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Inositol 1,4,5-trisphosphate and 5′-GTP induce calcium release from different intracellular pools

Volkmar Henne, Albrecht Piiper and Hans-Dieter Söling

Abteilung Klin. Biochemie, Universität Göttingen, Robert-Koch-Str. 40, D-3400 Göttingen, FRG

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It has been shown recently by several groups that 5′-GTP can release calcium from intracellular compartments independently from inositol 1,4,5-trisphosphate ($Ins(1,4,5)P_3$) by a mechanism which seems to be different from that used by $Ins(1,4,5)P_3$. We report here for the first time that the 5′-GTP-sensitive and the $Ins(1,4,5)P_3$-sensitive calcium pools reside in different intracellular compartments.

Ca^{2+} release; GTP; Inositol 1,4,5-trisphosphate; Endoplasmic reticulum; (Parotid)

1. INTRODUCTION

Receptor-mediated release of calcium from intracellular pools by inositol 1,4,5-trisphosphate ($Ins(1,4,5)P_3$) has been shown to occur in many different cell types. More recently, it has been reported that 5′-GTP can also stimulate the release of endogenous calcium. This has been shown for permeabilized cells [1,2] as well as for isolated microsomal fractions [3,4]. Both 5′-GTP and $Ins(1,4,5)P_3$ release calcium from intracellular compartments which accumulate calcium by an ATP-driven calcium pump [3]. 5′-GTP-mediated calcium release exhibits significant kinetic differences from that mediated by $Ins(1,4,5)P_3$. Thus, 5′-GTP-mediated calcium release is much more temperature-dependent and affected more greatly by the intravesicular concentration of free calcium [3].

Up to now it was not clear whether 5′-GTP releases calcium from the same compartment as $Ins(1,4,5)P_3$ but by a different mechanism, or whether the calcium released by 5′-GTP came from a different cellular compartment. We show here for the first time that the 5′-GTP-sensitive and $Ins(1,4,5)P_3$-sensitive calcium pools reside in different intracellular compartments, which seem to represent different functional domains of the endoplasmic reticulum.

Correspondence address: H.-D. Söling, Abteilung Klin. Biochemie, Universität Göttingen, Robert-Koch-Str. 40, D-3400 Göttingen, FRG

2. MATERIALS AND METHODS

Guinea pigs of the Pirbright White strain (200–250 g body wt) were obtained from Winkelmann (Dernbach, FRG). 5′-GTP (type III) was obtained from Sigma (Munich) and $Ins(1,4,5)P_3$ from Amersham Buchler (Braunschweig). All other biochemicals were from Boehringer Mannheim. Percoll was from Deutsche Pharmacia (Freiburg/Brg). All other chemicals (analytical grade) came from Merck (Darmstadt).

Guinea pig parotid gland acini were prepared as described [5]. For subcellular fractionation the acinar cells were washed 3 times in medium A (130 mM KCl, 2 mM $MgCl_2$, 2 mM K phosphate, 1 mM dithiothreitol, 1 mM benzamidine, 20 mM Hepes-KOH, pH 7.4). They were homogenized in a glass/glass Potter homogenizer. The homogenate was spun at 1000 × *g* for 10 min. The supernatant

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was used for subfractionation by either a Percoll gradient or sucrose gradient centrifugation. Percoll gradient centrifugation was carried out as follows: 5 ml of the 1000 × g supernatant were mixed with 10 ml medium A containing 40% (v/v) Percoll and spun for 20 min at 50000 × g_{av}. Starting from the top, seven fractions of about 2.1 ml were collected, each one being diluted with 8 ml medium A and spun for 30 min at 50000 × g_{av}. The pellets were resuspended in 0.4 ml medium A and used for determination of Ins(1,4,5)P_3- and 5′-GTP-mediated calcium release in the presence of 3% (w/v) polyethylene glycol (M_r 8000) as in [3]. Free calcium was determined with a calcium-sensitive electrode as in [3]. For sucrose gradient centrifugation, the 1000 × g supernatant was first spun for 10 min at 12000 × g_{av}. The pellet was resuspended in 1.3 ml of 0.3 M sucrose and 1 ml layered on top of a discontinuous gradient consisting of 1 ml of 2 M sucrose and 1.5 ml each of 1.4 and 1.1 M sucrose, and spun for 60 min at 370000 × g_{max} in a Ti SW65 swing-out rotor (Beckman Instruments). The membrane fractions banding at the 2 M/1.4 M sucrose interface (= IF3), at the 1.4 M/1.1 M sucrose interface (= IF2), and above the 1.1 M sucrose layer (= IF1) were removed with Pasteur pipettes and used for the determination of marker enzymes and Ins(1,4,5)P_3- and 5′-GTP-mediated calcium release. Aliquots from IF1 and IF3 were collected by centrifugation and fixed with Karnovsky solution. The fixed samples were contrasted with uranyl acetate and lead citrate, and embedded in Epon. Electron micrographs were taken on a Zeiss EM 9 electron microscope.

