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Inositol hexakisphosphate binding sites in rat heart and brain

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1 Inositol 1,4,5-trisphosphate ($Ins(1,4,5)P_3$) and inositol hexakisphosphate ($InsP_6$) are produced in response to stimulation of cardiac α_1-adrenoceptors. While the role of $Ins(1,4,5)P_3$ and $Ins(1,4,5)P_3$ receptors is well-defined in many tissues including brain, the functional role of the putative $InsP_6$-$InsP_6$ receptor system in cardiac function is less clear. Using quantitative autoradiography, this study examined the characteristics and regional localization of [^{3}H]-$InsP_6$ binding sites in rat heart and compared the affinity of a range of inositol polyphosphates for [^{3}H]-$InsP_6$ and [^{3}H]-$Ins(1,4,5)P_3$ binding sites in heart and brain.

2 [^{3}H]-$InsP_6$ bound to a single, high affinity site in sections of rat heart (K_D ranging from 22 ± 1.9 nM in right atria to 35 ± 2.6 nM in the interventricular septum, $n = 7$). The maximal number of binding sites (B_{max}) ranged from 5.1 ± 0.48 to 12 ± 1.8 pmol mg^{-1} protein in left atrium and left ventricle, respectively. Inositol phosphates inhibited binding of [^{3}H]-$InsP_6$ with the order of potency: $InsP_6 > Ins(1,4,5)PS_3 >$ inositol 1,3,4,5-tetrakisphosphate $\geqslant$ inositol pentakisphosphate $> Ins(1,4,5)P_3 >>$ inositol mono- and bisphosphates, consistent with the labelling of an $InsP_6$ binding site.

3 The $Ins(1,4,5)P_3$ analogue, $Ins(1,4,5)PS_3$, originally investigated as a putative selective radioligand for the $Ins(1,4,5)P_3$ receptor, was a potent inhibitor of [^{3}H]-$InsP_6$ binding in all heart regions ($K_I = 170$-260 nM). The K_I of $Ins(1,4,5)PS_3$ for the inhibition of [^{3}H]-$Ins(1,4,5)P_3$ binding in rat brain (60-220 nM) was similar to that observed for the inhibition of [^{3}H]-$InsP_6$ binding in heart, suggesting that $Ins(1,4,5)PS_3$ is not a specific ligand for either $Ins(1,4,5)P_3$ or $InsP_6$ receptor binding sites.

4 Previous studies have detected [^{3}H]-$InsP_6$ binding in mitochondrial and sarcoplasmic reticulum fractions of heart and links between $InsP_6$ and cardiac mitochondrial Ca^{2+} regulation have been proposed, suggesting further studies are warranted to determine the functional role(s) of $InsP_6$ and $InsP_6$ receptor binding sites in cardiac tissue.

Keywords: Inositol hexakisphosphate receptor; inositol 1,4,5-trisphosphate receptor; heart; brain; autoradiography

Introduction

Stimulation of α_1-adrenoceptors in rat heart results in the production of inositol 1,4,5-trisphosphate ($Ins(1,4,5)P_3$), a second messenger which in many cell types releases Ca^{2+} from an intracellular store to the cytosol (see Berridge & Irvine, 1989) and a transient increase in inositol hexakisphosphate ($InsP_6$; Scholz *et al.*, 1992). A high concentration of $InsP_6$ in rat heart (5-15 μM) has been determined by use of nuclear magnetic resonance (Szergold *et al.*, 1987). While the role of $Ins(1,4,5)P_3$ as a second messenger is well-defined in many tissues, if not in cardiac myocytes, the biological function of $InsP_6$ is unclear. Several cellular effects of $InsP_6$ have been described, including stimulation of Ca^{2+} influx into neurones (Nicoletti *et al.*, 1989), adrenal chromaffin cells (Regunathan *et al.*, 1992) and rat liver mitochondria (Copani *et al.*, 1991). Specific, high affinity binding sites for $InsP_6$ were identified in each of these tissues as well as in rat cerebellar homogenates (Hawkins *et al.*, 1990; Poyner *et al.*, 1993). In the only published study of cardiac [^{3}H]-$InsP_6$ binding, a 'receptor' protein with similar properties to that isolated from rat cerebellum (Thiebert *et al.*, 1992) was detected in a microsomal fraction of canine heart homogenates (Kijima & Fleischer, 1992).

