Inositol 1,3,4,5-tetrakisphosphate-induced Ca^{2+} sequestration into bovine adrenal-medullary secretory vesicles

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Ins(1,3,4,5) P_4 induced a rapid sequestration of Ca^{2+} into both secretory vesicles and microsomes of bovine adrenal medulla. The Ca^{2+} -sequestering role of Ins(1,3,4,5) P_4 contrasts with the Ca^{2+} -releasing role of Ins(1,4,5) P_3 in adrenal-medullary secretory vesicles and microsomes. The Ins(1,3,4,5) P_4 -induced Ca^{2+} sequestration into secretory vesicles was not inhibited by heparin (50 $\mu\text{g}/\text{ml}$), whereas Ins(1,4,5) P_3 -induced Ca^{2+} release was completely inhibited, indicating two different receptors for Ins(1,4,5) P_3 and Ins(1,3,4,5) P_4 . Furthermore, Ins(1,3,4,5) P_4 was as effective at 4 °C as at 24 °C in sequestering Ca^{2+} into secretory vesicles, implying Ca^{2+} sequestration through receptor-operated Ca^{2+} channels or activation of the Ca^{2+} -exchange mechanism by Ins(1,3,4,5) P_4 . The Ca^{2+} -sequestering activity of Ins(1,3,4,5) P_4 has also been demonstrated with $^{45}\text{Ca}^{2+}$; 10 μM -Ins(1,3,4,5) P_4 induced rapid uptake of $^{45}\text{Ca}^{2+}$ into secretory vesicles optimized for Ca^{2+} uptake, whereas 10 μM -Ins(1,4,5) P_3 induced $^{45}\text{Ca}^{2+}$ release from secretory vesicles in similar experiments.

INTRODUCTION

Ins(1,4,5) P_3 has been shown to induce Ca^{2+} release from intracellular stores in various cell lines [1]. Subsequent studies on the metabolic processing of Ins(1,4,5) P_3 showed the existence of various Ins(1,4,5) P_3 metabolites with potentially important functions [2]. Among these metabolites, Ins(1,3,4,5) P_4 has drawn a great deal of attention as a potential regulator of cytosolic Ca^{2+} concentration ($[\text{Ca}^{2+}]_i$) [2–4]. Ins(1,3,4,5) P_4 is formed very rapidly after agonist stimulation of animal tissues [5–7] via Ins(1,4,5) P_3 3-kinase. A rise in Ins(1,3,4,5) P_4 concentration follows immediately after the initial rise in Ins(1,4,5) P_3 and increased $[\text{Ca}^{2+}]_i$. The elevated Ins(1,4,5) P_3 level decreases rapidly concomitant with the increase in Ins(1,3,4,5) P_4 concentration [7].

Owing to the rapid appearance of Ins(1,3,4,5) P_4 and changes in $[\text{Ca}^{2+}]_i$, there has been considerable effort to find the potential role of Ins(1,3,4,5) P_4 in regulating $[\text{Ca}^{2+}]_i$. However, the exact role of Ins(1,3,4,5) P_4 in cellular Ca^{2+} homeostasis is yet to be determined. Suggested roles of Ins(1,3,4,5) P_4 include: (1) regulation of Ca^{2+} influx from outside the cell into an Ins(1,4,5) P_3 -sensitive Ca^{2+} pool [8]; (2) release of intracellular Ca^{2+} [4,9,10]; (3) control of Ca^{2+} transfer between intracellular pools [11,12]; and (4) sequestration of Ca^{2+} [13].

