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SPECIFIC BINDING OF INOSITOL HEXAKISPHOSPHATE (PHYTIC ACID) TO ADRENAL CHROMAFFIN CELL MEMBRANES AND EFFECTS ON CALCIUM-DEPENDENT CATECHOLAMINE RELEASE

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Abstract—In a membrane preparation of cultured bovine adrenal chromaffin cells, [^{3}H]inositol hexakisphosphate ([^{3}H]InsP_6) was shown to bind specifically with a K_d of 90 nM and a B_{max} of 700 fmol/mg protein. The Hill coefficient was not significantly different from unity. The association of [^{3}H]InsP_6 was slow, with equilibrium binding being reached within 10 min. The dissociation of [^{3}H]InsP_6 showed monophasic kinetics. In [^{3}H]InsP_6 competition binding experiments, we found that reduction in the number of phosphorylated sites in inositol resulted in a gradual loss of binding potency. In intact bovine adrenal chromaffin cells, InsP_6 elicited a concentration-dependent facilitation of $^{45}C^{2+}$ influx along with the release of the catecholamines, epinephrine and norepinephrine. The latter responses were slower and longer-lasting than responses to depolarizing stimuli, such as nicotine and high K^+. The catecholamine release required the presence of extracellular Ca^{2+}. In good agreement with the binding studies, lower inositol phosphates displayed reduced secretagogue potency. In conclusion, in bovine adrenal chromaffin cells, InsP_6 appeared to bind to specific sites and elicited Ca^{2+} influx and catecholamine release.

The role of inositol 1,4,5-triphosphate (Ins[1,4,5]-P_3)† as an intracellular second messenger, whose production is linked to various neurotransmitter receptors, has been well established [1]. The presence of more phosphorylated inositol analogs such as inositol 1,3,4,5,6-pentakisphosphate (Ins[1,3,4,5,6]-P_5) and inositol hexakisphosphate (InsP_6) in mammalian cells has been reported recently [2, 3]. These inositol polyphosphates have been identified and shown to be synthesized from *myo*-inositol in brain and in adrenal chromaffin cells [4, 5]. Hanley and co-workers [4] have proposed that Ins[1,3,4,5,6]P_5 and InsP_5 may act as neuromodulators by activating neurons in the area of the nucleus tractus solitarius upon microinjection in the rat, thereby regulating systemic blood pressure. More recent studies have shown the presence of high-affinity binding sites for InsP_6 in brain and pituitary cells in culture [6, 7]. The functional responses to InsP_6 in these cells include influx of calcium ions, release of D-[^{3}H]-aspartate from cerebellar neurons and release of prolactin from pituitary cells [8, 9].

The present study was undertaken to identify specific InsP_6 binding sites and investigate the possible extracellular effects of InsP_6 on adrenal chromaffin cells. Cultured bovine adrenal chromaffin cells provide an appropriate model for the catecholaminergic neuronal system, releasing catecholamines (CAs) in response to various stimuli. To assess whether InsP_6 could modulate the relase of catecholamines by stimulating the influx of calcium, the endogenous release of epinephrine and norepinephrine and the uptake of $^{45}Ca^{2+}$ were measured in bovine adrenal chromaffin cells. In addition, attempts were made to identify specific binding sites for [^{3}H]InsP_6 in chromaffin cell membranes.

MATERIALS AND METHODS

Primary culture of adrenal chromaffin cells. Monolayer primary cultures of chromaffin cells were prepared from bovine adrenal glands by the method of Wilson and Viveros [10], as modified by Ross *et al.* [11]. Bovine adrenal glands were obtained from a local slaughterhouse within 4 hr after the death of the animal. Glands were perfused with collagenase (Worthington) and DNAse (Sigma Chemical Co.) and medullae dissociated from cortex; minced tissue was digested in collagenase for 40–60 min and filtered through a 105 μm wire sieve. The cell suspension was loaded onto a step gradient of 15% and 7.5% Renografin (Squibb) and centrifuged for 20 min at 10,000 *g* Chromaffin cells were collected from the gradients, washed, and plated at required density in Dulbecco's Minimum Essential Medium (DMEM):F12 (1:1) supplemented with 10 mM HEPES (pH 7.4), 10% fetal bovine serum and

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† Abbreviations: Ins[1,4,5]P_3, inositol 1,4,5-triphosphate; InsP_6, inositol hexakisphosphate; Ins[1,2,4,5,6]P_5, inositol 1,3,4,5,6-pentakisphosphate; and CA, catecholamine.

antibiotics (penicillin and streptomycin). The cells were studied between days 3 and 5 of culture.