It was initially intended to explore the use of *myo*-D-[^{35}S(U)]-inositol 1,4,5-trisphosphorothioate ([^{35}S]-$Ins(1,4,5)PS_3$), an $Ins(1,4,5)P_3$ analogue (Challiss *et al.*, 1991), to study the $Ins(1,4,5)P_3$ receptor in rat heart. Following preliminary binding studies it became apparent that the binding specificity of [^{35}S]-$Ins(1,4,5)PS_3$ in myocardium was different from that described in brain and that in cardiac tissue this ligand predominantly labelled an $InsP_6$-sensitive binding site. We therefore decided to examine further the specificity of $Ins(1,4,5)PS_3$ and the nature of the $InsP_6$-sensitive site in rat heart. The aims of the present study were, by use of quantitative autoradiography, to characterize the cardiac [^{3}H]-$InsP_6$ binding site and to examine the affinity of a range of inositol polyphosphates for [^{3}H]-$Ins(1,4,5)P_3$ and [^{3}H]-$InsP_6$ binding sites in heart and brain.

Methods

Animals and tissue preparation

All animals were obtained from the Biological Research Laboratories, Austin Hospital (Heidelberg, Vic., Australia). Experiments were performed with the approval of the Animal Welfare Committee of the Austin Hospital and according to the ethical guidelines set out by the National Health and Medical Research Council of Australia. Male Wistar rats (200-300 g) were used for binding studies and were killed by stunning followed by cervical dislocation. Hearts and brains for use in binding characterization experiments were removed and frozen over liquid nitrogen. All tissue was stored at -70°C until processing (<2 weeks). Fresh, frozen sections (10 μm) were cut on a cryostat (Reichert-Jung Cryocut 1800; Leica Instruments, Nusslock, Germany), thaw-mounted onto gelatin-coated slides and stored at -20°C until used (~1-2 days).

Inositol polyphosphate binding

Optimal incubation conditions for [^{3}H]-$InsP_6$ binding were established in preliminary experiments by varying the pre-in-

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cubation and incubation time and the time of section washing post-incubation, to maximize specific binding levels and minimize non-specific binding levels. In all subsequent experiments, slide-mounted sections were brought to room temperature and pre-incubated in 25 mM Tris-HCl buffer, pH 7.0, at room temperature for 20 min. Incubation of tissue sections with [^{3}H]-$InsP_6$ was carried out at room temperature for 30 min in 25 mM Tris/1 mM EDTA, pH 7.4, with 3 nM [^{3}H]-$InsP_6$ and various concentrations of $InsP_6$ or other competing ligands. No di- or tri-valent cations were used in the incubation buffer. Non-specific binding was measured in the presence of 50 μM $InsP_6$ (Nicoletti *et al.*, 1990). Sections were washed in ice-cold incubation buffer for 2×30 s and either dried under a stream of cool air for autoradiography or wiped from the slides with Whatman GF/B glass fibre filters for liquid scintillation spectroscopy. Wiped sections were placed in scintillation vials with 3 ml Instagel scintillant (Amersham plc, Amersham, BK., U.K.) and radioactivity counted in a Delta 300 β-counter (Searle Analytical Inc., Des Plaines, IL., U.S.A.) Sections for autoradiography were apposed to Hyperfilm-^{3}H (Amersham) for 10 days, developed for 3 min at 17°C in D-19 developer (Kodak, Melbourne, Vic., Australia) and fixed in Rapid Fixer/Hypam hardener (Ilford-Anitec, Melbourne, Vic., Australia) for 5 min at room temperature. Slide-mounted brain or heart paste [^{3}H]-standards were co-exposed with tissue sections on each film for later quantitative densitometry.

For binding studies with [^{3}H]-Ins(1,4,5)P_3, tissue was incubated at 4°C for 30 min in 25 mM Tris/1 mM EDTA, pH 8.0, in the presence of 3 nM [^{3}H]-Ins(1,4,5)P_3 and various concentrations of unlabelled Ins(1,4,5)P_3 or other competing ligands. Sections were washed for 2×1 min in ice-cold 25 mM Tris/1 mM EDTA, pH 8.0 (see Worley *et al.*, 1987; 1989). Sections for autoradiography were apposed to Hyperfilm-^{3}H for 4 weeks and processed as described above.