Recently we discovered that Ins(1,4,5) P_3 at 10 μM induced release of 3.5–4.0 nmol of Ca^{2+} /mg of protein from secretory vesicles of adrenal medulla [14]. The Ins(1,4,5) P_3 -sensitive pools were refilled within 3 min, after which they were ready to be released again in response to a second addition of Ins(1,4,5) P_3 . The same amount of Ins(1,4,5) P_3 released 1.5 nmol of Ca^{2+} /mg of protein from adrenal-medullary microsomes. These results implied the role of chromaffin vesicles as the major intracellular Ca^{2+} store in chromaffin cells. In light of the facts that the secretory vesicles have Ins(1,4,5) P_3 -sensitive Ca^{2+} pools and the vesicles are the only known example of a major Ins(1,4,5) P_3 -sensitive intracellular Ca^{2+} store isolated in intact form, the vesicles provide a unique model system to test the Ca^{2+} -releasing activity of a variety of agents, including inositol phosphates. Hence I tested the effect of Ins(1,3,4,5) P_4 on Ca^{2+} flux of secretory

vesicles and found that Ins(1,3,4,5) P_4 rapidly sequestered Ca^{2+} into the vesicles.

MATERIALS AND METHODS

Materials

Ins1P, Ins(1,4) P_2 and heparin were obtained from Sigma. Ins(1,4) P_2 , Ins(1,4,5) P_3 , Ins(1,3,4,5) P_4 , Ins(1,4,5,6) P_4 and Ins P_5 [unspecified isomer form of Ins(1,3,4,5,6) P_5] were from Calbiochem. $^{45}\text{CaCl}_2$ (39.56 mCi/mg) was from DuPont–NEN, and Percoll was obtained from Pharmacia.

Adrenal-medullary secretory-vesicle preparation

Vesicles were isolated by the method described previously [14], except that Buffer I was 10 mM-Hepes/KOH (pH 7.2)/140 mM-KCl/0.22 M-sucrose, adjusted to a final free Ca^{2+} concentration of 0.3 μM with a minimal amount of Ca^{2+} or EGTA. EGTA added was usually less than 5 μM , and therefore did not interfere with the Ca^{2+} -release measurements.

Adrenal-medullary microsome preparation

Adrenal microsomes were prepared as described previously [14], except that Buffer I was 10 mM-Hepes/KOH (pH 7.2)/140 mM-KCl/0.22 M-sucrose, adjusted to a final free Ca^{2+} concentration of 0.3 μM with a minimal amount of Ca^{2+} or EGTA.

Measurement of Ca^{2+} flux by a Ca^{2+} -selective electrode

For Ca^{2+} -flux measurements, vesicles were resuspended in Buffer I at a concentration of 1 mg of protein/ml, and the amount of Ca^{2+} released or taken up in response to inositol phosphate was measured with a Ca^{2+} -selective electrode (Orion 93-20) connected to an ion/pH-meter (Fischer 420). Routinely 0.5 ml of vesicles was placed in an Orion micro sample dish, and 5 or 10 μl of appropriate inositol phosphate was added at a time. The voltage changes occurring owing to changes in Ca^{2+} concentration were recorded by a chart recorder, and the Ca^{2+} electrode was calibrated with a standard Ca^{2+} solution in every

Abbreviations used: Ins P_5 , unspecified isomer form (D/L mixture) of inositol 1,3,4,5,6-pentakisphosphate; $[\text{Ca}^{2+}]_i$, cytosolic Ca^{2+} concentration.

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experiment. Free Ca^{2+} concentrations in Buffer I with resuspended vesicles were determined by using fura-2 as described previously [14]. For microsome Ca^{2+} -flux measurements, microsomes were resuspended in Buffer I (1 mg/ml) containing 5 μg each of antimycin A and oligomycin/ml to prevent mitochondrial uptake.

$^{45}\text{Ca}^{2+}$ uptake/release assay

For $^{45}\text{Ca}^{2+}$ uptake/release experiments, purified vesicles were resuspended in an appropriate volume of Buffer I to give 0.5 mg of protein/ml. Ca^{2+} uptake was initiated by incubating vesicles at 24 °C or 37 °C in the presence of 1.5 μM free $^{45}\text{Ca}^{2+}$ (450 mCi/mmol). At various time points, 50 μl portions were added to 1 ml of ice-cold wash solution (Buffer I supplemented with 0.25 mM- LaCl_3) and filtered through a Millipore filter (HATF; 0.45 μm pore size). The filters were further washed with 2 $\times$ 2 ml of ice-cold wash solution. $^{45}\text{Ca}^{2+}$ remaining on the filter was measured by liquid-scintillation spectrometry.

