Regulation of the cerebellar inositol 1,4,5-trisphosphate receptor by univalent cations

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In the present study we investigated the effects of K^+ and other univalent cations on $[^3H]InsP_3$ $\{[^3H]Ins(1,4,5)P_3\}$ binding to sheep cerebellar microsomes. In equilibrium binding experiments performed over 4 s at pH 7.1 and 20 °C, the addition of K^+ to the binding medium decreased the affinity and increased the total number of binding sites for $InsP_3$ in a dose-dependent manner. At low $InsP_3$ concentration (0.5 nM) these effects resulted in a biphasic dose–response curve, with maximal binding at about 75 mM K^+ . In contrast, the dose–response curve calculated for $InsP_3$ at the physiological concentration of 5 μ M, was linear up to 200 mM K^+ . Univalent inorganic cations stimulated $[^3H]InsP_3$ binding to various extents, with the following descending order of efficiency at 75 mM: $Cs^+ \approx Rb^+ \approx K^+ > Na^+ > Li^+$. The effect of K^+ on $InsP_3R$ affinity was rapidly reversed upon cation removal. We

were therefore also able to demonstrate that K^+ increased B_{max} (maximal specific binding) by pre-treating microsomes with K^+ before measuring $[^3H]InsP_3$ binding in the absence of that cation. The increase in B_{max} was reversible, but this reversal occurred less rapidly than the change in affinity. These results are consistent with a process by which K^+ reversibly converted very low-affinity sites into sites with higher affinity, making them detectable in competitive binding experiments. They suggest that inter-conversion between these two affinity states constitutes the basis of a K^+ -controlled regulatory mechanism for cerebellar $InsP_3R$.

Key words: calcium channel, calcium signalling, cerebellum, inositol 1,4,5-trisphosphate [$Ins(1,4,5)P_3$], microsomes, potassium.

INTRODUCTION

$Ins(1,4,5)P_3$ receptors ($InsP_3Rs$) are intracellular ionic channels responsible for the mobilization of Ca^{2+} following the activation of the cell by various biological stimuli [1,2]. Functional $InsP_3Rs$ are tetrameric and are mostly located in the endoplasmic reticulum, from which Ca^{2+} is released after binding to $InsP_3$. $InsP_3Rs$ exist as three structurally and functionally similar isoforms (types 1–3) in mammalian cells. $InsP_3R$ isoforms can associate in homo- or hetero-meric structures. $InsP_3$ and Ca^{2+} are the major determinants of the channel activity of the three $InsP_3R$ isoforms. These two regulators may stimulate or inhibit $InsP_3R$ activity [2–7]. Control by these agents is thought to be essential for the creation of complex Ca^{2+} signals with various patterns [1].

The type 1 isoform of $InsP_3R$ is expressed at a very high level in the Purkinje cells of cerebellum, and type 1 homotetramers predominate in this tissue. Thus the relationship between structure and function has been most thoroughly characterized for cerebellar $InsP_3R$ 1. In mouse, this isoform consists of 2749 amino acids, organized into a large N-terminal cytoplasmic portion (positions 1–2275), a group of six-transmembrane regions near the C-terminal end (positions 2276–2589) that form an ion channel, and a short C-terminal domain (positions 2590–2749) [2,8]. The $InsP_3$ binding core domain lies between residues 226 and 578 and is highly conserved. It is thought to consist of two structurally different parts linked by a loop, forming a pocket with positive charges recognizing negatively charged $InsP_3$ [9,10]. Other regions of $InsP_3R$ 1 that do not bind $InsP_3$ (the first 225 N-terminal amino acids and C-terminal residues) seem to disrupt this minimal binding site, as indicated by the increase in affinity for $InsP_3$ resulting from their deletion [9–11]. Biochemical and electron-microscopy