[^{3}H]$InsP_6$ binding to chromaffin cell membranes. Washed cells (ice-cold 50 mM HEPES with 265 mM sucrose; pH 7.4) were suspended in 50 mM ice-cold Tris–HCl buffer (pH 7.4) with 5 mM EDTA and homogenized using a Polytron (Brinkmann; setting 6 for 10 sec. The homogenate was centrifuged at 40,000 *g* for 30 min at 4°. The resulting pellet was washed three times by resuspending in Tris–HCl buffer and recentrifuged. The final washed pellet was suspended in fresh binding buffer (137 mM NaCl, 5.4 mM KCl, 0.44 mM KH_2PO_4, 1.26 mM $CaCl_2$, 0.81 mM $MgSO_4$, 20 mM HEPES, 0.3% bovine serum albumin; pH 7.4) to give approximately 70 μg protein per assay tube with a final volume of 0.5 mL. Unless otherwise stated, samples were incubated on ice for 90 min and the incubations were terminated by centrifugation (Eppendorf Microfuge) for 2 min by washing the pellets' surface with ice-cold buffer. Pellets were dissolved in Soluene (NEN) mixed with Ready Organic (Beckman) and radioactivity was counted by liquid scintillation counting. Association and dissociation experiments were performed as detailed below and rates roughly estimated by semilogarithmic transformation. Binding data were analyzed in greater detail using LIGAND (Elsevier Biosoft), a parametric nonlinear regression routine.

As radioligand we employed *myo*-[inositol-2-^{3}H(N)]inositol hexakisphosphate ([^{3}H]$InsP_6$; New England Nuclear; 12.0 Ci/mmol). In most experiments, [^{3}H]$INsP_6$ was used at 2.0 nM. Nonspecific binding was determined by inclusion of 100 μM unlabeled $InsP_6$.

Release of catecholamines. The cells (1×10^6/well; 6 well plates) were washed once with Krebs–Ringer bicarbonate (KRB) buffer (120 mM NaCl, 6.0 mM KCl, 2.5 mM CaCl, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4$, 25 mM $NaHCO_3$, pH 7.4). The release of CAs was initiated by incubating the cells at 37° in a total volume of 1 mL of KRB with various concentrations of $InsP_6$. After the specified time, the medium was rapidly removed, mixed with 0.1 M perchloric acid and immediately stored (without alumina extraction) at −70° for assay of catecholamines. The cells were harvested using 0.4 M perchloric acid and the precipitated protein was removed by centrifugation. The catecholamines in the acid extract were purified by treatment with alumina as described [12]. The assay of catecholamines in the medium and the acid extract of cells was carried out using a high pressure liquid chromatography (HPLC) system as described [12]. Briefly, the catecholamines were separated using a reverse phase C_{18} column (Waters) and isocratic elution with 0.15 M monochloroacetate buffer (pH 3.0) containing 2 mM EDTA and 1 mM sodium octylsulfate. The catecholamines were detected using an LC-4B amperometric detector (Bioanalytical Systems, West Lafayette, IN) with the detector potential of +0.65 V versus the Ag/AgCl reference electrode. The samples were filtered using Millex filters (0.45 μm, Millipore) before injecting into the system. The results are expressed as percent released into the medium of the total cell hunt. The cell contents of epinephrine and norepinephrine were 24.5 to 28.6 and 18.5 to 21.3 nmol/10^6 cells, respectively.

Uptake of $^{45}Ca^{2+}$ by chromaffin cells. The cells (2×10^6 cells; 6 well plates) were washed with Krebs–Ringer solution containing 25 mM HEPES (pH 7.4) and preincubated for 1 min in the same buffer containing 1 μCi $^{45}Ca^{2+}$ at 37°. The drug was added to start the incubation and the uptake was terminated by rapid aspiration of the medium. The cells were washed four times with ice-cold, calcium-free buffer. The cells were solubilized using 1% Triton X-100 and collected into scintillation vials. The radioactivity was counted in a liquid scintillation system having 90% efficiency for $^{45}Ca^{2+}$. The amount of calcium taken up by the cells was calculated from the initial concentration of calcium in the medium (2.5 μmol/mL) and the results are expressed as nanomoles calcium/10)^6 cells.