RESULTS

Since $\text{Ins}(1,3,4,5)\text{P}_4$ and $\text{Ins}(1,4,5,6)\text{P}_4$ both have phosphate groups on the 1-, 4- and 5-positions, and $\text{Ins}(1,3,4,5)\text{P}_4$ was implicated in the Ca^{2+} flux of various cells [4,11,13], it was of great interest to determine what effect, if any, these compounds exert on the Ca^{2+} flux of adrenal secretory vesicles. As shown in Fig. 1, $\text{Ins}(1,4,5,6)\text{P}_4$ at 10 μM released about 30 % of the Ca^{2+} that can be released by the same concentration of $\text{Ins}(1,4,5)\text{P}_3$,

Fig. 1. Profile of Ca^{2+} flux from secretory vesicles in response to $\text{Ins}(1,4,5,6)\text{P}_4$ and $\text{Ins}(1,3,4,5)\text{P}_4$

$\text{Ins}(1,4,5)\text{P}_3$ (InsP_3), $\text{Ins}(1,4,5,6)\text{P}_4$ and $\text{Ins}(1,3,4,5)\text{P}_4$ were added at final concentrations of 10 μM . (a) Addition of $\text{Ins}(1,4,5)\text{P}_3$ followed by $\text{Ins}(1,4,5,6)\text{P}_4$. (b) Addition of $\text{Ins}(1,4,5)\text{P}_3$ followed by $\text{Ins}(1,3,4,5)\text{P}_4$. Traces indicate the changes in free Ca^{2+} concentration in the media and are representative of similar results repeated 10 times with 4 different vesicle preparations.

whereas $\text{Ins}(1,3,4,5)\text{P}_4$ induced sequestration of Ca^{2+} into the vesicles. To rule out the possibility of $\text{Ins}(1,3,4,5)\text{P}_4$ contamination by Ca^{2+} , several doses of $\text{Ins}(1,3,4,5)\text{P}_4$ from different batches were added to the media and found not to affect the Ca^{2+} -electrode readings, eliminating the possibility of $\text{Ins}(1,3,4,5)\text{P}_4$ contamination by Ca^{2+} .

Among all the inositol phosphates tested, which include $\text{Ins}1\text{P}$, $\text{Ins}(1,4)\text{P}_2$ and InsP_5 , only $\text{Ins}(1,3,4,5)\text{P}_4$ exhibited a Ca^{2+} -sequestering effect. Therefore, the possibility of synergistic effects between $\text{Ins}(1,4,5)\text{P}_3$ and $\text{Ins}(1,3,4,5)\text{P}_4$ on the Ca^{2+} flux of adrenal chromaffin vesicles was explored. As shown in Fig. 2, $\text{Ins}(1,3,4,5)\text{P}_4$ induced sequestration of Ca^{2+} into vesicles, regardless of its addition before or after addition of $\text{Ins}(1,4,5)\text{P}_3$, indicating an $\text{Ins}(1,4,5)\text{P}_3$ -independent role of $\text{Ins}(1,3,4,5)\text{P}_4$. $\text{Ins}(1,4,5)\text{P}_3$ elicited release of approx. 4 nmol of Ca^{2+} /mg of protein from the vesicles, and the $\text{Ins}(1,4,5)\text{P}_3$ -sensitive Ca^{2+} pools were repeatedly releasable by $\text{Ins}(1,4,5)\text{P}_3$. As has previously been shown [14,15], Ca^{2+} ionophore A23187 also induced Ca^{2+} uptake into the isolated secretory vesicles, and the uptake transient appeared to resemble the $\text{Ins}(1,3,4,5)\text{P}_4$ -induced transient (Figs. 2c and 2d). However, there was a slight difference between the A23187- and $\text{Ins}(1,3,4,5)\text{P}_4$ -induced Ca^{2+} -uptake patterns; the Ca^{2+} taken up by A23187 remained longer in the vesicles than that sequestered by $\text{Ins}(1,3,4,5)\text{P}_4$.