studies have shown that $InsP_3R$ 1 exists as at least two different structures: a compact square-shaped structure [12–16] and a pin-wheel structure with four protruding arms radiating from a central hub [17,18]. Evidence for a reversible Ca^{2+} -dependent transition from the compact conformation to the more extended conformation has been obtained [19]. The compact structure is thought to be maintained by non-covalent assembly of the various domains [15] and to involve direct interaction between the N- and C-terminal regions [15,16]. A recent study showed that, in the functional $InsP_3R$, individual binding sites may be located very close together, within 2 nm of each other [20]. Cytosolic Ca^{2+} controls channel activity in a biphasic manner [3,21] and inhibits $InsP_3$ binding to $InsP_3R$ 1 [3,7,22,23].

Although inorganic univalent cations such as K^+ have received relatively little attention, they are believed to play a crucial role in the mechanism of IICR [$Ins(1,4,5)P_3$ -induced Ca^{2+} release] from intracellular stores. This release has repeatedly been shown to require cytosolic K^+ or another univalent cation. Early studies on liver [24,25] and brain [26,27] microsomes and subsequent studies on blood platelets [28] have suggested that $InsP_3R$ activation induces a counterflow of K^+ ions into the endoplasmic reticulum membranes, preventing the build-up of a potential that would oppose Ca^{2+} release. It has also been suggested that K^+ causes desorption of Ca^{2+} from the matrix of the intracellular sequestering compartments [29]. However, studies on cerebellar microsomes have called into question the indirect role of K^+ in IICR and have instead suggested that this cation acts as a direct co-factor in the opening of the $InsP_3R$ channel [30]. Consistent with this suggestion, IICR has been observed in the presence of K^+ , with purified $InsP_3R$ reinserted into lipid vesicles [31]. Inorganic univalent cations are also known to affect $InsP_3R$ by inhibiting

Abbreviations used: $[Ca^{2+}]_i$, cytosolic Ca^{2+} ; IICR, $Ins(1,4,5)P_3$ -induced Ca^{2+} release; $InsP_3$, $Ins(1,4,5)P_3$; $InsP_3R$, $Ins(1,4,5)P_3$ receptor; $NMDG^+$, N-methyl-D-glucamine⁺.

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InsP₃ binding, by reducing the affinity of the receptor for InsP₃ [17,32,33]. Most studies of this inhibitory effect have been conducted under alkaline conditions. We recently showed that the dose-dependent effects of these cations on InsP₃ binding to cerebellar microsomes are different if experiments are performed at neutral pH [34]. Whereas K⁺ essentially inhibited InsP₃ binding under alkaline conditions, it had a biphasic effect at neutral pH, InsP₃ binding increasing and then decreasing with increasing K⁺ concentration. In the present study we sought to increase our understanding of the control exerted on InsP₃R by K⁺ and other inorganic univalent cations by studying in detail the effects of these cations on the binding of InsP₃ to sheep cerebellar microsomes.

EXPERIMENTAL

Preparation of cerebellar microsomes

Sheep cerebellum was homogenized and a microsomal fraction obtained by differential centrifugation, as described previously [35]. The tissue was homogenized in 4.5 vol. of an ice-cold medium composed of 5 mM Hepes, 250 mM sucrose, 10 mM KCl, 1 mM 2-mercaptoethanol, 10 µg/ml leupeptin and 0.2 mM PMSF, pH 7.4. The homogenate was diluted in 4.5 vol. of the same buffer and centrifuged successively at 1000 *g* for 5 min and 9000 *g* for 10 min to sediment the nuclear and mitochondrial fractions respectively. The resulting supernatant was centrifuged at 100 000 *g* for 75 min. The pellet was dispersed in a small volume of the initial medium to a protein concentration of 20–30 mg/ml. The suspension was homogenized, frozen in liquid nitrogen and stored at –80 °C.

