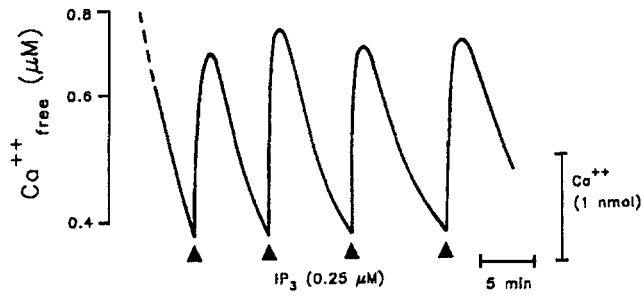

Fig. 1 Ca^{2+} release by IP_3 pulses in the presence of GTP from permeabilized pancreatic acinar cells.
Ca^{2+} fluxes of digitonin (10 μM) permeabilized pancreatic acinar cells (5.9 x 10^6 cells) were recorded with a Ca^{2+} selective electrode. The presence of 20 μM GTP in medium B (containing in mM: 2 Mg^{2+}/ATP, 5 NaN_3, 150 KCl, 20 MOPS pH 7.2) assured constant Ca^{2+} release during the repeated application of 0.25 μM IP_3

IP_3 (Fig. 2a). With 0.18 μM IP_3, half maximal inhibition occurred with around 5 μM decavanadate which was complete with 20 μM decavanadate (Fig. 2b). A similar half maximal effective decavanadate concentration was reported for rat insulinoma and rat pheochromocytoma cells [12].

The inhibition of the IP_3 induced Ca^{2+} release by decavanadate can not be explained by a decreased Ca^{2+} uptake, since decavanadate, unlike oligovanadate, did not influence the ATP driven Ca^{2+} uptake (Fig. 3). On the other hand, oligovanadate did not affect the IP_3 induced Ca^{2+} release (not shown). Thus decavanadate specifically affects the IP_3 induced Ca^{2+} release, while the action of oligovanadate is restricted to the Ca^{2+} uptake system. Furthermore, removing decavanadate by centrifugation and repeating the same experiment after resuspending the cells resulted in a full response to IP_3 (not shown). This indicates that the inhibition of the IP_3 induced Ca^{2+} release by decavanadate is reversible.

Decavanadate displaces IP_3 from its receptor

The structural similarity of decavanadate ($V_{10}O_{28}^{6-}$) [21, 22] with IP_3 suggested that they might interact with the same sites. Therefore, we investigated the effects of different vanadate species on binding of IP_3 to its receptor. These studies were also performed with permeabilized cells, i.e. with cellular preparations identical to that used for the investigation of Ca^{2+} fluxes. A 50% displacement of the labelled ligand (0.9 nM [^{3}H]-IP_3) was attained with 1.2 nM of unlabelled ligand (Fig. 4a). Scatchard-analysis of these data revealed a binding capacity of 140 fmol IP_3/mg cell protein (Fig. 4b). The binding of [^{3}H]-IP_3 by permeabilized pancreatic acinar cells was potently inhibited by decavanadate