For measuring Ins(1,4,5)P_3- or 5′-GTP-mediated calcium release, the subcellular fractions were first loaded with calcium in the following medium: 130 mM KCl, 2 mM $MgCl_2$, 2 mM K phosphate, 20 mM Hepes-KOH (pH 7.0), 2.5 mM ATP, 5 mM creatine phosphate, 40 U/ml creatine kinase, 1 mM benzamidine, 3% (w/v) polyethylene glycol (M_r 8000), 10 mM NaN_3, and 10 μg/ml each of antimycin A and oligomycin.

Marker enzyme activities, protein and RNA were determined by standard procedures (alkaline phosphodiesterase [6], succinic dehydrogenase [7], rotenone-insensitive NADH-cytochrome c reductase [8], UDP-galactosyltransferase [9], protein [10], RNA [11]).

3. RESULTS AND DISCUSSION

3.1. *Percoll gradient experiments*

Ins(1,4,5)P_3- and 5′-GTP-mediated calcium release was analyzed in the subcellular fractions obtained from the Percoll gradient after loading of the vesicles with calcium in the presence of ATP and Mg^{2+} as given in section 2. Calcium release was initiated when a steady state of free calcium had been reached (fig.1). Most of the Ins(1,4,5)-P_3-releasable calcium pool was associated with fractions having a low density and containing most of the plasma membranes (figs 1,2) as indicated by the activity of alkaline phosphodiesterase: in going from the low to the higher density fractions,

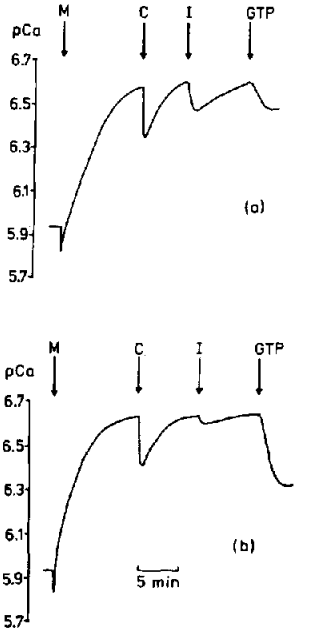

Fig.1. Ins(1,4,5)P_3- and 5′-GTP-induced calcium release from low (a) and high (b) density vesicles isolated from a post-nuclear fraction of guinea pig parotid gland acinar cells by Percoll-density gradient centrifugation. Acinar cells were prepared and used for subcellular fractionation by Percoll gradient centrifugation as given in section 2. The vesicles shown in panel a were taken from fraction 1 (lowest density) and those used in panel b were from fraction 7 (highest density) from the same gradient. M, addition of membrane vesicles (250 and 200 μg protein/ml in a and b, respectively); C, addition of 5 nmol $CaCl_2$; I, addition of 2.5 nmol Ins(1,4,5)P_3; GTP, addition of 5 nmol 5′-GTP. The total volume was 1 ml and the temperature, 37°C.