Determination of radioligand purity

Purity of [^{3}H]-Ins(1,4,5)P_3 and [^{3}H]-$InsP_6$ before and after incubation with tissue sections was established by ion exchange chromatography. [^{3}H]-Inositol phosphates were sequentially eluted from Dowex AG 1-X8 anion exchange columns (100–200 mesh; BioRad, Richmond, CA., U.S.A.) with increasing concentrations of ammonium formate in 0.1 M formic acid for [^{3}H]-Ins(1,4,5)P_3 (Berridge *et al.*, 1983) or increasing concentrations of HCl for [^{3}H]-$InsP_6$ (Spencer *et al.*, 1991). There was no evidence of breakdown of [^{3}H]-Ins(1,4,5)P_3 to lower [^{3}H]-inositol phosphates or of [^{3}H]-$InsP_6$ to [^{3}H]-inositol tetrakisphosphate or the lower [^{3}H]-inositol phosphates. Thin layer chromatography on polyethyleneimine-cellulose plates (Spencer *et al.*, 1991) showed no evidence of breakdown of [^{3}H]-$InsP_6$ to [^{3}H]-inositol pentakisphosphate.

Protein determination

Protein content of heart sections was measured by the method of Bradford (1976). Protein content of brain regions was determined by densitometry of sections stained for 3 min with 0.1% Coomassie blue G-250 (Sigma Chemical Co., St Louis, MO., U.S.A.) in 40% methanol/10% acetic acid and destained in solvent for 30 s. Protein standards were made by dilution of brain homogenate with Miles OCT embedding medium (Bayer Diagnostics, Melbourne, Vic., Australia).

Data analysis

Autoradiograms were analysed by quantitative densitometry using an MCID image analysis system (Imaging Research Inc., St. Catharines, Ontario, Canada). Binding parameters were determined by the iterative curve-fitting programme LIGAND (Munson & Rodbard, 1980). Binding affinities, densities and inhibition constants, expressed as apparent dissociation constant (K_D), bound ligand mg^{-1} tissue protein (B_{max}) and apparent inhibition constant (K_I) respectively, are shown as mean ± s.e.mean from simultaneous analyses using LIGAND. Hill coefficients (n_H) were obtained from analysis using the programme EBDA (McPherson, 1983).

Brain regions were identified by reference to the atlas of Paxinos & Watson (1986). Tritium activity may be quenched by up to 10–12% in heavily myelinated brain regions (Cincotta, unpublished data). No correction was made for any possible quenching during data analysis, however, as regions with a high percentage of grey matter were assessed and a fully quantitative comparison of binding density between brain regions and between heart and brain was not the intention of this study.

Materials

Ligands and chemicals used and their suppliers are listed below: (Ins(1,4,5)PS_3, [^{3}H]-$InsP_6$(15 Ci mmol^{-1}) and [^{3}H]-Ins(1,4,5)P_3 (21 Ci mmol^{-1}) were from NEN-DuPont, Boston, MA., U.S.A.; D-*myo*-inositol 1,4,5-trisphosphate, potassium salt from Amersham plc; $InsP_6$, barium salt, inositol pentakisphosphate ($InsP_5$), barium salt from Calbiochem, Alexandria, N.S.W., Australia; inositol 1,3,4,5-tetrakisphosphate, octasodium salt (Ins(1,3,4,5)P_4) from Research Biochemicals Inc., Natick, MA., U.S.A.; inositol 1-monophosphate, cyclohexylammonium salt, inositol 1,4-bisphosphate, potassium salt from Sigma; heparin sodium from BDH Merck, Melbourne, Vic., Australia. All other reagents were of analytical grade and were obtained from commercial suppliers.