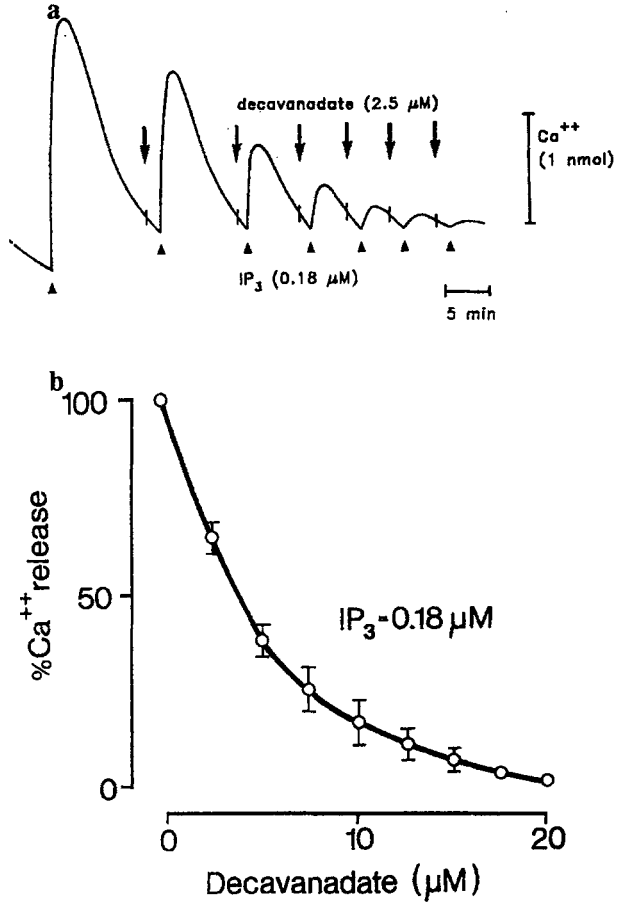

Fig. 2 Decavanadate inhibits IP_3 induced Ca^{2+} release.
(a) Ca^{2+} transients were recorded under identical conditions as described in Figure 1. Increasing amounts of decavanadate were added stepwise to the permeabilized cells immediately before stimulation with 0.18 μM IP_3. The addition of decavanadate did not change medium free Ca^{2+} concentration by itself, but it inhibited the subsequent Ca^{2+} release induced by IP_3
(b) Summary of 5 independent experiments as shown in Figure 2a (means ± SD). 100% corresponds to the Ca^{2+} release in the absence of decavanadate

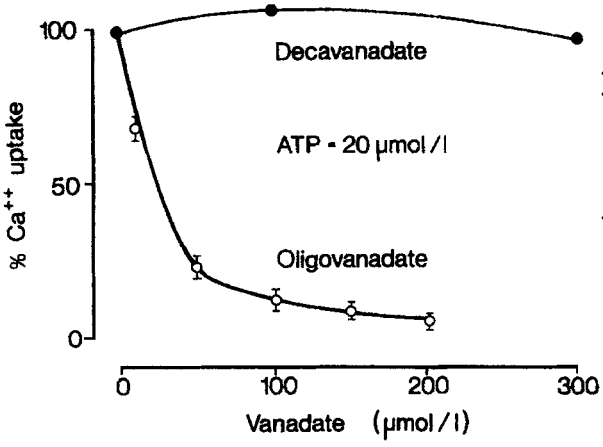

Fig. 3 Effect of oligo- and decavanadate on ATP driven Ca^{2+} uptake.

Effects on Ca^{2+} uptake were estimated by inducing transient Ca^{2+} uptake with low concentrations of ATP (20 µM; *see* also [12, 13]). 100% corresponds to a Ca^{2+} uptake of 2.5 to 3.8 nmol $Ca^{2+}/10^7$ cells (n = 5) which was carried out in the absence of vanadate. Oligo- or decavanadate were added in increasing amounts immediately prior to the additions of ATP. Vanadate concentrations refer to the monomeric form

(half maximal effective concentration of 0.5 µM), whereas oligovanadate was completely ineffective, even when tested at concentrations of 500 µM (Fig. 5). Thus, it can be concluded that the inhibition of the IP_3 induced Ca^{2+} release by decavanadate in pancreatic acinar cells is a consequence of the displacement of IP_3 from its binding site.

In order to establish the effects of decavanadate on IP_3 binding, we also performed IP_3 binding studies with isolated adrenal cortex microsomes, which are rich in IP_3 receptors and bind IP_3 with the same affinity as pancreatic acinar cells. We found a K_d of 1.4 nM and 2.3 pmol binding sites per mg protein. For adrenal cortical microsomes, half maximal displacement occurred around 0.5 µM decavanadate, whereas 500 µM oligovanadate was completely ineffective in displacing IP_3 (Fig. 6). Both 2 µM IP_3 and 25 µM decavanadate resulted in a very similar and rapid dissociation of labelled IP_3 from the receptor (Fig. 7).

The dissociation constant (1.2 nM) for the binding of IP_3 to its receptor in rat pancreatic acinar cells reported here is in the same order of magnitude as reported for permeabilized rat liver cells [23],