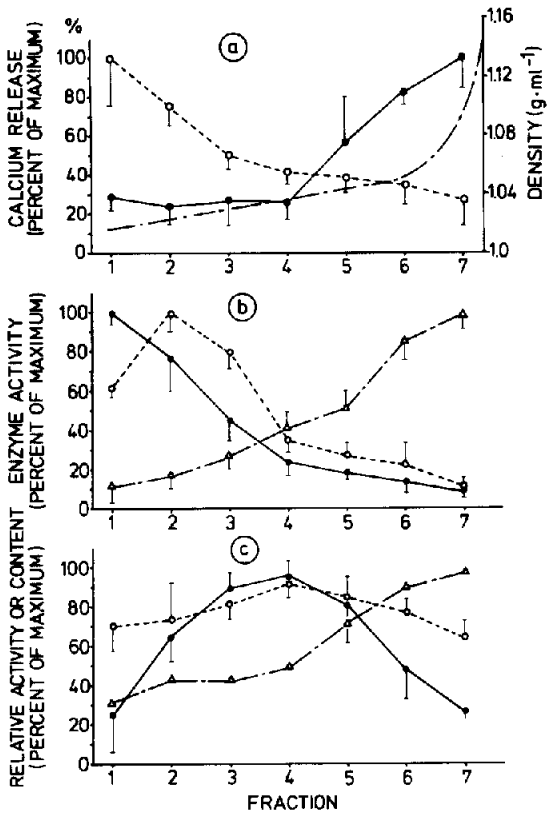

Fig.2. Density distribution of Ins(1,4,5)P_3- and 5′-GTP-releasable calcium pools and of marker enzymes in Percoll gradients from post-nuclear supernatants of guinea pig parotid acinar cells. The experiments were performed as given in section 2. The initial velocity of ATP-driven calcium uptake was monitored with a calcium-selective electrode. (a) Ins(1,4,5)P_3-mediated (○---○) and 5′-GTP-mediated (●——●) calcium release. The fractions showing maximal release were set to 100%. The maximal release mediated by Ins(1,4,5)P_3 was 12.7 ± 3.0 nmol/mg protein, the value for 5′-GTP being 27.4 ± 4.2 nmol/mg protein. The dashed line without symbols indicates the density along the gradient. (b) Distribution of specific activities of alkaline phosphodiesterase (●——●), galactosyltransferase (○---○) and succinic dehydrogenase (△——△). Same experiments as in panel a. The highest specific activities were set to 100% and the activities in the other fractions related to these values. The maximal activities were 158 nmol·mg protein^{-1}·min^{-1}, 2.2 nmol·mg protein^{-1}·h^{-1} and 40.8 nmol·mg protein^{-1}·min^{-1} for alkaline phosphodiesterase, galactosyltransferase and succinic dehydrogenase, respectively. (c) Initial rate of ATP-dependent calcium uptake (●——●) and rotenone-insensitive NADH-cytochrome *c* reductase (○---○), and RNA content (△——△). All values were related to the maximum value obtained in each single experiment which was set to 100%. The maximal initial calcium uptake rate was 101 nmol·mg protein^{-1}·min^{-1}, the maximal activity of NADH-cytochrome *c* reductase 98 nmol·mg protein^{-1}·min^{-1} and the maximal RNA content 148 μg·mg protein^{-1}. Values given are means ± SD from 6 experiments, except for RNA which was only measured in 2 experiments.

Ins(1,4,5)P_3-mediated calcium release decreased almost in parallel with the activity of alkaline phosphodiesterase (fig.2a). Ins(1,4,5)P_3-mediated calcium release from the fraction of highest density was only about 25% of that obtained with the fraction having the lowest density. Calcium release mediated by 5′-GTP behaved in the opposite way: maximal calcium release occurred from the most dense fraction, whereas 5′-GTP-mediated calcium release from the first 3–4 low density fractions was about 75% less (fig.2a). The fractions showing the highest response to 5′-GTP contained most of the mitochondria as indicated by the activity distribution of succinic dehydrogenase (fig.2b). They also contained most of the RNA, and substantial activities of NADH-cytochrome *c* reductase indicating a high content of rough endoplasmic reticulum (fig.2c). Ins(1,4,5)P_3- as well as 5′-GTP-mediated calcium release required previous calcium loading in the presence of ATP and Mg^{2+}. Moreover, the experiments were performed in the presence of 10 mM NaN_3 which is sufficiently high to block any interference with mitochondrial calcium metabolism. As indicated by the distribution of galactosyltransferase (fig.2b), the Ins(1,4,5)P_3-sensitive compartment does not seem to be Golgi vesicles. Furthermore, experiments with isolated Golgi vesicles from both parotid and liver indicate the complete absence of ATP-driven calcium uptake in this compartment. The same is the case for lysosomes which accumulate together with mitochondria in the most dense fractions (Piiper, A. and Söling, H.-D., unpublished). It seems likely, therefore, that the Ins(1,4,5)P_3-sensitive calcium pool resides in a section of the endoplasmic reticulum which is either of the same density as or tightly associated with the plasma membranes.