RESULTS

[^{3}H]$InsP_6$ binding to chromaffin cell membranes. Chromaffin cell membranes were prepared by standard methods and [^{3}H]$InsP_6$ binding assays were performed at 4° (to minimize hydrolysis of the ligand) in a final volume of 0.5 mL using extensively washed membranes from approximately 2×10^6 cells (30–35 μg protein).

Saturation analysis of [^{3}H]$InsP_6$ binding data with correction for isotopic dilution and nonspecific binding yielding a K_d of 90 nM and a B_{max} of 700 fmol/mg protein. The Hill coefficient was not significantly different from unity. A similar K_d value was estimated by determining association and dissociation rates in kinetic experiments (N = 3) with semi-logarithmic transformation of data sets. Association of [^{3}H]$InsP_6$ was slow and dissociation showed simple, monophasic dissociation kinetics (Fig. 1). The true association rate constant (K_{+1}) was 0.017 ($min^{-1} \cdot nM^{-1}$) as determined by the equation $K_{+1} = (K_{obs} - K_{-1})/L$. The K_{-1} of 0.96 ± 0.03 (min^{-1}) was determined experimentally, yielding a kinetically estimated K_d of 55 nM (K_{-1}/K_{+1}).

Competition experiments tested the potency of several unlabeled inositol phosphates to displace [^{3}H]$InsP_6$ binding (Fig. 2), yielding the following rank order of potency: $InsP_6$ > Ins[1,3,4,5,6]P_5 > Ins[1,3,4,5]P_4 > Ins[1,2,6]P_3 > Ins[1,4,5]P_3. In these experiments, the concentration of [^{3}H]$InsP_6$ was 2.0 nM with or without unlabeled inositol phosphates in various concentrations.

Effect of $InsP_6$ on catecholamine release. $InsP_6$ elicited a concentration-dependent release of endogenous epinephrine and norepinephrine from adrenal chromaffin cells (Fig. 3). The release of catecholamines was elevated at 0.1 μM $InsP_6$ and reached a maximum response at 10 μM. To test the calcium dependency of this response, the release of epinephrine and norepinephrine by $InsP_6$ upon Ca^{2+}. In Ca^{2+}-free buffer, $InsP_6$ up to 10 μM did not release CAs. However, at 100 μM $InsP_6$, a slight release of epinephrine and norepinephrine occurred also in Ca^{2+}-free medium. The time-course of release of CAs stimulated by $InsP_6$ (10 μM) is shown in Fig. 4. The release of both norepinephrine and epinephrine was increased markedly at 10 sec and

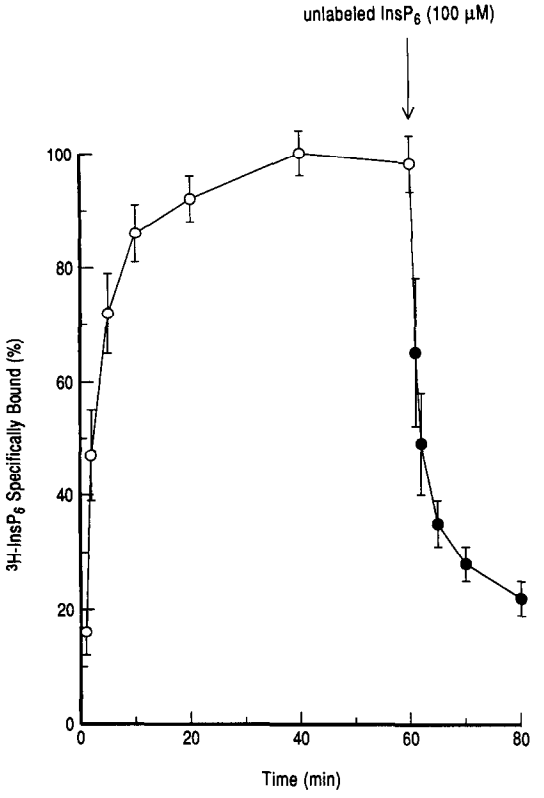

Fig. 1. [^{3}H]$InsP_6$ binding to adrenal chromaffin cell membranes. Association (○) and dissociation (●) are expressed as a function of time. At each time point nonspecific binding was assessed by coincubation with 100 μM unlabelled $InsP_6$. Radioligand concentration was always 2.0 nM. Dissociation was initiated by addition of 100 μM unlabeled $InsP_6$ (arrow). Values are expressed as a percentage of the specific binding as calculated from the mean (± SEM) of a representative experiment performed in triplicate.