InsP₃-binding studies

InsP₃ binding to cerebellar microsomes was measured at 20 °C by means of a perfusion protocol similar to that described elsewhere [7,33–35]. Microsomal membranes were thawed and diluted in an ice-cold medium (buffer A: 25 mM Hepes/Tris, pH 7.2, 1 mM H₃PO₄, 0.3 mM EDTA, 0.3 mM EGTA, 2 µg/ml leupeptin and 0.2 mM PMSF). A 120 µl aliquot of membrane preparation was diluted 1:10 in buffer A equilibrated at 20 °C. In some experiments, membranes were pre-treated with K⁺ by adding the chloride salt of this cation to this mixture. A 1 ml portion of the resulting suspension, containing 400–500 µg of protein, was layered on to a glass-fibre filter (GF/C). The adsorbed membranes were then manually perfused for 4 s at 20 °C with 1.2 ml of binding medium, composed of buffer A supplemented with 0.5 nM [³H]InsP₃, unlabelled InsP₃ and cations as indicated. To prevent the dissociation of [³H]InsP₃ from membranes, the radioactivity on the filter was counted without rinsing. Taking into account the rate of perfusion (0.3 ml/s) and the wet volume of the filter (40 µl), the medium surrounding adsorbed membranes should have been completely replaced by binding mixture within 150 ms. We estimated the *K*_d and *B*_{max} for InsP₃ binding in [³H]InsP₃ displacement experiments in which the concentration of unlabelled InsP₃ varied from 1 nM to 5 µM. Inplot software was used to fit the curves to the data points in these experiments. The following equation, expressing specific binding to the receptor plus non-specific binding, and corrected for the variable specific activity of [³H]InsP₃, was used [33]:

$$Y = \{B_{\max}/[1 + (K_d/C)^h]\} + \alpha C \cdot (C_0/C)$$

where *B*_{max} is the maximal specific binding, *K*_d the dissociation constant, *C* the free concentration of InsP₃, including the [³H]InsP₃ tracer, *h* the Hill coefficient, *α* the factor describing non-specific binding and *C*₀ the free concentration of the [³H]InsP₃

tracer. Non-specific binding was determined from the curve fitted to the data, and *B*_{max} was calculated as suggested by Swillens [36]. In other experiments, specific [³H]InsP₃ binding was estimated by subtracting radioactivity bound in the presence of 5 µM unlabelled InsP₃ from total bound radioactivity. The radioactivity corresponding to non-specific binding was mainly due to free [³H]InsP₃.

Materials

[³H]InsP₃ (21 Ci/mmol) was obtained from Du Pont–New England Nuclear, InsP₃ from Alexis Corporation (Cogen, France) and other reagents from Sigma or Alexis.

RESULTS

Dependence of InsP₃ binding on univalent cations

In previous studies, the effects on InsP₃ binding of univalent inorganic cations such as K⁺ have been studied mostly at basic pH values and at low temperatures [17,33]. In the present study we investigated [³H]InsP₃ binding to cerebellar microsomes under more physiological conditions, as commonly used to measure IICR (pH 7.1, 20 °C). These conditions can be applied to the binding assay by using a perfusion protocol, as previously shown [33,34]. In preliminary experiments with 0.5 nM [³H]InsP₃, binding doubled on addition of 100 mM K⁺ to the binding mixture. This effect could not be accounted for by incomplete equilibration or by a loss of microsomes from the filter in the absence of K⁺ in the binding medium. Binding equilibrium was reached within 4 s of perfusion, regardless of whether K⁺ was present in the binding medium. We therefore determined [³H]InsP₃ binding after 4 s of perfusion in all subsequent experiments.

