Influence of Mg^{2+} and pH on n.m.r. spectra and radioligand binding of inositol 1,4,5-trisphosphate

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We and others have shown that the binding of $Ins(1,4,5)P_3$ to its receptor is pH-sensitive and can be inhibited by Mg^{2+} . In the present study we have used 1H - and ^{31}P -n.m.r. spectroscopy to study whether these effects result from increased ionization of $Ins(1,4,5)P_3$ and a direct interaction with Mg^{2+} respectively. Under near-physiological conditions of ionic strength (100 mM-KCl), three ionizable groups were observed. The pH titration curve of the 1-phosphate was monophasic, with a pK_a of 6.3. The titration curves of the 4- and 5-phosphates were biphasic, suggesting that these groups interact; the pK_a values for the 4-phosphate determined by ^{31}P -n.m.r. were 5.7 and 7.8, and for the 5-phosphate they were 5.3 and 7.9. 1H - and ^{31}P -n.m.r. measurements suggest that Mg^{2+} binds weakly to $Ins(1,4,5)P_3$ at physiological pH. Mg^{2+} non-competitively inhibited binding of $Ins(1,4,5)P_3$ to its receptor in rat cerebellum and bovine adrenal cortex. Inhibition curves for rat cerebellum at pH 7.1 and 8.5, and also for bovine adrenal cortex at pH 8.5, appeared to be monophasic, with IC_{50} values (concn. of displacer giving 50% inhibition of specific binding) of 214 μM , 572 μM and 9.1 mM respectively. Scatchard analysis revealed that Mg^{2+} inhibited binding of $Ins(1,4,5)P_3$ to bovine adrenal cortex at pH 8.5 in a non-competitive manner. Our results suggest that the previously reported pH-sensitivity of the binding of $Ins(1,4,5)P_3$ may be caused by ionization of the phosphate groups in positions 4 and 5, and that the ability of Mg^{2+} to inhibit the binding of $Ins(1,4,5)P_3$ is not mediated by direct chelation but through a site located on, or close to, the $Ins(1,4,5)P_3$ receptor. Inhibition by Mg^{2+} is pH-sensitive and can vary at least 10-fold between tissues, suggesting possible receptor heterogeneity. Mg^{2+} may exert an important regulatory control on the release of Ca^{2+} by $Ins(1,4,5)P_3$.

INTRODUCTION

$Ins(1,4,5)P_3$ satisfies nearly all of the criteria necessary for it to be recognized as a second messenger regulating release of Ca^{2+} from non-mitochondrial stores (Berridge & Irvine, 1984). Activation of a wide variety of cell surface receptors leads to hydrolysis of phosphatidylinositol 4,5-bisphosphate by phospholipase C and rapid formation of $Ins(1,4,5)P_3$ (Berridge & Irvine, 1984). Binding sites for radiolabelled $Ins(1,4,5)P_3$ were first described by Baukal *et al.* (1985) and Spät *et al.* (1986), and were subsequently confirmed to be the $Ins(1,4,5)P_3$ receptor by purification to homogeneity from rat cerebellum (Supattapone *et al.*, 1988). The receptor has been cloned from mouse cerebellum (Furuichi *et al.*, 1989; Mignery *et al.*, 1989), and has a molecular mass of 250 kDa, is structurally similar to the ryanodine receptor and is thought to be active as a tetramer. Reconstitution of the $Ins(1,4,5)P_3$ receptor into phospholipid vesicles has revealed it to be a Ca^{2+} channel (Ferris *et al.*, 1989).

Paradoxically, however, basal levels of $Ins(1,4,5)P_3$ in many cells appear to be sufficient to stimulate maximal Ca^{2+} release. For example, the basal level of $Ins(1,4,5)P_3$ in rat cerebral cortex, estimated using an $Ins(1,4,5)P_3$ receptor binding assay, is approx. 2 μM (Challiss *et al.*, 1988), whereas significant release of intracellular Ca^{2+} by $Ins(1,4,5)P_3$ occurs at sub-micromolar concentrations in most tissues (Berridge & Irvine, 1984). Similar basal levels of $Ins(1,4,5)P_3$ have been measured in cells labelled to isotopic equilibrium with $[^3H]$ inositol (Nogimori *et al.*, 1990). These results suggest that the free cytosolic $Ins(1,4,5)P_3$ concentration is likely to be much lower than predicted from its intracellular mass.