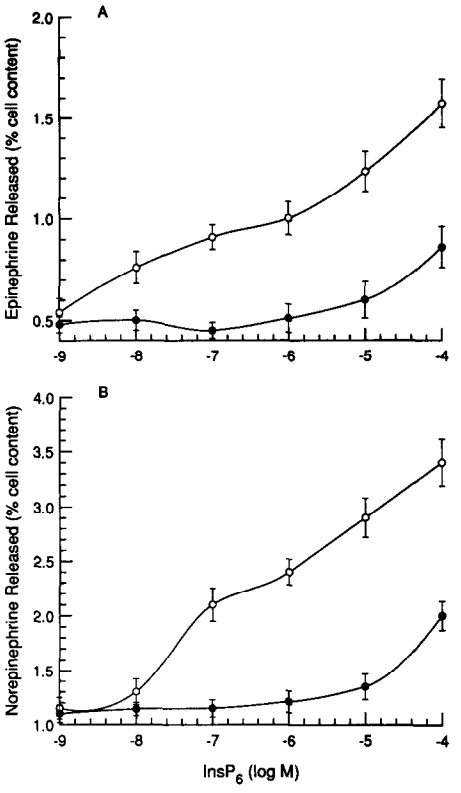

Fig. 3. Release of catecholamines from chromaffin cells stimulated by various concentrations of $InsP_6$. The release was measured in the presence (○—○) and absence (●—●) of calcium. The cell contents of epinephrine and norepinephrine were 24.5 to 28.6 and 18.5 to 21.3 nmol/10^6 cells, respectively. Values are expressed as a percentage of cell content released in 5 min and are means ± SEM of two experiments done in triplicate.

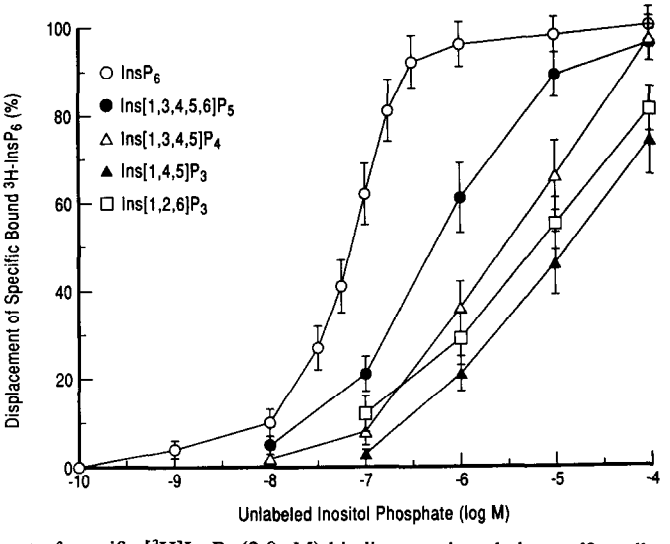

Fig. 2. Displacement of specific [^{3}H]$InsP_6$ (2.0 nM) binding to adrenal chromaffin cell membranes by five unlabeled inositol phosphate compounds: $InsP_6$; Ins[1,3,4,5,6]P_5; Ins[1,3,4,5]P_4; Ins[1,4,5]P_3; and Ins[1,2,6]P_3. Nonspecific binding was defined in the presence of 100 μM unlabeled $InsP_6$. Data points represent means ± SEM for a representative experiment performed in quadruplicate.