Increasing K⁺ concentration in the binding mixture from 5 to 300 mM resulted in a large increase in [³H]InsP₃ binding for K⁺ concentration of 50–100 mM (maximal stimulation 3.7-fold), followed by a gradual decrease for higher K⁺ concentrations (Figure 1A). The stimulation factor varied from 2.5 to 4, with a mean value of 2.9 ± 0.1 (*n* = 10). A similar biphasic dose–response curve was obtained with Li⁺, but the maximal stimulation observed (stimulation factor of 2.7; Figure 1A) was weaker than with K⁺. At concentrations exceeding 100 mM, Li⁺, like K⁺, inhibited [³H]InsP₃ binding. For both K⁺ and Li⁺, the optimal concentration for stimulation was 75 mM. Very similar concentration-dependence curves were obtained with potassium gluconate and KCl, suggesting that the anion played no role in this response (results not shown). We also determined the dose-dependent effects on [³H]InsP₃ binding of two organic univalent cations: NMDG⁺ (*N*-methyl-D-glucamine⁺) and Tris⁺ (Figure 1A). NMDG⁺ has often been added to cytosol-like solutions in the place of K⁺ to make it possible to study the dependence of IICR on this cation. Biphasic curves were also obtained with these agents, but their characteristics differed from those of the curves obtained with K⁺ and Li⁺. These agents increased [³H]InsP₃ binding to a lesser extent than K⁺, the maximal stimulation factors being only 1.9 and 1.4 for NMDG⁺ and Tris⁺ respectively. Optimal concentrations for the stimulation of [³H]InsP₃ binding (25 mM for NMDG⁺ and 5–10 mM for Tris⁺) were also smaller than for K⁺. Thus the potent effect of K⁺ on [³H]InsP₃ binding does not result simply from a non-specific effect of ionic strength or osmolarity. Adjustment of the osmolarity of binding mixtures to 270 mOsm with sucrose, had no effect on the dose-dependent stimulation by K⁺. The cation requirement of [³H]InsP₃ binding to cerebellar microsomes was further studied by comparing the effects of the chloride form of five univalent inorganic cations at a concentration of 75 mM.

Figure 1 Effects of univalent cations on [³H]InsP₃ binding to cerebellar microsomes

Cerebellar microsomes adsorbed on a filter were manually perfused for 4 s with binding mixture containing 0.5 nM [³H]InsP₃, unlabelled InsP₃ and cation, as indicated. (A) Dose-dependent effects of K⁺ (●), Li⁺ (▲), NMDG⁺ (■) in the chloride form or Tris gluconate (◆) at concentrations of 10–300 mM on [³H]InsP₃ binding. Results are corrected for non-specific binding and are expressed as a percentage of specific [³H]InsP₃ binding measured without added cation. Means from three experiments are presented. (B) [³H]InsP₃ displacement by increasing concentrations of unlabelled InsP₃ in the presence of K⁺ at 0 (○), 120 (▲) and 250 (■) mM. Data not corrected for non-specific binding are shown. Simulated curves were constructed with a one-site model. These analyses gave K_d values of 17, 55 and 269 nM and B_{max} values of 2.0, 14 and 25 pmol/mg of protein for K⁺ concentrations of 0, 120 and 250 mM respectively. (C) Dose-dependent effects of K⁺ on InsP₃-binding parameters. The InsP₃-binding parameters were determined as in Figure 1(B). Results are means for at least two experiments. Other experimental conditions are given in the Experimental section.

These cations stimulated [³H]InsP₃ binding in the following order of efficiency: Cs⁺ ≈ Rb⁺ ≈ K⁺ > Na⁺ > Li⁺.

Effects of K⁺ on InsP₃ binding parameters

We used various K⁺ concentrations in a series of competitive binding experiments with 0.5 nM [³H]InsP₃ and unlabelled ligand at concentrations ranging from 1 nM to 5 μM. The effects of K⁺ were studied at concentrations of 10–250 mM. Generally, non-linear regression analyses of the data suggested that there was one

type of binding site with a Hill slope of about 1. However, in some cases, especially at low K⁺ concentration or in the absence of added univalent cation, non-linear regression analysis suggested heterogeneity in InsP₃-binding sites. Nevertheless, no second type of binding site was reproducibly and satisfactorily characterized, so we used a one-site model to determine K_d and B_{max}. Figure 1(B) presents the results from three competitive binding experiments performed without or with K⁺ at a concentration of 120 or 250 mM. Assuming *h* = 1, K_d values of 17, 55 and 269 nM were determined for K⁺ concentrations of 0, 120 and 250 mM respectively. The corresponding B_{max} values were 2.0, 14 and 25 pmol/mg of protein respectively. Therefore the short treatment of cerebellar microsomes with K⁺ both decreased the affinity of InsP₃R for InsP₃ and dramatically increased the number of detectable binding sites.