Results

Validation of [^{3}H]-inositol polyphosphate binding methods in brain

To establish the validity of the radioligand binding methods used, preliminary studies were carried out with rat brain in which inositol polyphosphate binding sites have been previously characterized (Worley, *et al.*, 1987; 1989; Hawkins *et al.*, 1990; Donié & Reiser, 1991; Chadwick *et al.*, 1992). The relative density of [^{3}H]-Ins(1,4,5)P_3 binding was highest in the molecular layer of the cerebellum, cerebral cortex, caudate putamen and non-pyramidal layers of the hippocampus (Figure 1a) and two of these areas were chosen to assess the relative affinity of binding. Saturation experiments revealed that [^{3}H]-Ins(1,4,5)P_3 bound with high affinity and in a region-specific manner to high concentrations of sites in rat brain sections (Table 1). Hill coefficient values close to unity suggested that binding was to a single class of sites. The lower apparent binding affinity of [^{3}H]-Ins(1,4,5)P_3 in cerebellum (Table 1) probably results from the large difference in B_{max} in this region compared to other brain regions assessed (see Bürgisser, 1984). $InsP_6$ inhibited binding of [^{3}H]-Ins(1,4,5)P_3 with an $IC_{50} > 3$ μM. Competition binding studies thus showed the order of potency of inositol polyphosphates in competing for [^{3}H]-Ins(1,4,5)P_3 binding sites in different brain regions to be Ins(1,4,5)P_3 > Ins(1,4,5)PS_3 > $InsP_6$ (Table 1), consistent with binding to the Ins(1,4,5)P_3 receptor, as previously described (Worley *et al.*, 1987; 1989; Challiss *et al.*, 1991).

In contrast, [^{3}H]-$InsP_6$ bound with highest density in the caudate putamen, the granule cell layer of the cerebellum, the granule cell layer of the dentate gyrus and layers of the olfactory bulb (Figure 1b) and the affinity and maximal binding density of [^{3}H]-$InsP_6$ was assessed in the caudate putamen and cerebellum. The affinity of [^{3}H]-$InsP_6$ (80–130 nM) was 3–4 fold lower in brain sections than observed in heart sections (Tables 1 and 2). Unlike [^{3}H]-Ins(1,4,5)P_3 binding in different brain areas, however, this difference is probably not due to differences in B_{max}, as these are similar in the two tissues (*cf.* 14 fold difference in cerebellum and other brain regions). Other possible causes are an effect of tritium quenching in brain *cf.* heart, or the possible presence of tissue-specific co-factors that affect binding.

Table 1 Characteristics of [^{3}H]-$InsP_3$ and [^{3}H]-$InsP_6$ binding in rat brain sections

Brain region	K_D (nM)	B_{max} (pmol mg^{-1} protein)	n_H	K_I (nM) *Ins(1,4,5)PS$_3$*
[^{3}H]-$InsP_3$				
Molecular layer of the cerebellum	25±2.1	14±0.28	0.80±0.08	220±43
Non-pyramidal layers of hippocampus	5.1±1.0	0.85±0.07	1.07±0.04	59±8
Caudate putamen	7.6±1.1	0.93±0.07	1.03±0.02	64±7
[^{3}H]-$InsP_6$				
Granule cell layer of the cerebellum	79±13	4.7±0.6	1.01±0.01	–
Caudate putamen	130±24	8.4±2.2	1.02±0.01	–

Data were obtained by densitometry of autoradiograms. K_D and B_{max} were obtained by LIGAND analysis of 3 independent saturation binding experiments. n_H was obtained by EBDA analysis of saturation binding experiments. K_I values were obtained by analysis of 3 independent competition binding experiments using LIGAND. All values are shown as mean±s.e.mean.