It is known that $PtdIns(4,5)P_2$ binds Mg^{2+} when the cation is present at concentrations between 10^{-4} and 10^{-5} M (Veloso *et al.*,

1973; Gupta & Gupta, 1987; Toner *et al.*, 1988). Toner *et al.* (1988) have calculated that $PtdIns(4,5)P_2$ binds to Mg^{2+} at a ratio of 1:1.3 when the Mg^{2+} concentration is 10^{-3} M. It is also possible that $Ins(1,4,5)P_3$ exists in a Mg^{2+} -bound form in the cytosol and is therefore unable to bind to its receptor. In support of this, we have recently reported that Mg^{2+} inhibits the binding of $Ins(1,4,5)P_3$ to its receptor in human uterine membranes with an IC_{50} (concn. of displacer giving 50% inhibition of specific binding) of approx. 100 μM (Rivera *et al.*, 1990). A similar result has also been reported by Volpe *et al.* (1990) in dog cerebellum, although the inhibitory effect of Mg^{2+} was non-competitive, suggesting that it is mediated through an allosteric site, possibly located on the $Ins(1,4,5)P_3$ receptor itself.

In the present study, we have investigated directly the interaction between $Ins(1,4,5)P_3$ and Mg^{2+} using 1H - and ^{31}P -n.m.r., and have carried out detailed studies on the effect of Mg^{2+} on the binding of $Ins(1,4,5)P_3$ to its receptor in rat cerebellum and bovine adrenal cortex. In addition, n.m.r. spectroscopy was used to determine pH titration curves for $Ins(1,4,5)P_3$ over the pH range 4–11, as we and others have shown that the binding of $Ins(1,4,5)P_3$ to its receptor is pH-sensitive (Varney *et al.*, 1990; Rivero *et al.*, 1990).

MATERIALS AND METHODS

D- $[^3H]$ $Ins(1,4,5)P_3$ was obtained from Amersham International and Du Pont (U.K.) Ltd. (specific radioactivity 17–54 Ci/mmol). $Ins(1,4,5)P_3$, purified by a modified Folch extraction (Folch, 1949; Brown & Stewart, 1966; Irvine *et al.*, 1986), was kindly donated by Dr. Robin Irvine (Babraham, Cambridge, U.K.) or synthesized as described by Reese & Ward (1987). Dowex AG 1-X8 formate resin was from Bio-Rad. Soluene was obtained

from Packard Instrument Co. Other standard laboratory reagents were obtained from Sigma Chemical Co.

Tissue preparation

Bovine adrenal glands were obtained fresh from the abattoir and adrenal cortices were dissected at 4 °C. Rat cerebella were dissected from freshly killed male Sprague-Dawley rats (150–300 g). Membranes were prepared as described by Varney *et al.* (1990) and finally resuspended at 2–3 mg/ml in buffer containing 20 mM-NaCl, 100 mM-KCl, 20 mM-Tris and 1 mM-EDTA, at pH 8.5 or 7.1.

[³H]Ins(1,4,5)P₃ binding

These assays were performed in quintuplicate, essentially as described by Varney *et al.* (1990). Experiments were performed in a final volume of 250 µl containing 20 mM-NaCl, 100 mM-KCl, 100 mM-Tris/Hepes, 0.1 mM-EDTA, approx. 1–3 nM-[³H]Ins(1,4,5)P₃ and various concentrations of Ins(1,4,5)P₃ (0–5 µM) and Mg²⁺ (0–40 mM) at pH 7.1 or 8.5; non-specific binding was determined in the presence of 10 µM-Ins(1,4,5)P₃. Experiments were started by addition of membrane suspension [0.2 mg of protein (rat cerebellum) or 0.5 mg of protein (bovine adrenal cortex)] and left for 5 min at 4 °C. The reaction was stopped by centrifugation at 13000 g for 5 min. Supernatants were discarded and pellets were washed with distilled water. Tissue was solubilized using 100 µl of Soluene-100 and radioactivity was determined by liquid-scintillation counting. Free Mg²⁺ concentrations in the EDTA-containing buffers were calculated using the computer program Freecalc version 2.1, kindly donated by Dr. L. Brass (University of Philadelphia). This program is based on affinity constants derived by Fabiato & Fabiato (1979).