To determine whether the $\text{Ins}(1,3,4,5)\text{P}_4$ -induced Ca^{2+} sequestration can also be demonstrated at a lower temperature, secretory vesicles were chilled on ice for 20 min, and the effect of $\text{Ins}(1,3,4,5)\text{P}_4$ on the Ca^{2+} flux was determined while the vesicles were kept on ice. Fig. 3 shows that $\text{Ins}(1,3,4,5)\text{P}_4$ -induced sequestration of Ca^{2+} into the vesicles at 4 °C as well as it did at

Fig. 3. Ca^{2+} uptake into secretory vesicles in response to $\text{Ins}(1,3,4,5)\text{P}_4$ at 24 °C and 4 °C

(a) Addition of $\text{Ins}(1,3,4,5)\text{P}_4$ (InsP_4) at a final concentration of 10 μM at 24 °C. (b) Same as (a), but carried out at 4 °C. Traces indicate the changes in free Ca^{2+} concentration in the media and are representative of results repeated 5 times.

Fig. 2. Ca^{2+} release/uptake profile from secretory vesicles in response to $\text{Ins}(1,3,4,5)\text{P}_4$, $\text{Ins}(1,4,5)\text{P}_3$ or A23187

$\text{Ins}(1,3,4,5)\text{P}_4$ (InsP_4) and $\text{Ins}(1,4,5)\text{P}_3$ (InsP_3) were added at final concentrations of 10 μM , and A23187 was added at a final concentration of 2.5 μM . (a) When the calcium electrode reading stabilized, $\text{Ins}(1,3,4,5)\text{P}_4$ and then $\text{Ins}(1,4,5)\text{P}_3$ were added to the vesicle-containing medium. (b) Addition of $\text{Ins}(1,4,5)\text{P}_3$ followed by $\text{Ins}(1,3,4,5)\text{P}_4$. (c) Two successive additions of $\text{Ins}(1,4,5)\text{P}_3$ followed by A23187. (d) Two successive additions of $\text{Ins}(1,4,5)\text{P}_3$ followed by $\text{Ins}(1,3,4,5)\text{P}_4$. Traces indicate the changes in free Ca^{2+} concentration in the media and are representative of results repeated 10 times with 5 different vesicle preparations.

Fig. 4. Ca^{2+} uptake into microsomes in response to Ins(1,3,4,5) P_4

Ins(1,3,4,5) P_4 (Ins P_4) and Ins(1,4,5) P_3 (Ins P_3) were added at final concentrations of 10 μM , and A23187 was added at a final concentration of 2.5 μM . (a) Addition of Ins(1,4,5) P_3 followed by Ins(1,3,4,5) P_4 . (b) Addition of A23187. (c) Addition of Ins(1,3,4,5) P_4 . Traces indicate the changes in free Ca^{2+} concentration in the media and are representative of similar results repeated 5 times.

Fig. 5. Effect of Ins(1,3,4,5) P_4 on Ca^{2+} flux of secretory vesicles in the presence of heparin

Heparin was added to the vesicles at a final concentration of 50 $\mu g/ml$, and incubated for 20 min at 24 $^{\circ}C$ before addition of Ins(1,4,5) P_3 (Ins P_3) or Ins(1,3,4,5) P_4 (Ins P_4), which were each added at final concentrations of 10 μM . (a) Two successive additions of Ins(1,4,5) P_3 . (b) Addition of Ins(1,4,5) P_3 followed by Ins(1,3,4,5) P_4 . Traces indicate the changes in free Ca^{2+} concentration in the media and are representative of similar results repeated 5 times.

24 $^{\circ}C$. The extent of Ca^{2+} sequestration at 4 $^{\circ}C$ even appeared to be slightly more pronounced than that at 24 $^{\circ}C$.