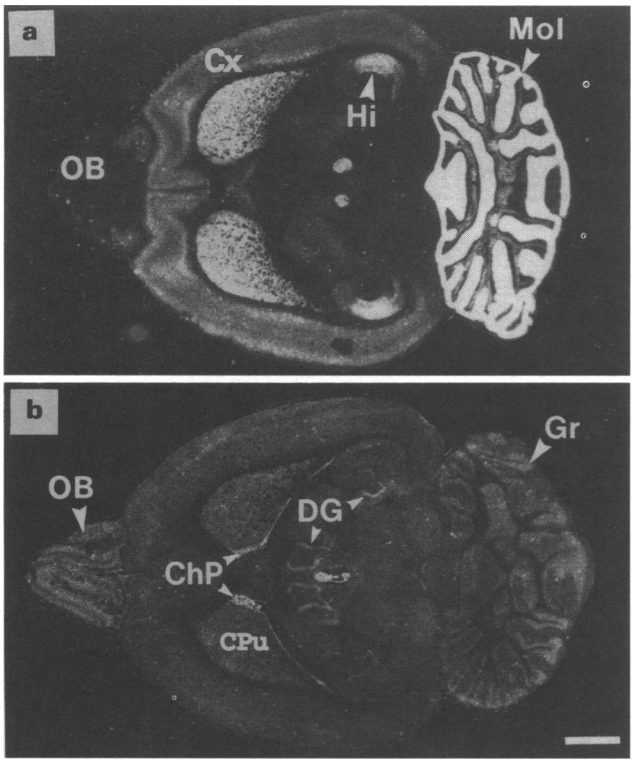

Figure 1 Autoradiographs of inositol polyphosphate binding in rat brain sections. Images were produced by using the Hyperfilm image as a negative and binding sites are represented by accumulations of white grains. Total binding of each radioligand is illustrated, while non-specific binding was negligible (i.e. indistinguishable from film background) in each case (data not shown). (a) [^{3}H]-Ins(1,4,5)P_3; (b) [^{3}H]-$InsP_6$. ChP: choroid plexus; CPu: caudate putamen; Cx: cerebral cortex; DG: granule cell layer of the dentate gyrus; Gr: granule cell layer of the cerebellum; Hi: non-pyramidal cell layers of the hippocampus; Mol: molecular layer of the cerebellum; OB: olfactory bulb. Bar = 2 mm.

Localization and characterization of [^{3}H]-$InsP_6$ binding in rat heart

Autoradiographic studies revealed that [^{3}H]-$InsP_6$ binding to rat heart sections was of a high density and distributed evenly over the ventricular myocardium, with somewhat lower levels of binding over atrial myocardium (Figure 2a). High levels of specific binding and low levels of non-specific binding (measured in the presence of 50 μM $InsP_6$) were present in both atrial and ventricular muscle (Figure 2b). Micromolar concentrations of Ins(1,4,5)PS_3 (1 μM) inhibited >90% of [^{3}H]-$InsP_6$ binding (Figure 2c), while Ins(1,4,5)P_3 (10 μM) inhibited 50% of binding (Figure 2d). Binding of [^{3}H]-$InsP_6$ also occurred in the region of the aorta but this was not inhibited by competing inositol polyphosphates and thus represents non-specific binding (Figure 2). In subsequent equilibrium saturation binding experiments in heart sections, in the absence of added cations, [^{3}H]-$InsP_6$ bound saturably and with high affinity to a single class of sites as evidenced by the monophasic inhibition of [^{3}H]-$InsP_6$ binding by $InsP_6$ (Figure 3a), the linear Scatchard transformation of the saturation data (data not shown) (Figure 3b) and n_H values close to unity (Table 2). Binding affinity and pharmacological specificity were similar in all heart regions but binding density was about 2 fold higher in ventricles than in atria (Table 2).

Autoradiographic competition binding studies in heart showed that inositol polyphosphates competed for binding of [^{3}H]-$InsP_6$ with the rank order of potency: $InsP_6$ > Ins(1,4,5)PS_3 > Ins(1,3,4,5)P_4 ≥ InsP_5 > Ins(1,4,5)P_3 >> inositol mono- and bisphosphates (Figure 3a, Table 2). Neither inositol 1-monophosphate (10 μM) nor inositol 1,4-bisphosphate (10 μM) inhibited binding of [^{3}H]-$InsP_6$ in heart sections (data not shown). The affinity of Ins(1,4,5)P_3 was over 300 fold lower than $InsP_6$ in all heart regions. Ins(1,4,5)PS_3 had a similar potency for inhibition of [^{3}H]-$InsP_6$ binding in heart (Table 2) and brain (IC_{50} < 500 nM; data not shown), but in heart Ins(1,4,5)PS_3 was 20 fold more potent at the [^{3}H]-$InsP_6$ binding site than was Ins(1,4,5)P_3 (Table 2). These results are consistent with binding of [^{3}H]-$InsP_6$ to an $InsP_6$ receptor in rat heart (see Thiebert *et al.*, 1992; Kijima & Fleischer, 1992) and a lack of selectivity of Ins(1,4,5)PS_3 for [^{3}H]-Ins(1,4,5)P_3 compared with [^{3}H]-$InsP_6$ sites.