Metabolism of [³H]Ins(1,4,5)P₃

Metabolism of [³H]Ins(1,4,5)P₃ at pH 7.1 and at pH 8.5 was measured as described by Varney *et al.* (1990). Membranes were incubated for 5 min with [³H]Ins(1,4,5)P₃ in the presence of Mg²⁺ (0–40 mM) under the same conditions as those used for the binding experiments. Incubations were terminated on ice by addition of an equal volume of 10% HClO₄. After 15 min, the samples were centrifuged at 1000 g for 5 min. Freon/octylamine (1:1, v/v; 0.5 ml) and 125 µl of EDTA (10 mM) were added to 400 µl of the supernatant. After vortex mixing for 30 s, samples were centrifuged at 13000 g for 5 min. Inositol phosphates were separated on Dowex AG 1-X8 anion-exchange resin. Inositol and glycerophosphoinositol were eluted with 16 ml of 60 mM-ammonium formate/5 mM-sodium tetraborate, and 16 ml of 400 mM ammonium formate/0.1 M-formic acid eluted inositol mono- and bis-phosphates. Ins(1,4,5)P₃ was eluted with 16 ml of 800 mM-ammonium formate/0.1 M-formic acid. Fractions were collected for liquid-scintillation counting.

¹H-n.m.r.

¹H-n.m.r. spectra were recorded on a Bruker AM500 spectrometer. A pulse angle of 60° (approx. 6 µs) was used, with a relaxation delay of 2.0 s between pulses. A pre-saturation pulse for suppression of the residual H²O resonance was applied during the delay. Prior to Fourier transformation, the free induction decay signals were zero-filled to 16384 points and resolution was enhanced by Gaussian multiplication (Ernst, 1966), with the maximum of the Gaussian function set to 0.10 and a line broadening factor of –10 Hz. All spectra were recorded at a temperature of 27 °C. The chemical shifts were determined using acetone as an internal reference at 2.214 p.p.m. The Ins(1,4,5)P₃ concentration was 4–6 mM in ²H₂O. pH adjustments of the Ins(1,4,5)P₃ samples were made with either

NaO²H or ²HCl (0.4%, w/w). The pH values quoted are uncorrected meter readings on a radiometer PHM 84 Research pH meter, using an Ingold combination electrode inserted directly into a 5 mm n.m.r. tube. No adjustment was made for isotope effects between H⁺ and ²H⁺, as it has been shown that, in solution, ionization effects are compensated for by isotope effects at the glass electrode (Glasoe & Long, 1960).

¹H-n.m.r. spin decoupling

Spin-decoupling experiments were performed on a sample of Ins(1,4,5)P₃ (2 mM, in ²H₂O, pH 7.12). The spin-decoupling experiments involved the acquisition of two sets of spectra. For the first set, there was an initial 0.02 s irradiation of the selected resonance, which was followed by a normal acquisition sequence. The second set of spectra was collected by applying the 0.02 pulse 'off-resonance', i.e. at a frequency which did not correspond to any proton resonance in the spectrum. The program was designed to perform this cycle of irradiations for a selected number of scans. In order to process the spectra, the 'off-resonance' free induction decay signal was subtracted from the 'on-resonance' free induction decay signal, and a Fourier transform was applied to the resulting decay signal. This produced a difference spectrum which only contained the resonances from the proton involved in a particular spin system.

³¹P-n.m.r.

³¹P-n.m.r. spectra were recorded at 145.77 MHz using a GE digital data acquisition system interfaced with a spectrometer constructed at the University of Oxford. Sweep widths of 3 kHz were collected using 4 k data points. Spectra were processed using pre-Fourier-transform exponential multiplication equivalent to 2–6 Hz line broadening. All measurements were performed on 2.5 ml samples in 10 mm outer diam. n.m.r. tubes. The Ins(1,4,5)P₃ concentration was 3–5 mM with 100 mM-KCl in ²H₂O. The pH is quoted uncorrected for the small deuterium isotope effect (Kalinichenko & Lobyshev, 1976). pH titrations were effected by the addition of microlitre aliquots of 50 mM aqueous solutions of NaOH or HCl. MgCl₂ was added in microlitre quantities as an aqueous solution diluted from a 1 M stock. Chemical shifts are quoted relative to external 85% orthophosphoric acid (0.0 p.p.m.). The probe temperature was 25.0 ± 0.1 °C.

Data analysis

Curves were analysed by non-linear regression using an iterative curve-fitting program 'GraphPad' (ISI Software). Binding experiments were performed in quintuplicate and results are shown as means ± s.e.m. from 3–5 experiments.