The detailed relationship between K⁺ concentration and InsP₃ binding parameters is illustrated in Figure 1(C). K⁺ increased K_d over the entire range of cation concentrations tested (0–250 mM). The K_d value measured for 250 mM K⁺ (258 nM) was 14 times higher than that measured in the absence of added cation. The increase in K_d was not constant over the entire range of K⁺ concentrations. The slope of the curve was shallow at low K⁺ concentrations, but became much steeper at K⁺ concentrations exceeding 100 mM. Similarly, B_{max} was increased over the whole range of K⁺ concentrations, with a much higher value of B_{max} (9.5 times higher) at 250 mM K⁺ than in the absence of this cation (Figure 1C). However, the dose-response curve for this parameter differed from that for K_d. Unlike the K_d curve, the B_{max} curve had a near-constant slope over most of the range of K⁺ concentrations and reached a maximum at 200–250 mM. These differences in the K⁺-dependence of K_d and B_{max} account for the biphasic nature of the dose-dependence curve of K⁺ effects on [³H]InsP₃ binding at low ligand concentration (Figure 1A).

We also carried out equilibrium binding in the presence of 150 mM Li⁺ or K⁺ to determine which binding parameter caused the difference between these cations at concentrations exceeding 25 mM (Figure 1A). This difference was found to be mainly due to Li⁺ having a lower capacity to increase B_{max}. Comparison of effects of NMDG⁺ and K⁺ at a concentration of 125 mM showed that the B_{max} values obtained were similar for NMDG⁺ and K⁺, but K_d was 220% higher with NMDG⁺.

In many studies, InsP₃R channel activity in several cells and tissues, including the cerebellum, has been determined in a 110 mM Tris/250 mM Hepes buffer devoid of inorganic univalent cation. Using this medium in [³H]InsP₃ binding measurements, Kaftan et al. [37] found the presence of at least two binding sites in canine cerebellar microsomes. The data obtained with this medium in the present study also suggested binding-site heterogeneity, although a model with two binding components did not give a significantly better fit than a one-site one. The InsP₃-binding parameters determined in Tris/Hepes medium were similar to those determined at low K⁺ concentration.

Reversibility of K⁺ effects: increase in B_{max} with no change in affinity

The effects of K⁺ on InsP₃ binding to cerebellar microsomes were further characterized by assessing their reversibility. We treated membranes with K⁺ for about 10 s at 20 °C and transferred them to filters, which were perfused for 4 s with cation-free binding mixture. Results from an experiment in which K⁺ concentration varied from 25 to 300 mM, and binding was measured with 0.5 nM [³H]InsP₃, are presented in Figure 2(A). With membranes pre-treated with K⁺ concentrations of up to about 50 mM, binding was similar to that for control membranes (membranes pre-incubated