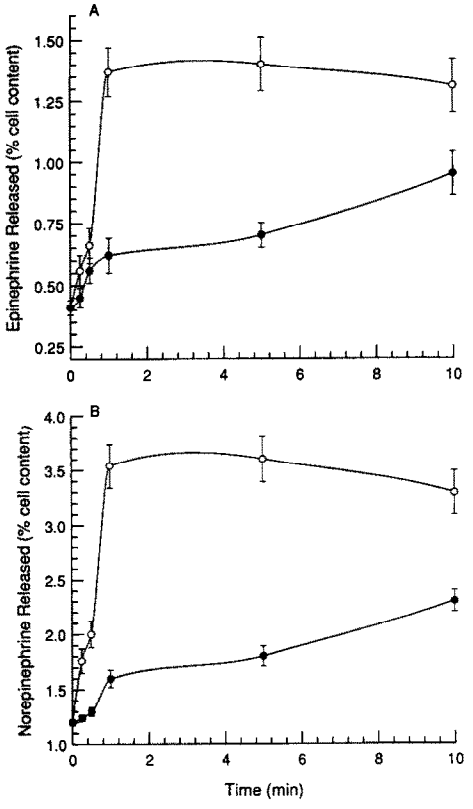

Fig. 4. Release of norepinephrine (A) and epinephrine (B) in the presence (○—○) and absence (●—●) of 10 μM $InsP_6$ measured at various time points. Values are means ± SEM for a representative experiment done in triplicate.

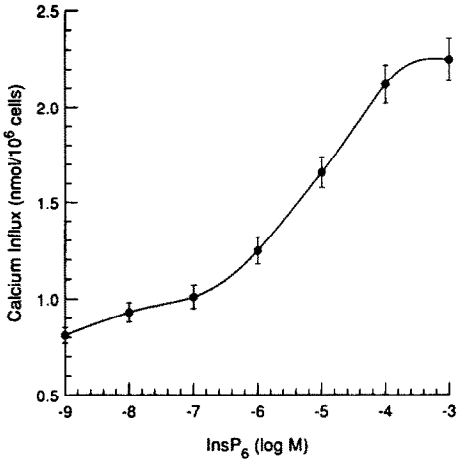

Fig. 5. Influx of $^{45}Ca^{2+}$ into chromaffin cells stimulated by various concentrations of $InsP_6$. The incubation conditions are given in Materials and Methods. Values are means ± SEM of two experiments each done in triplicate. All values except 10^{-7} and 10^{-8} were significantly different from basal influx ($P < 0.001$).

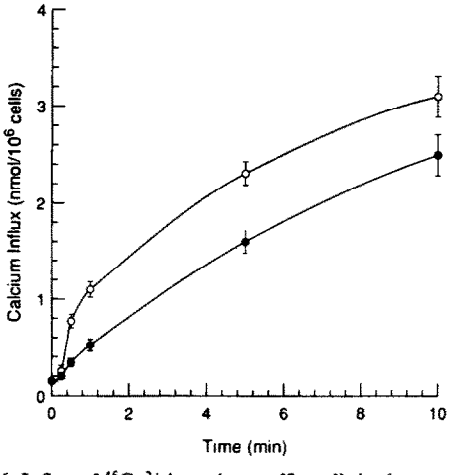

Fig. 6. Influx of $^{45}Ca^{2+}$ into chromaffin cells in the presence (○—○) and absence (●—●) of 10 μM $InsP_6$ measured at various time points. Values are means ± SEM for a representative experiment done in triplicate. All values except that at 15 sec were significantly different from control ($P < 0.01$).

reached a maximum response at 1 min. The percent increase in release of both epinephrine and norepinephrine over the basal release began to decline after 5 min.

Effect of $InsP_6$ on $^{45}Ca^{2+}$ uptake. In bovine adrenal chromaffin cells, addition of $InsP_6$ produced a concentration-dependent increase in the uptake of $^{45}Ca^{2+}$, significant at 1 min (Fig. 5). The uptake of $^{45}Ca^{2+}$ was elevated significantly at 1 μM $InsP_6$ and the maximal response, occurring at 100 μM, corresponded to a 2- to 3-fold increase over basal uptake. The time-course of $^{45}Ca^{2+}$ uptake, stimulated by 10 μM $InsP_6$, was rather slow with a significant increase occurring at 30 sec, and the specific uptake (the difference between the presence and absence of $InsP_6$) reached a plateau at 5 min (Fig. 6).