RESULTS

¹H- and ³¹P-n.m.r. of Ins(1,4,5)P₃

Initial ¹H-n.m.r. spectra of Ins(1,4,5)P₃ at pH 7.1 in ²H₂O were examined in order to verify its conformation (results not shown). Resonance peaks could be assigned to the six –CH groups on the basis of chemical shifts and coupling constants (Table 1); values were similar to those determined by Lindon *et al.* (1986). Assignments were confirmed by spin decoupling (results not shown). The data are consistent with Ins(1,4,5)P₃ having five equatorial substitutions and one axial substitution in position 2 (Grado & Ballou, 1961; Tomlinson & Ballou, 1961).

At pH > 2, each phosphate will have lost one proton. A further increase in pH causes the loss of a second proton from each phosphate group and has been investigated by monitoring chemical shifts of –CH protons (Fig. 1). No detectable changes

Table 1. N.m.r. parameters and assignments for $\text{Ins}(1,4,5)\text{P}_3$

Resonance (p.p.m.)	Multiplicity	Assignments	Coupling constants (Hz)
4.22	Triplet	H-2	$J_{\text{H-2-H-3}}$ 2.6
4.19	Quartet	H-4	$J_{\text{H-4-H-3}}$ 8.8 $J_{\text{H-4-H-5}} \sim 9.2$ $J_{\text{H-4-P}} \sim 9.2$
3.93	Quartet	H-5	$J_{\text{H-5-H-4}}$ 8.5 $J_{\text{H-5-H-6}} \sim 9.2$ $J_{\text{H-5-P}} \sim 8.0$
3.91	Multiplet	H-1	$J_{\text{H-1-H-2}}$ 2.6 $J_{\text{H-1-H-6}} \sim 8.5$ $J_{\text{H-1-P}} \sim 8.6$
3.86	Triplet	H-6	$J_{\text{H-6-H-1}} \sim 8.5$ $J_{\text{H-6-H-5}} \sim 8.5$
3.65	Doublet of doublets	H-3	$J_{\text{H-3-H-4}}$ 8.8 $J_{\text{H-3-H-2}}$ 2.6

Fig. 1. Change in chemical shift as a function of pH for $\text{Ins}(1,4,5)\text{P}_3$

(a) Structure of $\text{Ins}(1,4,5)\text{P}_3$. (b) The curves represent the H-1 (Δ), H-2 ($\blacktriangle$), H-3 ($\blacksquare$), H-4 ($\square$), H-5 ($\blacklozenge$) and H-6 ($\bullet$) proton resonances respectively. ^1H -n.m.r. spectra were recorded in $^2\text{H}_2\text{O}$ at 27 °C.

in coupling constants were observed over this pH range, suggesting no change in ring conformation (results not shown). All six resonances corresponding to the six $-\text{CH}$ groups shifted over the pH range 5–10. The resonance peak corresponding to H-2 moved in the opposite direction to the other resonances, consistent with its axial location. The shifting of the H-4 and H-5 resonances appeared to be biphasic (Fig. 1), possibly reflecting an interaction between the 4- and 5-phosphates (see ^{31}P -n.m.r. spectra below). The change in the position of resonance for the

Fig. 2. ^{31}P -n.m.r. spectra of $\text{Ins}(1,4,5)\text{P}_3$

^{31}P -n.m.r. was performed on approx. 5 mM- $\text{Ins}(1,4,5)\text{P}_3$ in the presence of 0, 3 and 10 mM- Mg^{2+} in 50 mM-Tris/100 mM-KCl, pH 7.1, at 25 °C.

Fig. 3. Chemical shift as a function of pH for $\text{Ins}(1,4,5)\text{P}_3$

The curves represent the 1-phosphate ($\bullet$), 4-phosphate ($\square$) and 5-phosphate ($\circ$) groups. ^{31}P -n.m.r. spectra (also see Fig. 2) were recorded in 100 mM-KCl (in $^2\text{H}_2\text{O}$) at 25 °C (145 MHz). The three phosphate peaks were assigned using published n.m.r. data on PtdInsP and PtdInsP_2 (von Paridon *et al.*, 1986) and by comparison with our ^1H -n.m.r. data.