Figure 2 K⁺-dependent increase in $B_{\max}$ with no inhibitory effect

(A) Effect of prior incubation of microsomes with various concentrations of K⁺ on subsequent [³H]InsP₃ binding. The membrane suspension was diluted in buffer A supplemented with K⁺ to obtain the indicated final concentration. After 10 s pretreatment, [³H]InsP₃ binding to membranes was measured over 4 s perfusion, with a binding mixture containing 0.5 nM [³H]InsP₃, but no K⁺. Results are corrected for non-specific binding and are expressed as a percentage of specific [³H]InsP₃ binding determined for the sample pretreated without cation. Means from three experiments are presented. Other experimental conditions are given in the Experimental section. (B) Determination of InsP₃-binding parameters of membranes pretreated with K⁺. [³H]InsP₃ displacement by increasing concentrations of unlabelled InsP₃ was performed with membranes pretreated with K⁺ at 0 (○), 125 (▲) or 300 (■) mM. Data not corrected for non-specific binding are presented. Simulated curves were constructed with a one-site model. These analyses gave K_d values of 18, 15 and 12 nM and $B_{\max}$ values of 2.5, 5.3 and 9.7 pmol/mg of protein for K⁺ concentrations of 0, 125 and 300 mM respectively. (C) Simulations of dose-dependent effects of K⁺ on InsP₃ binding obtained for increasing InsP₃ concentrations (●, 10 nM; ▲, 100 nM; ■, 5 μM). InsP₃ binding was calculated for a one-site model, using binding characteristics shown in Figure 1(C). Increasing InsP₃ concentration decreased the inhibitory effect of K⁺ on InsP₃ binding, clearly demonstrating the activating component of the K⁺ effect.

in the absence of the univalent cation). Comparison with the results of experiments in which InsP₃ binding was measured in the presence of K⁺ (Figure 1A) suggested that the effects of K⁺ were completely reversed during the 4 s period of perfusion with binding mixture devoid of K⁺. By contrast, increasing K⁺ beyond 100 mM in the preincubation medium resulted in a very

sharp increase in [³H]InsP₃ binding. Maximal [³H]InsP₃ binding, 4.4 times higher than that for the control, was attained at a concentration of about 200–250 mM K⁺, with about half this increase observed at 150 mM K⁺ (Figure 2A). In additional experiments, increasing K⁺ to 800 mM had no further effect on [³H]InsP₃ binding. Given that high K⁺ concentrations in the binding mixture strongly inhibited [³H]InsP₃ binding (Figure 1A), we concluded that the K⁺-induced decrease in the affinity of InsP₃R for InsP₃ is rapidly reversed when the cation is removed. We therefore hypothesized that the dose-response curve in Figure 2(A) reflects the $B_{\max}$ level at the end of perfusion with K⁺-free binding mixture.

We tested this hypothesis by determining the InsP₃-binding parameters of membranes pretreated as described for the experiment shown in Figure 2(A). Membranes were pretreated with 0, 125 or 300 mM K⁺, and displacement curves were constructed on the basis of a 4 s perfusion (as indicated in Figure 1B, except that K⁺ was absent from the binding mixtures). Results from a typical experiment are illustrated in Figure 2(B). Assuming $h = 1$, non-linear regression analyses provided K_d values of 18, 15 and 12 nM for membranes pre-incubated in the presence of 0, 125 and 300 mM K⁺ respectively. The corresponding $B_{\max}$ values were 2.5, 5.3 and 9.7 pmol/mg of protein respectively. Therefore the increase in [³H]InsP₃ binding to membranes pretreated with high concentrations of K⁺ (Figure 2A) was attributed to an increase in $B_{\max}$. This result, obtained with membranes pre-exposed to K⁺ for short periods of time, confirms the results of equilibrium binding experiments performed in the presence of this cation (Figures 1B and 1C). However, the level of [³H]InsP₃ binding in experiments carried out in the presence of K⁺ was much lower, owing to the large decrease in the affinity of the InsP₃R. Another important result of the pretreatment experiments was the dissociation of the effects of K⁺ on binding parameters, the increase in $B_{\max}$ being observed with no change in affinity.