Ins(1,3,4,5) P_4 also induced Ca^{2+} uptake into adrenal-medullary microsomes, which have been shown to release Ca^{2+} in response to Ins(1,4,5) P_3 (Fig. 4). However, unlike chromaffin vesicles, Ins(1,3,4,5) P_4 and A23187 had opposite effects on Ca^{2+} flux in microsomes. Ins(1,3,4,5) P_4 induced Ca^{2+} uptake, whereas A23187 induced Ca^{2+} release, clearly demonstrating the Ca^{2+} -sequestering role of Ins(1,3,4,5) P_4 . Release of Ca^{2+} by A23187 in the adrenal-medullary microsomes is not unexpected, considering that microsomal fractions from various sources have been shown to release Ca^{2+} in the presence of A23187 [16,17]. Since all the Ca^{2+} -flux experiments with microsomes were carried out in the presence of mitochondrial Ca^{2+} -uptake blockers (oligomycin and antimycin A), the uptake induced by Ins(1,3,4,5) P_4 cannot be attributed to mitochondrial Ca^{2+} uptake.

To determine whether Ins(1,4,5) P_3 and Ins(1,3,4,5) P_4 bind the same sites on the vesicles, the effect of heparin, which is known to bind to the high-affinity Ins(1,4,5) P_3 receptor to inhibit Ins(1,4,5) P_3 -mediated Ca^{2+} release [18,19], was studied. As shown in Fig. 5, heparin completely inhibited Ins(1,4,5) P_3 -induced Ca^{2+} release from the vesicles, but Ins(1,3,4,5) P_4 -mediated Ca^{2+} sequestration remained unaffected. During the course of incubation in the presence of heparin, Ca^{2+} concentration in the media rose steadily, probably owing to heparin-induced Ca^{2+} leakage from the vesicles, reaching about 1.8 μM after 20 min of incubation. However, it was found in parallel experiments that this level of Ca^{2+} alone does not inhibit Ins(1,4,5) P_3 -induced Ca^{2+} release from vesicles, consistent with other reports [4,13]. This result shows that Ins(1,3,4,5) P_4 and Ins(1,4,5) P_3 bind to different receptors.

In order to determine whether the Ca^{2+} -sequestering activity of Ins(1,3,4,5) P_4 can also be demonstrated with $^{45}Ca^{2+}$, secretory vesicles were incubated in the presence of 1.5 μM free $^{45}Ca^{2+}$, and the effect of Ins(1,3,4,5) P_4 on $^{45}Ca^{2+}$ uptake was measured. As shown in Fig. 6(a), Ins(1,3,4,5) P_4 induced a rapid uptake of

Fig. 6. $^{45}Ca^{2+}$ uptake and release in response to Ins(1,3,4,5) P_4 and Ins(1,4,5) P_3 by bovine adrenal chromaffin vesicles

$^{45}Ca^{2+}$ uptake into secretory vesicles was measured in the presence of Ins(1,3,4,5) P_4 , Ins(1,4,5) P_3 and A23187. (a) Effect of Ins(1,3,4,5) P_4 on Ca^{2+} uptake. Purified vesicles (0.5 mg/ml) were incubated at 24 $^{\circ}C$ in the presence of 1.5 μM free $^{45}Ca^{2+}$. At the indicated time, 10 μM -Ins(1,3,4,5) P_4 (Ins P_4 ; $\blacktriangle$) was added to the uptake mixture (see the Materials and methods section for details). Data are representative of experiments repeated 4 times with similar results. (b) Effect of Ins(1,4,5) P_3 and A23187 on Ca^{2+} uptake. Purified vesicles (0.5 mg/ml) were incubated at 37 $^{\circ}C$ in the presence of 1.5 μM free $^{45}Ca^{2+}$. At the indicated times, 1 μM -A23187 ($\bullet$), 10 μM -Ins(1,4,5) P_3 (Ins P_3 ; $\blacktriangle$) and 2.5 μM -A23187 ($\blacksquare$) were added to the uptake mixture. Data are representative of experiments repeated 4 times with similar results.