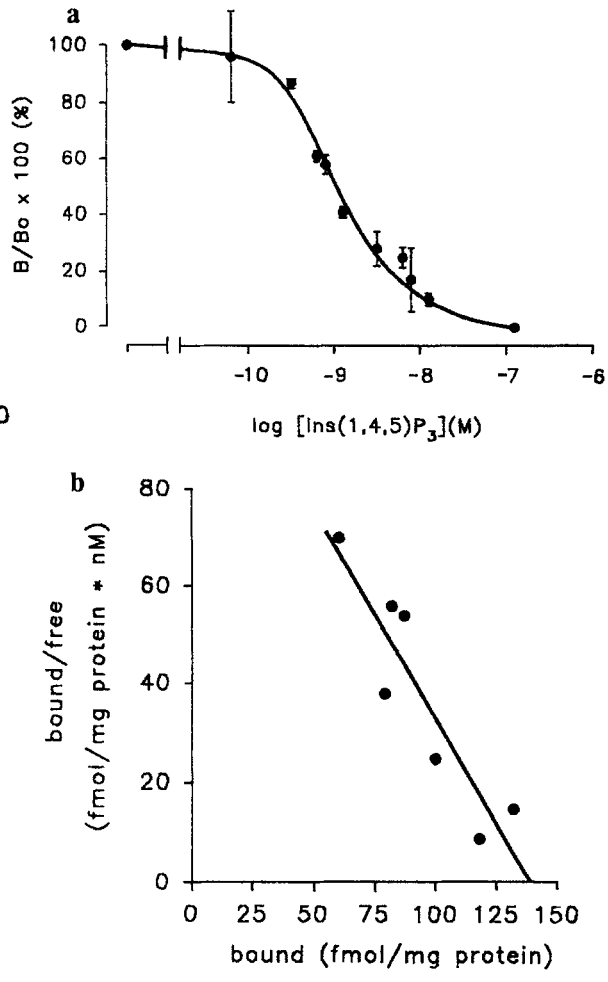

Fig. 4 Binding of IP_3 to permeabilized pancreatic acinar cells.

(a) Digitonin permeabilized cells (500 µg cell protein) were incubated with 0.9 nM [^{3}H]-IP_3 and increasing concentrations of unlabelled IP_3. Specific binding is expressed as the percentage of [^{3}H]-IP_3 bound in the presence of various concentrations of unlabelled IP_3 (B) and in the absence of unlabelled IP_3 (Bo), with each value corrected for nonspecific binding (NSB). Nonspecific binding was defined in the presence of 2 µM unlabelled IP_3 and was typically 30% of total binding. The graph shows one out of three similar experiments carried out in triplicate

(b) Scatchard plot of a single representative experiment with triplicate determinations of each point. The data were fitted by linear regression. In three experiments a K_d = 1.2 nM and B_{max} = 140 fmol/mg of protein were estimated

whereas submicromolar dissociation constants have been reported for permeabilized guinea pig hepatocytes and rabbit neutrophils [24]. Similarly the dissociation constant (1.4 nM) for the binding of

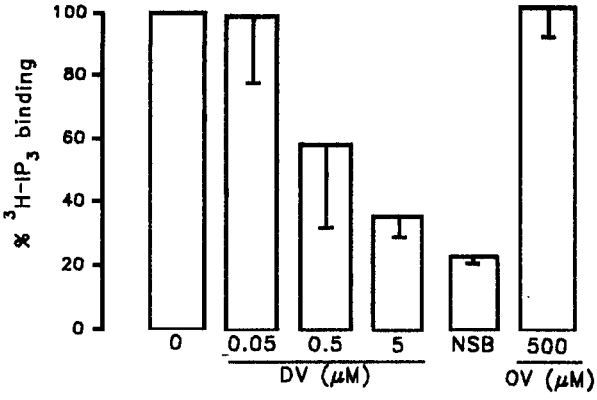

Fig. 5 Decavanadate inhibits binding of IP_3 to its receptor in permeabilized pancreatic acinar cells.
Permeabilized cells were incubated with 0.9 nM [^{3}H]-IP_3 and different concentrations of decavanadate (DV) and oligovanadate (OV). Nonspecific binding (NSB) was defined as binding in the presence of 2 μM unlabelled IP_3. 100% corresponds to the binding of [^{3}H]-IP_3 in the absence of vanadate species and in the absence of unlabelled IP_3. The figure shows the mean (± SD) of 3 independent experiments carried out in triplicate. Oligovanadate refers to the monomeric form

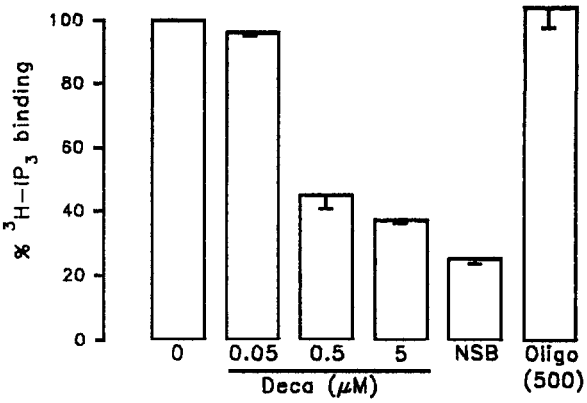

Fig. 6 Decavanadate inhibits binding of IP_3 to its receptor from adrenal cortex microsomes.
Isolated adrenal cortex microsomes (0.4 mg protein) were incubated in a final volume of 400 μl with 0.9 nM [^{3}H]-IP_3 and different concentrations of decavanadate (Deca) and oligovanadate (Oligo). For experimental details, see Materials and Methods. The figure shows the mean (± SD) of 3 independent experiments carried out in triplicate. Oligovanadate refers to the monomeric form