To determine whether the action of $InsP_6$ was specific for this isomer, the effects of Ins[1,3,4,5,6]-P_5 and Ins[1,4,5]P_3 on catecholamine release and $^{45}Ca^{2+}$ uptake were tested in the chromaffin cells. As shown in Table 1, the larger response was produced by $InsP_6$ (10 μM) while Ins[1,3,4,5,6]P_5 (100 μM), although less effective, produced a significant release of catecholamines and uptake of $^{45}Ca^{2+}$. However, Ins[1,4,5]P_3 (100 μM) was completely ineffective in causing either the release of catecholamines or the uptake of $^{45}Ca^{2+}$. The ability of various calcium channel blockers to inhibit the uptake of $^{45}Ca^{2+}$ caused by $InsP_6$ was also tested. As shown in Table 2, ω-conotoxin, neomycin or nifedipine, at concentrations that are known to inhibit calcium channels [13], failed to inhibit the $InsP_6$-mediated increase in $^{45}Ca^{2+}$ uptake. However, cobalt, which blocks the entry of calcium into the cell by any type of channel, completely inhibited the uptake of $^{45}Ca^{2+}$ stimulated by $InsP_6$. None of these

Table 1. Effects of inositol phosphates on catecholamine release and $^{45}Ca^{2+}$ uptake in adrenal chromaffin cells

	Catecholamine released (% of cell content)		
	Epinephrine	Norepinephrine	$^{45}Ca^{2+}$ uptake (nmol/10^6 cells)
Control	0.512 ± 0.08	1.05 ± 0.12	0.616 ± 0.04
$InsP_6$ (10 μM)	1.26 ± 0.08*	2.71 ± 0.15*	1.72 ± 0.12*
Ins[1,3,4,5,6]P_5 (100 μM)	1.12 ± 0.09*	2.31 ± 0.15*	1.51 ± 0.11*
Ins[1,4,5]P_3 (100 μM)	0.615 ± 0.05	1.12 ± 0.11	0.713 ± 0.07

Catecholamine release was measured for 5 min at 37° in KRB medium. $^{45}Ca^{2+}$ uptake was measured for 1 min following a 1-min preincubation with $^{45}Ca^{2+}$ using Krebs–HEPES buffer at 37°. Values are means ± SEM of two experiments done in triplicate.
* $P < 0.001$ compared to control.

Table 2. Effects of various calcium channel blockers on $^{45}Ca^{2+}$ uptake in adrenal chromaffin cells

	$^{45}Ca^{2+}$ uptake (nmol/10^6 cells)
Control	0.474 ± 0.11
$InsP_6$ (10 μM)	1.15 ± 0.23*
+ Cobalt (10 μM)	0.581 ± 0.18†
+ ω-Conotoxin (10 μM)	1.02 ± 0.15*
+ Neomycin (10 μM)	1.09 ± 0.19*
+ Nifedipine (1 μM)	1.1 ± 0.21*

The uptake of $^{45}Ca^{2+}$ was measured for 1 min following a 1-min preincubation with $^{45}Ca^{2+}$ using Krebs–HEPES buffer at 37°. When antagonists were used, they were added along with $^{45}Ca^{2+}$. Values are means ± SEM of a representative experiment done in triplicate.
* $P < 0.001$ compared to control.
† $P < 0.001$ compared to $InsP_6$ group.

blockers modifed the basal $^{45}Ca^{2+}$ uptake (data not presented).

DISCUSSION

The roles of Ins[1,4,5]P_3 and Ins[1,3,4,5]P_4 as intracellular second messengers mediating the effects of various neurotransmitters are well established [1]. Recently higher phosphates of inositol, i.e. Ins[1,3,4,5,6]P_5 and $InsP_6$, have been identified in mammalian cells [3, 4]. The functional importance of these inositol phosphates is only beginning to emerge [4] and a role as endogenous extracellular neuromodulators has been proposed [4, 9].

$InsP_6$ is likely to act at specific receptors since the present study identified specific high affinity [^{3}H]-$InsP_6$ binding sites in chromaffin cell membranes. The population of binding sites was homogenous but exhibited lower affinity (90 nM) than do many other sites for putative extracellular messengers. The relatively low affinity of [^{3}H]$InsP_6$ sites should, however, be considered in relation to the high concentrations of inositol penta- and hexakisphosphate that have been estimated to be present in chromaffin [5] and other [14, 15] cells. Moreover, our binding data are largely consistent with data obtained by other investigators in brain [6, 7].