Fig. 4. Inhibition by Mg^{2+} of $\text{Ins}(1,4,5)\text{P}_3$ binding to membranes from (a) bovine adrenal cortex and (b) rat cerebellum

Bovine adrenal cortical and rat cerebellum membranes were incubated with $[\text{H}]\text{Ins}(1,4,5)\text{P}_3$ at pH 7.1 (● and ▲ respectively) and at pH 8.5 (○ and △ respectively) in the presence of various concentrations of Mg^{2+} . Free Mg^{2+} concentrations were calculated using the computer program Freecal (see the Materials and methods section) and are plotted against the specific binding, expressed as a percentage of maximal binding. Points represent means $\pm$ S.E.M. for four experiments performed in quintuplicate.

other -CH protons appeared to be monophasic, although the small changes in chemical shift observed prevented an accurate fitting of these curves. It is particularly noticeable that curves corresponding to the H-3 and H-6 positions are shallow.

These results suggest that H-2, H-3 and H-6 are affected by ionization of phosphate groups at positions 1, 4 and 5. It therefore follows that the spectra of the H-1, H-4 and H-5 protons will be influenced by ionization of distal phosphates in addition to the adjacent phosphate and that the corresponding pH curves may be multicomponent rather than mono- or biphasic. In view of these possible additional influences on the ^1H -n.m.r. spectra, and its ability to monitor directly the individual phosphates, we have used ^{31}P -n.m.r. to investigate further the ionization of $\text{Ins}(1,4,5)\text{P}_3$. These experiments have been carried out in the presence of 100 mM-KCl to mimic intracellular ionic strength.

Three resonance peaks corresponding to the three phosphates were observed (Fig. 2). pH titrations of these peaks over the pH range 4–11 produced changes in chemical shift corresponding to the dissociation of second protons from the 1-, 4- and 5-phosphates (Fig. 3). One titration curve appeared to be monophasic (Hill slope = 1.1) with an estimated pK_a value of

Fig. 5. Effect of Mg^{2+} on $[\text{H}]\text{Ins}(1,4,5)\text{P}_3$ binding in bovine adrenal cortical membranes at pH 8.5

Membranes were incubated with $[\text{H}]\text{Ins}(1,4,5)\text{P}_3$ (approx. 1 nM) and various concentrations of unlabelled $\text{Ins}(1,4,5)\text{P}_3$ in the absence (●) or presence (○) of 5 mM- Mg^{2+} . The displacement curves were transformed into Scatchard plots which are plotted as B/F , i.e. the ratio of specifically bound to free ligand, in fmol of $\text{Ins}(1,4,5)\text{P}_3$ $[\text{fmol} \cdot (\text{mg of protein})^{-1} \cdot \text{nM}^{-1}]$, against bound, i.e. the amount of specifically bound ligand, in fmol of $\text{Ins}(1,4,5)\text{P}_3 \cdot (\text{mg of protein})^{-1}$. In absence of Mg^{2+} , the K_d is 5 nM and the B_{max} is 629 fmol·mg of protein $^{-1}$; in presence of 5 mM- Mg^{2+} , the K_d is 5 nM and the B_{max} is 247 fmol·(mg of protein) $^{-1}$.

6.3. The remaining two curves appeared to be biphasic, with estimated pK_a values of 5.7 (61.7%) and 7.8 (38.3%), and 5.3 (30.5%) and 8.0 (69.5%), respectively (Fig. 3). Their biphasic nature suggests that they reflect an interaction between the two adjacent phosphates in positions 4 and 5. They can be assigned to the phosphates in positions 4 and 5 by comparison with ^{31}P -n.m.r. data for $\text{PtdIns}4\text{P}$ and $\text{PtdIns}(4,5)\text{P}_2$ (von Paridon *et al.*, 1986) and ^1H -n.m.r. data for $\text{Ins}(1,4,5)\text{P}_3$. The monophasic curve represents the phosphate in position 1.

Effect of Mg^{2+} on ^1H - and ^{31}P -n.m.r. spectra of $\text{Ins}(1,4,5)\text{P}_3$

The possible interaction between Mg^{2+} and $\text{Ins}(1,4,5)\text{P}_3$ was investigated initially using ^1H -n.m.r. in $^2\text{H}_2\text{O}$ at pH 6.9. The H-4 and H-5 resonances showed the largest changes on addition of Mg^{2+} , and yielded linear Lineweaver-Burk plots indicative of 1:1 binding of Mg^{2+} to $\text{Ins}(1,4,5)\text{P}_3$, with a K_d of 3.56 ± 0.02 mM. It is probable that Mg^{2+} binds equally between the 4- and 5-phosphates.