$^{45}Ca^{2+}$ from the medium, despite the high intravesicular Ca^{2+} concentration, confirming the Ca^{2+} -selective-electrode data. Ca^{2+} uptake into the secretory vesicles against a concentration gradient has also been observed with A23187 under similar conditions to Fig. 6(a) while Ins(1,4,5) P_3 mediated Ca^{2+} release (Fig. 6b). Although the secretory vesicles contain large amounts of Ca^{2+} , almost all (more than 99.9%) of the Ca^{2+} stays bound to the intravesicular matrix, including chromogranin A [15], so that accumulated $^{45}Ca^{2+}$ ions do not freely equilibrate with the intravesicular Ca^{2+} . The effect of A23187 became more prominent as the concentration of ionophore was increased from 1 μM to 2.5 μM .

DISCUSSION

Unlike the well-established Ca^{2+} -mobilizing role of Ins(1,4,5) P_3 , the potential second-messenger role of Ins(1,3,4,5) P_4 in the control of $[Ca^{2+}]_i$ has yet to be established. One of the difficulties of finding a possible role of Ins(1,3,4,5) P_4 in the control of $[Ca^{2+}]_i$ was the lack of good model system. We have

recently identified secretory vesicles of adrenal medulla as the major $\text{Ins}(1,4,5)\text{P}_3$ -sensitive intracellular Ca^{2+} store of chromaffin cells [14], thereby allowing us to study specific effects of various inositol phosphates on the Ca^{2+} flux of intracellular store directly.

In the present system a strict stereospecific requirement has clearly been demonstrated by the contrasting effects of $\text{Ins}(1,3,4,5)\text{P}_4$ and $\text{Ins}(1,4,5,6)\text{P}_4$ (Fig. 1). Despite the presence of phosphate groups on the 1-, 4- and 5-positions, an additional phosphate on the 3- or 6-position drastically changed the effect of these inositol phosphates. Of all the inositol phosphates tested, including $\text{Ins}1\text{P}$, $\text{Ins}(1,4)\text{P}_2$, $\text{Ins}(1,4,5\text{-CH}_2\text{-})\text{P}_3$ [an $\text{Ins}(1,4,5)\text{P}_3$ analogue in which the oxygen atom linking the inositol ring to 5-phosphate is replaced with a methylene group; S. H. Yoo, unpublished work] and InsP_5 , for their effects on the Ca^{2+} flux of secretory vesicles, $\text{Ins}(1,3,4,5)\text{P}_4$ was the only agent that elicited Ca^{2+} uptake; all the others were either totally ineffective or induced Ca^{2+} release. Addition of $\text{Ins}(1,3,4,5)\text{P}_4$ to the incubation media which did not contain secretory vesicles (Buffer I only) did not affect the reading of the Ca^{2+} -selective electrode, eliminating any artefactual contribution to the Ca^{2+} signal by $\text{Ins}(1,3,4,5)\text{P}_4$. Therefore the Ca^{2+} -sequestering activity of $\text{Ins}(1,3,4,5)\text{P}_4$ is assumed to be the genuine effect of the inositol phosphate.

The transient nature of $\text{Ins}(1,3,4,5)\text{P}_4$ -induced Ca^{2+} influx, i.e. a rapid uptake followed by a slow return to the pre-uptake level, in the low- Ca^{2+} media implies that $\text{Ins}(1,3,4,5)\text{P}_4$ opens Ca^{2+} channels. Furthermore, the effect of $\text{Ins}(1,3,4,5)\text{P}_4$ on sequestration of Ca^{2+} into vesicles was not affected by temperature, and was as effective at 4 °C as at 24 °C (Fig. 3), implying either that $\text{Ins}(1,3,4,5)\text{P}_4$ sequesters Ca^{2+} through receptor-operated Ca^{2+} channels or that $\text{Ins}(1,3,4,5)\text{P}_4$ activates some sort of Ca^{2+} -exchange mechanism, analogous to that for A23187. However, unlike A23187-mediated $\text{Ca}^{2+}/\text{H}^+$ exchange, $\text{Ins}(1,3,4,5)\text{P}_4$ did not mediate $\text{Ca}^{2+}/\text{H}^+$ exchange in the vesicles (results not shown). In light of the fact that there is a very active $\text{Na}^+/\text{Ca}^{2+}$ exchange mechanism in the secretory vesicles of chromaffin cells [20–22], there exists a possibility that $\text{Ins}(1,3,4,5)\text{P}_4$ activates a $\text{Na}^+/\text{Ca}^{2+}$ exchange mechanism in the vesicles. Nevertheless, the effect of $\text{Ins}(1,3,4,5)\text{P}_4$ on Ca^{2+} sequestration remained the same even in the presence of 2–5 mM- Na^+ in the media.