Discussion

The present studies revealed a high density of [^{3}H]-$InsP_6$ binding sites throughout the heart, while parallel studies demonstrated a more localized distribution of [^{3}H]-$InsP_6$ sites in brain, which differed from that of [^{3}H]-Ins(1,4,5)P_3 sites (see Worley *et al.*, 1987; 1989; Parent & Quirion, 1994). The apparent binding affinity of [^{3}H]-$InsP_6$ (K_D ranging from 22–35 nM) in different regions of the heart was similar to that reported for the isolated $InsP_6$ receptor protein (10 nM; Thiebert *et al.*, 1992) and for binding sites present in cardiac microsomes (12 nM; Kijima & Fleischer, 1992). In the present study, competition binding studies demonstrated that the cardiac [^{3}H]-$InsP_6$ binding site possessed a 200 fold selectivity for $InsP_6$ over Ins(1,4,5)P_3 and an approximately 20 fold se-

lectivity for $InsP_6$ compared to $Ins(1,3,4,5)P_4$ and $InsP_5$, which is similar to that reported for the $InsP_6$ binding site in cerebellar homogenates (Hawkins *et al.*, 1990; Poyner *et al.*, 1993).

The current studies with [^{3}H]-$InsP_6$ have also extended our knowledge of [^{3}H]-$InsP_6$ binding in brain, confirming a specific binding site for [^{3}H]-$InsP_6$ in cerebellum and identifying similar sites enriched in dentate gyrus and caudate putamen (see also Parent & Quirion, 1994). The binding site in brain had lower affinity (79–130 nM) and similar densities (5–10 pmol mg^{-1} protein) to that seen in heart, indicating possible differences