Mg^{2+} -phosphate interactions decrease with increasing ionic strength, and therefore the interaction of Mg^{2+} with $\text{Ins}(1,4,5)\text{P}_3$ was investigated further under the more physiological conditions of 100 mM-KCl at pH 7.1 using ^{31}P -n.m.r. In the presence of 3 or 10 mM- Mg^{2+} , $\text{Ins}(1,4,5)\text{P}_3$ spectra exhibited marked broadening. Some shifting of peaks was also observed, with changes being greatest for the 4- and 1-phosphates. These results are consistent with an interaction with Mg^{2+} (Fig. 2). However, subsequent titration with Mg^{2+} up to 200 mM (results not shown) failed to produce an endpoint, possibly because of the ever-increasing ionic strength generating ever-diminishing binding constants.

Effect of Mg^{2+} on the binding of $\text{Ins}(1,4,5)\text{P}_3$ to its receptor

The binding of $\text{Ins}(1,4,5)\text{P}_3$ to its receptor has been shown to be pH-dependent in several tissues, including bovine adrenal cortex (Varney *et al.*, 1990) and rat cerebellum (Worley *et al.*, 1987). Since maximal binding is observed at pH 8.5, the effect of Mg^{2+} on $\text{Ins}(1,4,5)\text{P}_3$ binding was investigated at both pH 8.5

Fig. 6. $[\text{}^3\text{H}]\text{Ins}(1,4,5)\text{P}_3$ metabolism in membranes from (a) bovine adrenal cortex and (b) rat cerebellum

Incubations at pH 7.1 (conditions as for binding experiments) were terminated after 5 min on ice by addition of an equal volume of 10% HClO_4 . Inositol phosphates were extracted as described in the Materials and methods section and radioactivity was measured by liquid-scintillation counting. $\circ$, $[\text{}^3\text{H}]\text{InsP}_1 + [\text{}^3\text{H}]\text{InsP}_2$; $\bullet$, $[\text{}^3\text{H}]\text{InsP}_3$.

and pH 7.1 (physiological pH) in the presence of 100 mM-KCl. Mg^{2+} inhibited $\text{Ins}(1,4,5)\text{P}_3$ binding in both tissues (Figs. 4a and 4b). In rat cerebellum, Mg^{2+} produced a monophasic inhibition with IC_{50} values of 214 μM and 572 μM at pH 7.1 and 8.5 respectively. In bovine adrenal cortex Mg^{2+} inhibited the binding of $\text{Ins}(1,4,5)\text{P}_3$ at pH 8.5 with an IC_{50} of 9.1 mM and with a Hill slope of 1.0. At pH 7.1, however, the inhibition curve for Mg^{2+} was shallow (Hill slope = 0.5), suggesting the presence of two sites. In support of this, a better fitting of the curve was obtained using a two-site model ($r^2 = 0.995$). This yielded IC_{50} values of 257 μM (54.9%) and 10.2 mM (45.2%) (Fig. 4a).

The inhibitory effect of Mg^{2+} on the binding of $\text{Ins}(1,4,5)\text{P}_3$ to its receptor in bovine adrenal cortical membranes was investigated further by carrying out competition studies with $\text{Ins}(1,4,5)\text{P}_3$ in the presence of 5 mM- Mg^{2+} at pH 8.5. Scatchard analysis showed that the data could be fitted to a single site with a K_d of 6.2 ± 0.5 nM in the absence and 6.2 ± 0.8 nM in the presence of 5 mM- Mg^{2+} ($n = 4$; e.g. see Fig. 5). The density of $\text{Ins}(1,4,5)\text{P}_3$ binding sites was decreased in the presence of 5 mM- Mg^{2+} from 774 ± 85 fmol/mg of protein to 323 ± 41 fmol/mg of protein.

Metabolism of $[\text{}^3\text{H}]\text{Ins}(1,4,5)\text{P}_3$

Since $\text{Ins}(1,4,5)\text{P}_3$ phosphatase is both pH- and Mg^{2+} -dependent (Joseph & Williamson, 1985; Sasaguri *et al.*, 1985), with

optimum activity at approx. pH 7.0 and 1 mM- Mg^{2+} , we investigated $[\text{}^3\text{H}]\text{Ins}(1,4,5)\text{P}_3$ breakdown in the presence of various Mg^{2+} concentrations at pH 7.1 and pH 8.5. Under the same conditions as those used for the binding assays, metabolism of $[\text{}^3\text{H}]\text{Ins}(1,4,5)\text{P}_3$ was found to be no greater than 10% in both the adrenal cortex and the rat cerebellum, and was not affected by Mg^{2+} (0–40 mM) (e.g. Figs. 6a and 6b).