The contrasting effects of $\text{Ins}(1,4,5)\text{P}_3$ and $\text{Ins}(1,3,4,5)\text{P}_4$ on the Ca^{2+} flux of vesicles clearly demonstrate two independent effects of $\text{Ins}(1,4,5)\text{P}_3$ and $\text{Ins}(1,3,4,5)\text{P}_4$, eliminating uncertainties regarding conversion of $\text{Ins}(1,3,4,5)\text{P}_4$ into $\text{Ins}(1,4,5)\text{P}_3$ and vice versa during experiments involving whole cells or cellular extracts. The Ca^{2+} -sequestering property has not been observed with nucleotides such as ATP, GTP, UTP, CTP and TTP; it was only obtained with $\text{Ins}(1,3,4,5)\text{P}_4$ in both vesicles and microsomes of adrenal medulla.

Recently Ely *et al.* [9] reported $\text{Ins}(1,3,4,5)\text{P}_4$ -induced Ca^{2+} release from crude microsomal fractions of bovine adrenal medulla (adrenal particulate). Owing to the crude nature of the adrenal particulate preparation used in the study, it is not possible to compare their results directly even with the microsome results of the present study. Nevertheless, it appeared to me that some of the experimental conditions in which the experiments had been carried out might have led to the observed Ca^{2+} release. For example, the effect of $\text{Ins}(1,3,4,5)\text{P}_4$ on Ca^{2+} flux was measured in the extremely-low- Ca^{2+} (as low as 20 nM) environment, thereby favouring Ca^{2+} efflux to the media when Ca^{2+} channels were opened by $\text{Ins}(1,3,4,5)\text{P}_4$. A similar result has been observed previously with secretory vesicles of bovine adrenal medulla, where addition of A23187 to the vesicles in a low- Ca^{2+} environment caused release of Ca^{2+} from the vesicles [15], contrary to the usual A23187-induced Ca^{2+} uptake into secretory vesicles. In addition, it was reported that 3% of $\text{Ins}(1,3,4,5)\text{P}_4$

was metabolized to $\text{Ins}(1,4,5)\text{P}_3$ within seconds of its addition to the adrenal particulate preparation. Since the $\text{Ins}(1,3,4,5)\text{P}_4$ concentration used in those experiments was 20 μM , it is estimated that approx. 0.6 μM - $\text{Ins}(1,4,5)\text{P}_3$ is available to Ca^{2+} stores within seconds of $\text{Ins}(1,3,4,5)\text{P}_4$ introduction, which is a high enough concentration to induce Ca^{2+} release. In fact, it has previously been noticed that $\text{Ins}(1,3,4,5)\text{P}_4$ added to permeabilized mouse lymphoma cells caused release of Ca^{2+} by its conversion into $\text{Ins}(1,4,5)\text{P}_3$ [23].