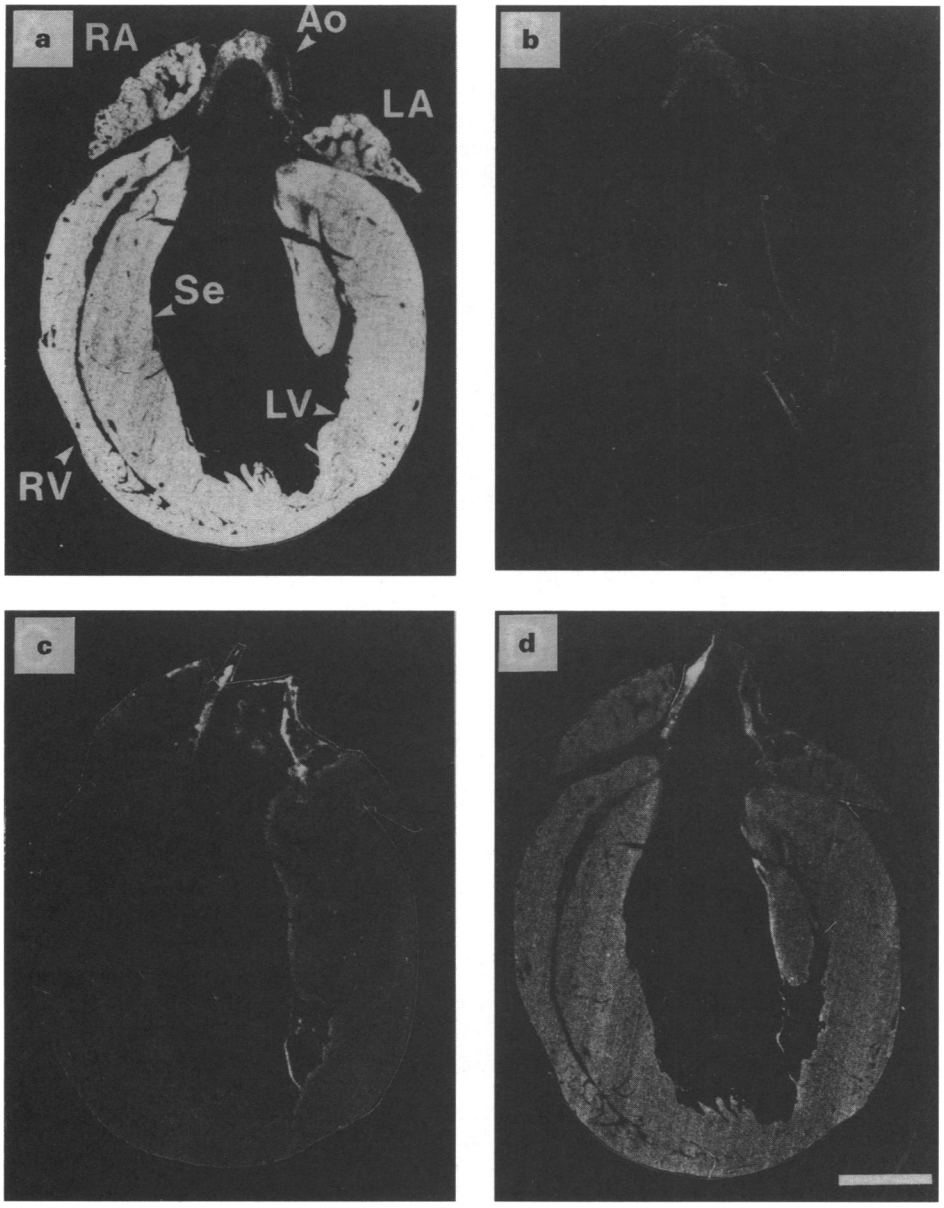

Figure 2 Autoradiographs of [^{3}H]-$InsP_6$ binding in rat heart. (a) Total binding; (b) non-specific binding in the presence of 50 μM $InsP_6$; (c) binding in the presence of 1 μM $Ins(1,4,5)PS_3$; (d) binding in the presence of 10 μM $Ins(1,4,5)P_3$. Binding also occurred in the region of the aorta, but was not inhibited by inositol polyphosphates and therefore represents non-specific binding. Ao: aorta; LA: left atrium; LV: left ventricular free wall; RA: right atrium; RV: right ventricular free wall; Se: interventricular septum. Bar = 2 mm.

Table 2 Characteristics of [^{3}H]-$InsP_6$ binding in rat heart sections

Heart region	K_D (nM)	B_{max} (pmol mg^{-1} protein^{-1})	n_H	K_I (nM) *Ins(1,4,5)PS$_3$*	*InsP$_4$*	*InsP$_5$*	*Ins(1,4,5)P$_3$*
Left atrium	24±1.6	5.1±0.49	0.84±0.05	253±30	453±47	818±55	5066±432
Right atrium	22±1.9	5.2±0.44	0.89±0.05	261±30	535±61	745±96	5650±509
Left ventricle	30±1.2	11±1.8	0.90±0.05	173±15	475±70	567±29	6293±324
Right ventricle	51±1.9	11±1.3	0.92±0.03	198±17	387±40	553±38	4649±281
Interventricular septum	35±2.6	11±1.4	0.84±0.08	187±14	376±38	478±4	5608±323

Data were obtained by densitometry of autoradiograms. K_D and B_{max} were obtained by LIGAND analysis of 7 independent saturation binding experiments. n_H was obtained by EBDA analysis of saturation binding experiments. K_I values were obtained by analysis of 3–4 independent competition binding experiments using LIGAND. All values are shown as mean±s.e.mean.

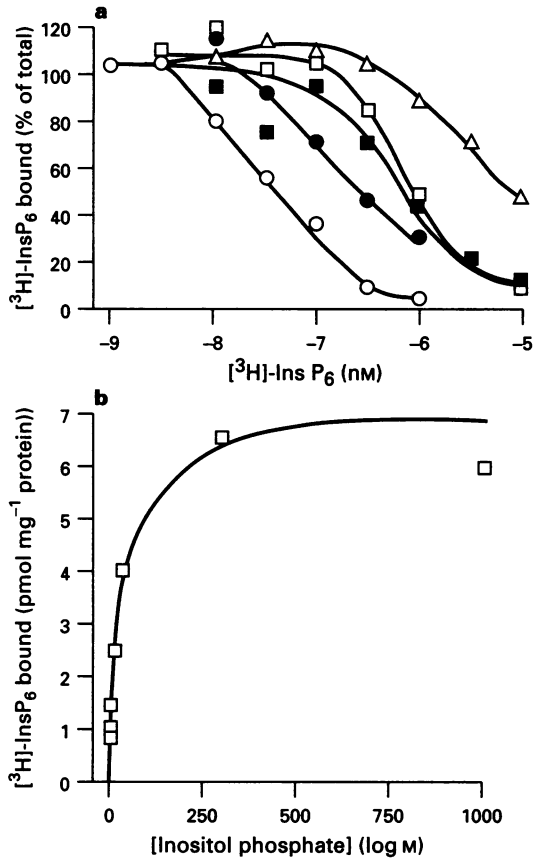

Figure 3 [^{3}H]-InsP$_6$ binding in rat heart. (a) Equilibrium competition binding studies in left atrial sections showing binding inhibition by InsP$_6$ (○), Ins(1,4,5)PS$_3$ (●), InsP$_4$ (■), InsP$_5$ (□), and Ins(1,4,5)P$_3$ (△). Curves are representative of 3–4 independent experiments and left atrial binding characteristics are typical of other heart regions. (b) Saturation binding isotherm of [^{3}H]-InsP$_6$ binding to left atrium. Data were obtained in duplicate by quantitative autoradiography and are representative of 7 independent experiments.