Pretreatment experiments confirmed that K⁺ affected $B_{\max}$, but the values of this binding parameter in these experiments were only one-half to one-third those obtained in equilibrium binding experiments in the presence of K⁺. This difference may result from a large fraction of the K⁺-dependent increase in $B_{\max}$ being reversed during the 4 s perfusion with K⁺-free binding mixture. Consistent with this, the increase in $B_{\max}$ was completely reversed when microsomes were pretreated with low concentrations of K⁺ (< 100 mM) (Figure 2A). We took advantage of the rapid reversal of the K⁺ effect on the InsP₃R affinity to investigate the kinetics of this reversal in the increase in $B_{\max}$ at high K⁺ concentration. Membranes were pretreated with 300 mM K⁺ and then washed with buffer A for various lengths of time before assessing [³H]InsP₃ binding during 4 s of perfusion. Following the removal of K⁺, a large proportion of InsP₃-binding sites became undetectable within seconds. The half-time for this rapid reversal was estimated at about 1.5 s. However, as binding measurements were made at 4 s, this value should be taken with caution. Nevertheless, these kinetic results are consistent with the reversal of the increase in $B_{\max}$, accounting for the lower $B_{\max}$ values obtained in pretreatment experiments.

DISCUSSION

We show here that K⁺, the predominant inorganic cation in cells, has a strong effect on the binding of InsP₃ to sheep cerebellar microsomes, a subcellular fraction containing mostly type 1 InsP₃R [7,38]. Increasing the concentration of K⁺ or other inorganic cations in the medium surrounding the membranes increased both the K_d and $B_{\max}$ for InsP₃ binding. In contrast with the decrease in affinity [17,33], the increase in the number of binding sites has not been described before, and this increase is indeed puzzling.

Several pieces of evidence indicated that the effects of K⁺ on these two binding parameters were distinct: (i) $B_{\max}$ increased gradually with K⁺ concentration, whereas the increase in K_d was much more pronounced at concentrations above 100 mM K⁺ (Figure 1C); (ii) the effect of K⁺ on $B_{\max}$ was reversed much more slowly than that on K_d ; (iii) similar $B_{\max}$ values were measured in the presence of K⁺ and NMDG⁺ at concentrations of 125 mM, whereas the K_d value with NMDG⁺ was double that with K⁺. The combined effects of K⁺ on InsP₃ binding parameters account for the biphasic dose-response curve at low InsP₃ concentrations (Figure 1A). Figure 2(C) shows simulations of dose-response curves obtained for higher InsP₃ concentrations using binding constants from Figure 1(C). Increasing InsP₃ concentration, which reduces the inhibitory effect of K⁺, increases the stimulation of binding and shifts the binding peak to higher K⁺ concentrations. With 5 μ M InsP₃, a concentration occurring in cells stimulated with hormones, InsP₃ binding increased over a large range of K⁺ concentrations (0–200 mM).

Consistent with almost all previous studies on cerebellar membranes [20,22,33,35,39,40], our competitive binding experiments suggested that [³H]InsP₃ bound to one type of site. However, the potent increase of the total number of binding sites in response to K⁺ suggests that very low-affinity (and therefore undetectable) sites are present in membranes that are converted by this cation to sites with higher affinity, making them detectable. The lack of evidence of binding-site heterogeneity in these competitive experiments is in keeping with the demonstration by Swillens et al. [41] that homologous displacement curves are generally unable to reveal multiple binding sites. Strong evidence in favour of the conversion of undetectable binding sites into higher-affinity detectable sites on the addition of K⁺ was obtained in experiments in which membranes were pretreated with K⁺ before measuring [³H]InsP₃ binding without univalent cation. Conversely, the removal of K⁺ causes a rapid reversal of the increase in $B_{\max}$, suggesting that the high-affinity sites are reconverted into low-affinity undetectable sites. Thus InsP₃R is present in cerebellar membranes in at least two interconvertible states with different affinities for InsP₃ (independently of the affinity regulators, Ca²⁺ [7] and InsP₃ [35]). K⁺ favours a state with nanomolar affinity, whereas, in the absence of this cation, InsP₃R is mainly in a conformation with a lower affinity (with a K_d presumably > 1 μ M). Kaftan et al. [37] assessed [³H]InsP₃ binding at neutral pH, in the absence of alkaline univalent cation (Hepes/Tris buffer) in InsP₃R preparations from canine cerebellum. They observed two types of InsP₃-binding site, with K_d in the nanomolar and micromolar ranges. They proposed that there are two different InsP₃-binding sites on each monomer of the tetrameric InsP₃R. By contrast the present study suggests that the low- and high-affinity sites are interconvertible and therefore not different. It has been reported that the C-terminal domain of the InsP₃-binding site in mouse InsP₃R 1 binds InsP₃ with a very low affinity, which is strongly increased by the N-terminal domain, itself devoid of binding capacity [10]. The conversion of low- to high-affinity sites presently described may be related to this interaction between the two different parts of the InsP₃-binding site. Another isoform of InsP₃R, the type 2 receptor, is well-known to bind InsP₃ in different affinity states [32,42].