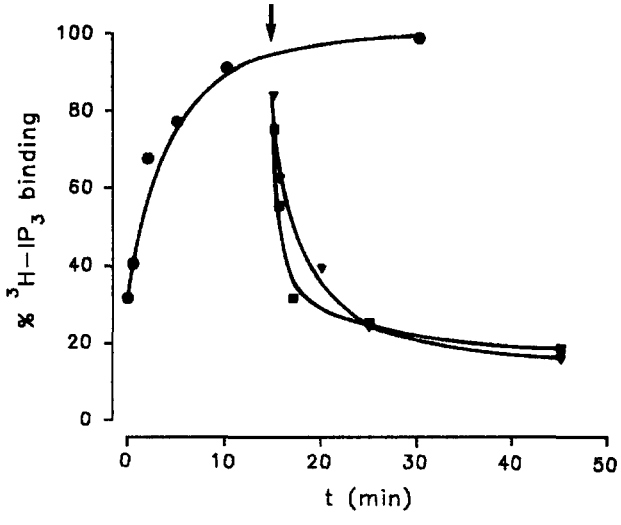

Fig. 7 Displacement of [^{3}H]-IP_3 from adrenal cortex microsomes by unlabelled IP_3 and decavanadate.
The time dependent association of [^{3}H]-IP_3 (0.9 nM) with the receptor of adrenal cortex microsomes (circles) was determined. After 15 min, either 2 μM unlabelled IP_3 (squares) or 25 μM decavanadate (triangles) were added as indicated by the arrow. 100% corresponds to the binding of [^{3}H]-IP_3 as determined after 30 min. The data in this figure are representative of 1 out of 3 independent experiments

IP_3 to isolated adrenal cortex microsomes is in accordance with the K_d value reported for isolated microsomes from different cell types [20, 25, 26].

Although the estimated IP_3 concentration which causes half maximal Ca^{2+} release (approx. 0.2 μM at 0.2 μM free Ca^{2+}; *see* also [13]) is in accordance with the observation of other authors (*see* [1, 27, 13]) it contrasts with the dissociation constant for IP_3 (1.2 nM) found within the same cellular preparation. A similar discrepancy, when comparing displacement of IP_3 from its receptor with its potency in Ca^{2+} mobilization, was also observed with microsomes from the adrenal cortex [26, 28]. In this context, the higher amounts of decavanadate (5 μM) required for a half maximal inhibition of the IP_3 induced Ca^{2+} release compared to the inhibition of IP_3 binding (0.5 μM decavanadate) are not unexpected. A similar behaviour was also reported for heparin which inhibits binding of IP_3 to its receptor protein and IP_3 induced Ca^{2+} release, as does decavanadate [10]. Certainly some of these differences may be explained by the different experimental conditions used for the different assays. Besides the difference in ionic strength and pH, it should be noted that the Ca^{2+} release experiments (to keep Ca^{2+} in the store) must be

carried out in the presence of millimolar concentrations of Mg^{2+} and ATP, while displacement assays are carried out in their absence. Both Mg^{2+} and ATP affect IP_3 metabolism [29, 30] and binding of IP_3 [7, 20, 23]. Under our experimental conditions, 1 mM ATP reduced IP_3 binding to about 10% of that measured in the absence of ATP. Identical values for IP_3 binding and Ca^{2+} releasing potency have recently been reported using identical conditions for both assays [23].

Conclusions

In conclusion, decavanadate appears to be the most useful inhibitor of IP_3 induced Ca^{2+} release and inhibitor of IP_3 binding to its receptor protein. Compared to decavanadate, rather high concentrations of other polyanions (patent blue, cibacron blue) have been reported to displace IP_3 from its receptor [31], but the effects of these substances on subsequent steps leading to IP_3 induced Ca^{2+} release have not been elucidated. Additionally, decavanadate-mediated inhibition provides a sensitive marker to assess IP_3 binding sites in permeabilized cells, microsome fractions and IP_3 receptor preparations. Finally, the small molecular size and well defined chemical structure [21, 22] of decavanadate furnish important advantages over heparin for further characterization of the IP_3 binding site on its receptor.

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