between the cardiac and brain [^{3}H]-InsP$_6$ binding sites or the differential presence of cofactors which modify binding in these tissues. The similar affinity of Ins(1,4,5)PS$_3$ for both the Ins(1,4,5)P$_3$ receptor binding site in brain (see also Challiss *et al.*, 1991) and the InsP$_6$ binding site in heart reveals that this compound does not distinguish these two binding sites.

An inositol polyphosphate receptor in brain with equal affinities for InsP$_6$ and Ins(1,3,4,5)P$_4$ has been previously described (Chadwick *et al.*, 1992), as have specific Ins(1,3,4,5)P$_4$ receptors (Donié & Reiser, 1991). We have not directly investigated the presence of Ins(1,3,4,5)P$_4$ receptor binding sites in heart using [^{3}H]-Ins(1,3,4,5)P$_4$, however, a recent report describing a differential distribution of [^{3}H]-Ins(1,3,4,5)P$_4$ and [^{3}H]-InsP$_6$ binding in brain (Parent & Quirion, 1994) and the present results demonstrating the differential affinities of the various inositol polyphosphates, including Ins(1,3,4,5)P$_4$, suggest that the [^{3}H]-InsP$_6$ binding site in heart is a specific site for InsP$_6$. Confirmation of the presence of an InsP$_6$ binding protein in heart could presumably be achieved in future studies employing immunolocalization and/or detection of mRNA encoding the protein as reported for the cardiac Ins(1,4,5)P$_3$ receptor (Gorza *et al.*, 1993; Kijima *et al.*, 1993; Moschella & Marks, 1993).

The current study has not localized binding of [^{3}H]-InsP$_6$ to a specific cellular location in heart. However, the even distribution throughout the myocardium and high density of binding suggest binding to cardiac myocytes, which are the major component by volume of myocardium. In addition, selective or enriched binding to other tissue components such as endothelial cells, myocardial vessels, nerves, or the conducting system, would produce very different autoradiographic binding patterns as seen for other receptor systems, e.g. atrial natriuretic peptide and neuropeptide Y/peptide YY receptor binding to cardiac blood vessels (Currie *et al.*, 1989; Allen *et al.*, 1993). The broad and even distribution of sites does not, however, preclude some [^{3}H]-InsP$_6$ binding to these various tissue components, particularly in light of the probable ubiquitous distribution of such a binding protein (see below).

In liver, InsP$_6$ binding has been localized to a mitochondrial fraction in which InsP$_6$ stimulated the influx of Ca^{2+} (Copani *et al.*, 1991). Functionally, cardiac α_1-adrenoceptor stimulation has been linked to both InsP$_6$ production (Scholz *et al.*, 1992) and regulation of mitochondrial Ca^{2+} levels (Crompton *et al.*, 1983). Mitochondria contain Fe^{3+}, for which InsP$_6$ has extremely high affinity (affinity constant 10^{25}–10^{30}; Poyner *et al.*, 1993), as a component of the cytochrome system, but it is unclear if this Fe^{3+} is accessible to InsP$_6$ in binding experiments. If it were, the high [^{3}H]-InsP$_6$ binding density in heart would be consistent with the large number of mitochondria in cardiac myocytes, the mitochondria occupying 32% by volume of rat cardiac muscle (Barth *et al.*, 1992).

An alternative intracellular binding site for InsP$_6$ is the sarcoplasmic reticulum since high levels of [^{3}H]-InsP$_6$ binding activity have been observed in a sarcoplasmic reticular fraction of canine heart (Kijima & Fleischer, 1992). The functional role of this binding site is unclear although it may be assumed that it is involved in ionic regulation (Timerman *et al.*, 1992). In light of the present findings and those of recent reports (e.g. Scholz *et al.*, 1992) the nature of the InsP$_6$ binding site in heart and its possible functional role(s) are clearly worthy of further investigation.

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