Based on competition studies, we propose that [^{3}H]$InsP_6$-labeled sites are relatively specific for [^{3}H]$InsP_6$ and unlabeled $InsP_6$. Less phosphorylated analogs showed a gradual loss of potency, thus indicating that $InsP_6$ binding sites are different from, for example, the recently characterized intracellular Ins[1,4,5]P_3 receptor [16]. The relative ability of Ins[1,3,4,5,6]P_5 to displace [^{3}H]$InsP_6$ correlates with its actions on CA release and $^{45}Ca^{2+}$ uptake.

In the present study, it was observed that extracellular addition of $InsP_6$ stimulates the release of epinephrine and norepinephrine from bovine adrenal chromaffin cells in a concentration-dependent manner. In adrenal chromaffin cells, depolarization with high K^+ or cholinergic stimulation causes a massive exocytotic release of CAs (20–30% of cell content) by activating voltage-dependent and nicotinic ion channels, respectively [17]. In contrast, agents such as bradykinin, angiotensin II or endothelin-1 cause a smaller release of CAs (<10% of cell content) by mobilizing intracellular calcium through an inositol triphosphate-dependent mechanism [18, 19]. The maximal release of catecholamines stimulated by $InsP_6$ was still slightly lower, i.e. about 3–4% of cell content, indicating that (i) depolarizing, (ii) G-protein-coupled and (iii) $InsP_6$-elicited responses all occur through different mechanisms.

The absence of calcium ions in the incubation medium completely abolished the effect of $InsP_6$, providing evidence that the influx of calcium is required for the release of CAs. To further investigate the mechanism of CA release stimulated by $InsP_6$, the influx of $^{45}Ca^{2+}$ into chromaffin cells was measured. $InsP_6$ increased the uptake of $^{45}Ca^{2+}$, although this response was less pronounced than the CA-releasing effect of $InsP_6$. For example, 0.1 μM $InsP_6$ caused a significant release of both epinephrine and norepinephrine, whereas this concentration was not effective in increasing the uptake of $^{45}Ca^{2+}$. It cannot be excluded, however, that this may be due to lower sensitivity of the $^{45}Ca^{2+}$ uptake as a measure of Ca^{2+} influx relative to the measurement of

endogenous CA release. The time-course of uptake of $^{45}Ca^{2+}$ was relatively slow (compared to depolarization stimuli) and long-lasting, suggesting that the influx of $^{45}Ca^{2+}$ occurs either secondary to a moderate release of intracellular calcium or through a non-voltage sensitive, but $InsP_6$-sensitive receptor/ion channel. To test the involvement of any known calcium channels in the actions of $InsP_6$, the effect of $InsP_6$ was tested in the presence of nifedipine, ω-conotoxin and neomycin. As none of these blockers inhibited the effect of $InsP_6$ on $^{45}Ca^{2+}$, influx may not involve any of the channels sensitive to these agents. Since cobalt blocks the effect of $InsP_6$ on $^{45}Ca^{2+}$ uptake, the effect of $InsP_6$ could be physiologically relevant and may not be due to nonspecific actions of $InsP_6$ on chromaffin cells. Similar findings have been reported by Nicoletti *et al.* [9] where $InsP_6$-mediated $^{45}Ca^{2+}$ uptake was not blocked by Ca^{2+} channel blockers in cultured cerebellar neurons. The uptake of $^{45}Ca^{2+}$ correlates with the release of CAs as maximum uptake and release were both obtained at 1 min. However, at a shorter time point, such as 15 sec, the release of CAs was significant but without apparent increase in $^{45}Ca^{2+}$ uptake. This could be due to practical difficulty in accurately measuring the uptake of $^{45}Ca^{2+}$ during very short time periods.

Both stimulation of the release of CAs and the influx of $^{45}Ca^{2+}$ by $InsP_6$ seem to be specific for higher inositol phosphates. Ins[1,3,4,5,6]P_5 was relatively less potent compared to $InsP_6$, whereas the well-characterized intracellular second messenger Ins[1,4,5]P_3 was inactive.

In summary, the present study has demonstrated a Ca^{2+}-dependent action of $InsP_6$ on catecholamine release in chromaffin cells. The kinetics of $InsP_6$-elicited release appear to differ from several previously characterized extracellularly acting secretagogues. Specific [^{3}H]$InsP_6$ recognition sites on chromaffin cell membranes have been identified and characterized.

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