DISCUSSION

In agreement with the conformational structure of $\text{Ins}(1,4,5)\text{P}_3$ determined by Grado & Ballou (1961) and Tomlinson & Ballou (1961), later confirmed by Lindon *et al.* (1986), ^1H -n.m.r. demonstrated that $\text{Ins}(1,4,5)\text{P}_3$ has five equatorial substituents and one axial substituent. No detectable change in conformation was observed over the pH range 4–11.

$\text{Ins}(1,4,5)\text{P}_3$ undergoes further ionization over the pH range 5–9, and this has been investigated by ^1H - and ^{31}P -n.m.r. Slight differences in the pK_a values of the individual phosphates were observed using ^1H -n.m.r. and ^{31}P -n.m.r. spectroscopy. The latter technique monitors directly the loss of protons from the phosphate groups, whereas ^1H -n.m.r. is an indirect monitor and is influenced by ionization of distal phosphates in addition to adjacent phosphates. In addition, different conditions of ionic strength were used in the two sets of experiments, and this is also likely to affect the pK_a values obtained. The increase in affinity of $\text{Ins}(1,4,5)\text{P}_3$ for its receptor with increasing pH (Varney *et al.*, 1990) may therefore be explained by ionization of the 4- and 5-phosphate groups. However, these data do not rule out an additional effect of pH on the conformation of the active site of the $\text{Ins}(1,4,5)\text{P}_3$ receptor.

^1H -n.m.r. studies performed in $^2\text{H}_2\text{O}$ indicated that Mg^{2+} interacts predominantly with the 4- and 5-phosphates, with a stoichiometry of 1:1 for Mg^{2+} binding to $\text{Ins}(1,4,5)\text{P}_3$. Mg^{2+} has a K_d of 3.56 ± 0.02 mM for $\text{Ins}(1,4,5)\text{P}_3$ at pH 6.9 under these conditions of low ionic strength. ^{31}P -n.m.r. of $\text{Ins}(1,4,5)\text{P}_3$ indicated that under more physiological conditions of ionic strength (100 mM-KCl) $\text{Ins}(1,4,5)\text{P}_3$ has a lower affinity for Mg^{2+} . The high K_d argues against $\text{Ins}(1,4,5)\text{P}_3$ chelation with Mg^{2+} being an important factor in the decrease of free cytosolic $\text{Ins}(1,4,5)\text{P}_3$ in the cell. These data are consistent with the non-competitive nature of inhibition of $\text{Ins}(1,4,5)\text{P}_3$ binding to Mg^{2+} (see below).

We have shown that Mg^{2+} strongly inhibits $\text{Ins}(1,4,5)\text{P}_3$ binding to its receptor in bovine adrenal cortex in a non-competitive manner, suggesting that inhibition is mediated by an effect on the $\text{Ins}(1,4,5)\text{P}_3$ receptor rather than by chelation of $\text{Ins}(1,4,5)\text{P}_3$. Non-competitive inhibition by Mg^{2+} has also been observed in dog cerebellum (Volpe *et al.*, 1990) and is also likely in rat cerebellum, especially in view of the low IC_{50} value of Mg^{2+} . These results suggest that Mg^{2+} inhibits the binding of $\text{Ins}(1,4,5)\text{P}_3$ through a site located next to [i.e. on a separate protein which interacts with the $\text{Ins}(1,4,5)\text{P}_3$ receptor], or on, the $\text{Ins}(1,4,5)\text{P}_3$ receptor and, that inhibition is unrelated to the weak interaction between $\text{Ins}(1,4,5)\text{P}_3$ and Mg^{2+} observed with ^1H - and ^{31}P -n.m.r.

Mg^{2+} has an approximately 10-fold greater affinity for the $\text{Ins}(1,4,5)\text{P}_3$ receptor in rat cerebellum than bovine adrenal cortex and this may represent further evidence that these tissues express different subtypes of this receptor. The possibility of $\text{Ins}(1,4,5)\text{P}_3$ receptor subtypes has already been alluded to by a number of groups, on account of the variation in dissociation constants and affinity of heparin and $\text{Ins}(1,4,5)\text{P}_3$ for $\text{Ins}(1,4,5)\text{P}_3$ receptors from different tissues (Willcocks & Nahorski, 1990; Varney *et al.*, 1990). Alternatively, however, this difference may be due to the Ca^{2+} -binding protein, calmodin, which is present in cerebellum (Danoff *et al.*, 1988) but absent from bovine adrenal cortex (Varney *et al.*, 1990). Ca^{2+} -bound calmodin non-com-

petitively inhibits $\text{Ins}(1,4,5)\text{P}_3$ binding (IC_{50} 300 nM) and it is possible that Mg^{2+} inhibition could also be mediated by this protein. However, the strong inhibition in bovine adrenal cortex, where there is no evidence that calmodin is present, suggests a binding site for Mg^{2+} on the receptor itself.