$\text{Ins}(1,3,4,5)\text{P}_4$ -induced Ca^{2+} uptake was also demonstrated with $^{45}\text{CaCl}_2$, in agreement with the Ca^{2+} -electrode data. $\text{Ins}(1,4,5)\text{P}_3$ induced release of $^{45}\text{Ca}^{2+}$ in the vesicles optimized for $^{45}\text{Ca}^{2+}$ uptake, whereas A23187 caused $^{45}\text{Ca}^{2+}$ uptake, consistent with all the previous results [14,15]. In view of the fact that attempts to demonstrate the Ca^{2+} -releasing effect of $\text{Ins}(1,3,4,5)\text{P}_4$ utilizing permeabilized whole cells such as rat hepatocytes [24] and neutrophils [25] have not been successful, it appears that $\text{Ins}(1,3,4,5)\text{P}_4$ has a role clearly distinguishable from the Ca^{2+} -releasing role of $\text{Ins}(1,4,5)\text{P}_3$. The Ca^{2+} -sequestering effect of $\text{Ins}(1,3,4,5)\text{P}_4$ has been previously reported in permeabilized rat liver epithelial cells by Hill *et al.* [13,26]. The present results are in agreement with those of Hill *et al.* [13,26] and point to the role of $\text{Ins}(1,3,4,5)\text{P}_4$ as a Ca^{2+} -sequestering messenger. In this regard, there remains a possibility that the synergistic effects of $\text{Ins}(1,3,4,5)\text{P}_4$ and $\text{Ins}(1,4,5)\text{P}_3$ to control cytosolic Ca^{2+} concentration [2] might in fact be the results of $\text{Ins}(1,3,4,5)\text{P}_4$ -induced Ca^{2+} sequestration into intracellular Ca^{2+} stores and $\text{Ins}(1,4,5)\text{P}_3$ -induced Ca^{2+} release from the stores.

Although $\text{Ins}(1,4,5)\text{P}_3$ and $\text{Ins}(1,3,4,5)\text{P}_4$ were known to undergo rapid metabolism in whole cells, with half-lives of 7.6 s [27] and 15–43 s [6,27] respectively, this has not been studied with an isolated intact $\text{Ins}(1,4,5)\text{P}_3$ -sensitive Ca^{2+} store. My preliminary h.p.l.c. analysis, on a SAX column, of [^3H] $\text{Ins}(1,3,4,5)\text{P}_4$ which had been recovered from the vesicle-containing media after 2 min of incubation showed that there were no detectable metabolites of [^3H] $\text{Ins}(1,3,4,5)\text{P}_4$ (S. H. Yoo, unpublished work). Lack of metabolism has also been observed for $\text{Ins}(1,4,5)\text{P}_3$ in the identical experiments in which there was neither a decrease in the total concentration of $\text{Ins}(1,4,5)\text{P}_3$ nor any detectable metabolites of [^3H] $\text{Ins}(1,4,5)\text{P}_3$ (S. H. Yoo, unpublished work). In this regard, it is not clear why a second addition of $\text{Ins}(1,4,5)\text{P}_3$ induces a new round of Ca^{2+} release while there is still enough $\text{Ins}(1,4,5)\text{P}_3$ present. It may be that the $\text{Ins}(1,4,5)\text{P}_3$ receptor exists in several different affinity states for $\text{Ins}(1,4,5)\text{P}_3$. In other words, the initial affinity of the $\text{Ins}(1,4,5)\text{P}_3$ receptor for $\text{Ins}(1,4,5)\text{P}_3$ might be changed to a lower affinity after the first round of opening and closure in response to the first addition of $\text{Ins}(1,4,5)\text{P}_3$, thereby rendering the receptor unresponsive to the existing $\text{Ins}(1,4,5)\text{P}_3$ until more $\text{Ins}(1,4,5)\text{P}_3$, in the form of second addition, is introduced. It is very conceivable that the altered affinity of $\text{Ins}(1,4,5)\text{P}_3$ for its agonist might gradually revert back to its original affinity state as the surrounding $\text{Ins}(1,4,5)\text{P}_3$ concentration diminishes.

The high degree of structural selectivity demonstrated by the contrasting effects of $\text{Ins}(1,3,4,5)\text{P}_4$ and $\text{Ins}(1,4,5)\text{P}_3$ or $\text{Ins}(1,4,5,6)\text{P}_4$ on the Ca^{2+} fluxes of secretory vesicles appears to reflect a delicate and fine-tuned control mechanism for the cellular homeostasis of Ca^{2+} in chromaffin cells.

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