The 5′-GTP-releasable calcium, on the other hand, seems to reside in the rough endoplasmic reticulum or a specialized fraction of it.

3.2. *Sucrose gradient experiments*

The experiments performed with sucrose gradients confirmed the results obtained with Percoll gradient centrifugation, indicating that the separation of a preferentially 5′-GTP-sensitive compartment from the Ins(1,4,5)P_3-sensitive compartment was not an artifact resulting from the Percoll. Sucrose gradient centrifugation led to the separation of 3 distinct membrane fractions (IF1–IF3) (table 1 and fig.3). Again the fraction with the lowest density had the highest activity of the plasma membrane marker alkaline phosphodiesterase and the largest pool of Ins(1,4,5)P_3-releasable calcium, whereas 5′-GTP-mediated calcium release was measured mainly in IF3, the fraction with the highest density and the highest RNA content (table 1). Electron microscopy reveals that IF3 indeed represents mainly rough endoplasmic reticulum, whereas IF1 contains mostly plasma membrane vesicles, smooth endoplasmic reticulum vesicles and Golgi cisternae (fig.3). Experiments with skeletal muscle sarcoplasmic reticulum have shown that the distribution of the activity of ATP-driven calcium uptake capacity was different from that of calcium release activity. While most of the releasable calcium resided in the 'heavy' sarcoplasmic reticulum, the maximal activity of ATP-driven calcium uptake was measured in the 'light' sarcoplasmic reticulum [12]. As depicted in fig.2c, membrane vesicles from parotid gland acinar cells also exhibited a dissociation between calcium uptake activity and calcium release. The highest activities of ATP-driven calcium uptake were found in the Percoll fractions of intermediate density and not in the top or bottom fractions which showed the highest calcium release mediated by Ins(1,4,5)P_3 and 5′-GTP, respectively.

Table 1

Distribution of Ins(1,4,5)P_3- and 5′-GTP-releasable calcium pools, marker enzymes, and RNA between membrane fractions from guinea pig parotid acinar cells separated by sucrose density gradient centrifugation as in fig.3

Parameter analyzed	Fraction		
	IF1	IF2	IF3
Ins(1,4,5)P_3-releasable calcium	100 ± 10	21 ± 5	22 ± 3
5′-GTP-releasable calcium	22 ± 7	44 ± 20	100 ± 15
Initial calcium uptake	28 ± 7	89 ± 14	100 ± 2
Alkaline phosphodiesterase	100 ± 4	28 ± 6	10 ± 1
Galactosyltransferase	100 ± 25	7 ± 3	5 ± 2
Rotenone-insensitive NADH-cytochrome *c* reductase	45 ± 10	94 ± 15	87 ± 16
Succinic dehydrogenase	9 ± 2	71 ± 8	100 ± 5
RNA	14 ± 6	46 ± 30	100 ± 21

The different parameters were determined as given in section 2. All values are expressed as percent. The fractions exhibiting the highest activity or amount were set to 100%. The mean absolute values were as follows: Ins(1,4,5)P_3-mediated calcium release, 16 nmol/mg protein; 5′-GTP-mediated calcium release, 41 nmol/mg protein; initial calcium uptake, 70 nmol/mg protein per min; alkaline phosphodiesterase, 152 mU/mg protein; galactosyltransferase, 3.3 nmol/mg per h; rotenone-insensitive NADH-cytochrome *c* reductase, 110 mU/mg protein; succinic dehydrogenase, 51 mU/mg protein; RNA, 71 μg/mg protein. Mean values ± SD; $n = 6$

It appears, therefore, that Ins(1,4,5)P_3 and 5′-GTP differ not only with respect to the mechanism by which they induce calcium efflux from internal stores [3], but also with respect to the pools from which they release calcium. The finding that the Ins(1,4,5)P_3-sensitive calcium pool was recovered together with the plasma membrane fraction would be in line with the concept that Ins(1,4,5)P_3 formed in the plasma membrane by receptor-induced activation of a specific phospholipase C gains access to a portion of the endoplasmic reticulum which is in close apposition to the plasma membrane. The 5′-GTP-sensitive calcium pool on the other hand, appears to be located in a subfraction of the rough endoplasmic reticulum. The physiological importance of 5′-GTP-mediated calcium release is still a matter of debate as – in contrast to Ins(1,4,5)P_3-mediated calcium release – it requires the presence of polyethylene glycol [1–4]. This argument however may be of minor importance as we have recently shown that 5′-GTP also exerts its effect when polyethylene glycol is replaced by bovine serum