The results presented here provide no information about the sites and mechanisms through which K⁺ modifies InsP₃-binding constants in sheep cerebellar microsomes. However, the observation of biphasic Na⁺-dependence of InsP₃ binding to the N-terminal 604 amino acids of mouse InsP₃R 1 [43] strongly suggests that these K⁺ effects result from direct interactions with the InsP₃-binding region of the sheep receptor. This also suggests that these effects are exerted on the InsP₃R, independently of accessory

regulators. The K⁺-dependent conversion of InsP₃R may result from the removal of putative intramolecular inhibition, reducing the affinity of the binding core. The 223 N-terminal amino acids known to inhibit binding are a clear candidate for this inhibitory function [9–11]. A role for the C-terminal region, which has been shown to interact with the N-terminal region [15,16] and to also inhibit binding [11], cannot be excluded.

The dependence of IICR on cytosolic inorganic univalent cations such as K⁺ is well established. The mechanisms proposed to account for this K⁺ effect include an influx of K⁺ in the endoplasmic reticulum [24–29] and a role of K⁺ as a cofactor of InsP₃R, as proposed by Mezna and Michelangeli [30]. As this latter mechanism, our studies are in favour of a direct control of InsP₃R by K⁺. However, it is unclear whether the K⁺-dependent conversion of InsP₃R is involved in the K⁺-dependence of IICR and which of the high- or low-affinity form is the most active. With an InsP₃ concentration similar to those observed under conditions of hormonal stimulation (5 μ M), the proportion of InsP₃-binding sites occupied depended directly on K⁺ concentration (Figure 2C). This correlation is strikingly reminiscent of that reported by Mezna and Michelangeli between IICR and K⁺ concentration under similar conditions of InsP₃ concentration and pH [30]. We therefore propose that the K⁺-promoted conversion of InsP₃R into the higher-affinity state is involved in the dependence of IICR on K⁺.

Our results highlight the importance of the ionic composition of the medium in studies of InsP₃R. Differences in ionic composition, pH and temperature may be largely responsible for the highly divergent values published for the InsP₃ binding parameters of InsP₃R in the cerebellum and other tissues. K⁺ may also affect other properties of InsP₃R. For example, we found that the extent of inhibition of InsP₃ binding by Ca²⁺ [7] was dependent on K⁺ concentration (results not shown).

Our results suggest that physiological or pathophysiological conditions modifying intracellular concentrations of K⁺ near InsP₃R 1 in cells would also affect the mobilization of stored Ca²⁺. A variety of agonists induce membrane depolarization and the activation of potential-dependent K⁺ channels. Stimulation of these conductances inhibits Ca²⁺ influx via voltage-dependent Ca²⁺ or receptor-operated channels, limiting the accumulation of intracellular Ca²⁺. The results presented here suggest that the stimulation of these conductances may also prevent cytosolic Ca²⁺ increases by inhibiting IICR. Very large losses of cellular K⁺ have been observed in shrunken cells undergoing apoptosis [44]. Such decreases could also lead to a decrease in InsP₃R activity. The overexpression of certain K⁺ channels has been shown to be associated with various cancers [45,46]. Changes in InsP₃R activity may therefore occur in these cancers as a result of changes in K⁺ concentration modifying the control mechanisms.

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