We have also shown that inhibition of $\text{Ins}(1,4,5)\text{P}_3$ binding by Mg^{2+} is strongly pH-dependent. The IC_{50} for Mg^{2+} increased 5-fold and 2.5-fold for bovine adrenal cortex and the rat cerebellum respectively on raising the pH from 7.1 to 8.5. For bovine adrenal cortex at pH 8.5 and rat cerebellum at pH 7.1 and pH 8.5, the inhibition curves for Mg^{2+} were monophasic, suggesting a single binding site in each case. However, in bovine adrenal cortex at pH 7.1, inhibition was biphasic (IC_{50} 257 μM and 10.2 mM respectively), and this may be related to the presence of two binding sites for $\text{Ins}(1,4,5)\text{P}_3$ in this tissue under these conditions. Scatchard plots of $\text{Ins}(1,4,5)\text{P}_3$ binding to membranes from bovine adrenal cortex at pH 7.1, in contrast with those at pH 8.5, are biphasic (results not shown).

Physiological significance

Our results suggest that Mg^{2+} may be an important factor in the regulation of the cytosolic Ca^{2+} concentration through its influence on the binding of $\text{Ins}(1,4,5)\text{P}_3$ to its receptor. Only limited information exists concerning the free Mg^{2+} concentration in the cell; fluorimetric and n.m.r. measurements suggest values of 0.3–1 mM (Rink *et al.*, 1982; Murphy *et al.*, 1989; Brookes & Bachelard, 1989). If the value were as high as 1 mM, the IC_{50} for Mg^{2+} inhibition of $\text{Ins}(1,4,5)\text{P}_3$ binding in rat cerebellum suggests that most of the receptor is unavailable for $\text{Ins}(1,4,5)\text{P}_3$ binding, whereas in bovine adrenal cortex, where the IC_{50} is 10-fold lower, the majority of the receptors would be available.

The concentration of Mg^{2+} is not thought to fluctuate in the cell (Alvarez-Leefmans *et al.*, 1987); however, pH is known to change upon cell activation and this is thought to be an important regulator of cellular function. For example, it has been found that intracellular pH is increased by growth factors and hormones that stimulate the phosphoinositide cycle, and by phorbol esters that stimulate protein kinase C (Berridge & Irvine, 1984; Berridge, 1984; Macara, 1985). Physiological pH changes, however, are usually no greater than 0.1–0.2 pH units, although local pH changes may be greater. An increase in intracellular pH is known to increase the ability of $\text{Ins}(1,4,5)\text{P}_3$ to release Ca^{2+} (Brass & Joseph, 1985) and this may be explained, at least in part, by an increase in the affinity of $\text{Ins}(1,4,5)\text{P}_3$ for its receptor, together with a decrease of the inhibitory effect of cellular levels (0.3–1 mM) of Mg^{2+} with modest increases in alkalinity. This influence of pH on $\text{Ins}(1,4,5)\text{P}_3$ binding could result in a sensitive regulation of $\text{Ins}(1,4,5)\text{P}_3$ actions in response to small alterations in pH whose sensitivity is augmented by the alleviation of the Mg^{2+} -mediated inhibition at slightly alkaline pH.

In conclusion, it is unlikely that the apparently high concentration of $\text{Ins}(1,4,5)\text{P}_3$ under basal conditions can be explained by chelation to Mg^{2+} . However, Mg^{2+} may play an important role in the regulation of $\text{Ins}(1,4,5)\text{P}_3$ -stimulated Ca^{2+} release through a site located on or close to the receptor.

We are very grateful to Robin Irvine for supplying $\text{Ins}(1,4,5)\text{P}_3$ and to Dr. Ian Campbell for help with the planning and interpretation of the ^{31}P -n.m.r. studies. A. M. W. and M. A. V. are M.R.C. Scholars. S. P. W. is

a Royal Society University Research Fellow. R. J. P. W. is a member of the Oxford Centre for Molecular Sciences. H. C. G. thanks the Royal Commission for the Exhibition of 1851 for financial support. C. B. R. thanks the M.R.C. for the award of a Project Grant.

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