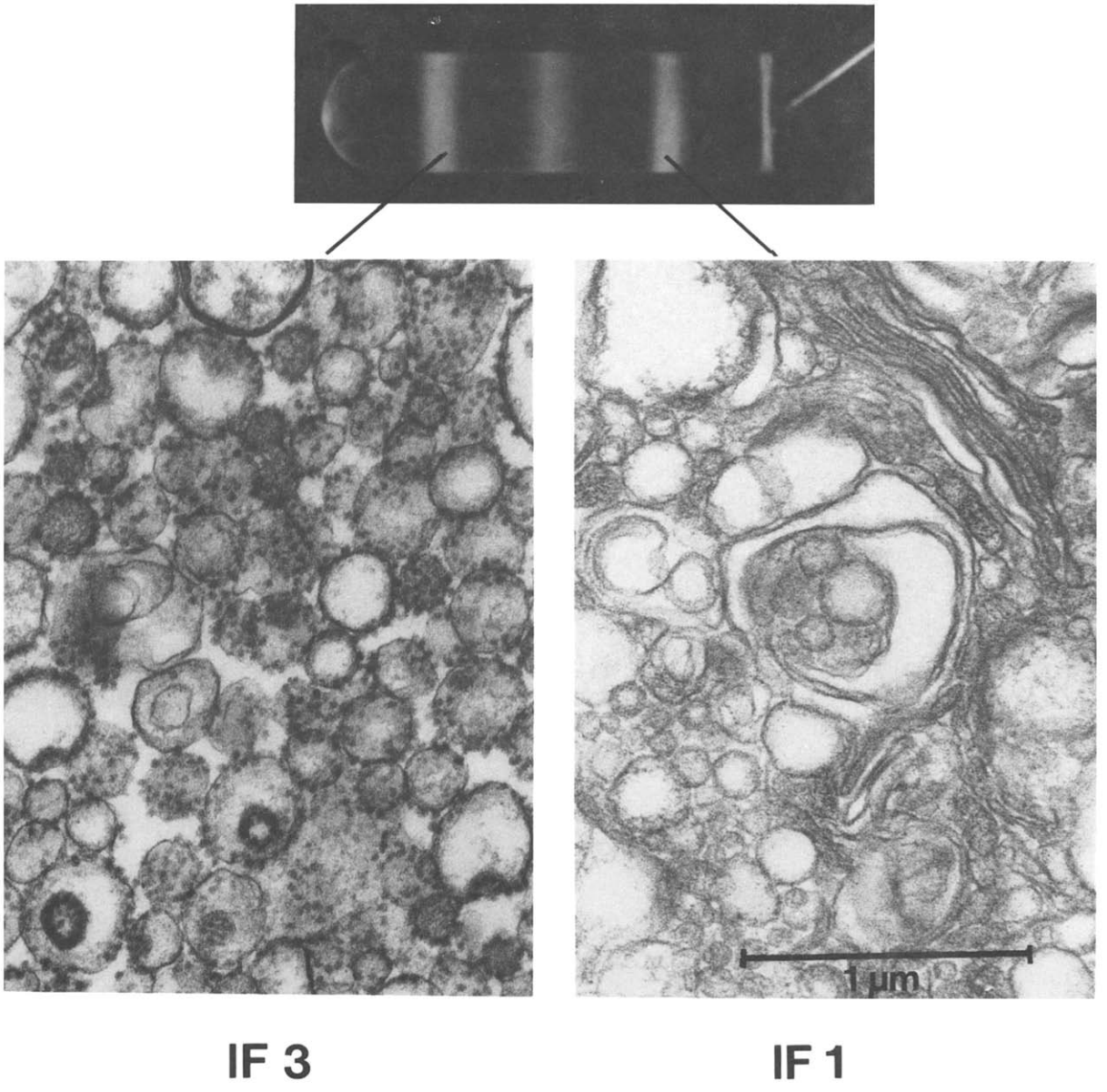

Fig.3. IF1 and IF3 membranes from guinea pig parotid acinar cells separated by sucrose gradient centrifugation.

albumin [3]. Calcium release from the 5′-GTP-sensitive pool may therefore be involved in specific functions different from those controlled by calcium released from the Ins(1,4,5)P_3-sensitive calcium pool.

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