Evidence that a low-molecular-mass GTP-binding protein is required for store-activated Ca^{2+} inflow in hepatocytes

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The roles of a monomeric GTP-binding regulatory protein in the activation of store-activated plasma membrane Ca^{2+} channels and in the release of Ca^{2+} from the smooth endoplasmic reticulum (SER) in rat liver parenchymal cells were investigated with the use of freshly isolated rat hepatocytes and rat liver microsomes. A low concentration (approx. 130 μM intracellular) of guanosine 5'-[γ -thio]triphosphate (GTP[S]) activated Ca^{2+} inflow in intact hepatocytes in the absence of an agonist, whereas a high concentration (approx. 530 μM intracellular) of GTP[S] or guanosine 5'-[β -imido]triphosphate (p[NH]ppG) inhibited the Ca^{2+} inflow induced by inhibitors of the activity of the endoplasmic-reticulum Ca^{2+} -ATPase (SERCA) and by vasopressin. GTP (530 μM) prevented the inhibition of Ca^{2+} inflow by GTP[S] and p[NH]ppG. Brefeldin A and the peptide human Arf-1-(2–17), which inhibit many functions of ADP ribosylation factor (Arf) proteins, inhibited the Ca^{2+} inflow induced by SERCA inhibitors and vasopressin, and altered the profile of Ca^{2+} release

from the SER. These effects were observed at concentrations of Brefeldin A and Arf-1-(2–17) comparable with those that inhibit the functions of Arf proteins in other systems. Succinylated Arf-1-(2–17) had a negligible effect on Ca^{2+} inflow. GTP[S] and Arf-1-(2–17) completely inhibited the synergistic action of GTP and $\text{Ins}(1,4,5)\text{P}_3$ in releasing $^{45}\text{Ca}^{2+}$ from rat liver microsomes loaded with $^{45}\text{Ca}^{2+}$. AlF_4^- (under conditions expected to activate trimeric G-proteins) and succinylated Arf-1-(2–17) had no effect on GTP/ $\text{Ins}(1,4,5)\text{P}_3$ -induced $^{45}\text{Ca}^{2+}$ release, and a mastoparan analogue caused partial inhibition. Arf-1-(2–17) did not inhibit $^{45}\text{Ca}^{2+}$ release induced by either thapsigargin or ionomycin. It is concluded that a low-molecular-mass G-protein, most probably a member of the Arf protein family, is required for store-activated Ca^{2+} inflow in rat hepatocytes. The idea that the role of this G-protein is to maintain a region of the SER in the correct intracellular location is discussed briefly.

INTRODUCTION

Receptor-activated Ca^{2+} channels in the plasma membrane have a major role in the regulation by extracellular signals of the functions of 'non-excitable' and some 'excitable' animal cells [1]. In rat hepatocytes, extracellular signals that activate receptor-activated Ca^{2+} channels include vasopressin, α_1 -adrenergic agonists, angiotensin II and epidermal growth factor (EGF) [2–11]. There are several types of receptor-activated Ca^{2+} channel, which most probably serve different physiological functions. These are the store-activated Ca^{2+} channels (SACCs), which are activated by the release of Ca^{2+} from a region of the smooth endoplasmic reticulum (SER) and comprise the Ca^{2+} -specific Ca^{2+} -release-activated Ca^{2+} channels as well as non-specific cation channels; second-messenger-activated non-specific cation channels; and ligand-gated non-specific cation channels [12,13]. SACCs can be activated artificially by agents such as thapsigargin, 2,5-di-*t*-butylhydroquinone (DBHQ) and cyclopiazonic acid (CPA), which inhibit the endoplasmic-reticulum Ca^{2+} -ATPase (SERCA) and thus induce the release of Ca^{2+} from the SER (reviewed in [14]).

The mechanisms of activation of SACCs are not well understood. At present there are two main hypotheses: (1) a conformational coupling hypothesis, which proposes the direct

interaction of one or more proteins in the SER with the plasma membrane SACC protein [12,13,15,16], and (2) an intracellular messenger hypothesis, which proposes that a mobile intracellular messenger, such as calcium influx factor, is generated by the Ca^{2+} -depleted SER, diffuses through the cytoplasmic space and activates the SACCs [17].

We have previously obtained evidence that the trimeric GTP-binding regulatory protein (G-protein) G_{i2} is required for the activation of hepatocyte SACCs [18–21]. Experiments with some other cell types have also implicated G_{i2} , or another trimeric G-protein, in the activation of both receptor-activated [22–26] and voltage-operated [27] Ca^{2+} channels. Studies with mouse lacrimal acinar cells and rat basophilic leukaemic cells have suggested the involvement of a monomeric G-protein in store-activated Ca^{2+} inflow [28,29]. It was proposed that the putative monomeric G-protein in some way mediates the fusion of membranes of the SER with the plasma membrane, or an interaction between these membranes, a step thought to be required for the activation of SACCs [13,29]. Another process that is thought to involve the fusion of regions of the SER membrane and to require a monomeric G-protein has been observed in isolated rat liver microsomes [30] and in permeabilized smooth muscle cells [31]. This is the enhancement by GTP of the $\text{Ins}(1,4,5)\text{P}_3$ -induced release of Ca^{2+} from microsomes or the SER.

Abbreviations used: Arf, ADP ribosylation factor; CPA, cyclopiazonic acid; DBHQ, 2,5-di-*t*-butylhydroquinone; EGF, epidermal growth factor; $\text{G}_{i2\alpha}$ peptide, $\text{G}_{i2\alpha}$ -(345–355); $\text{G}_{i3\alpha}$ peptide, $\text{G}_{i3\alpha}$ -(344–354); GTP[S], guanosine 5'-[γ -thio]triphosphate; IGF-II, insulin-like growth factor II; p[NH]ppG, guanosine 5'-[β -imido]triphosphate; SACC, store-activated Ca^{2+} channel; SER, smooth endoplasmic reticulum; SERCA, endoplasmic-reticulum Ca^{2+} -ATPase.

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The aim of the present experiments was to investigate the role of a monomeric G-protein in the activation of SACCs in rat hepatocytes. Initially we used guanosine 5'-[γ -thio]triphosphate (GTP[S]) as a probe. GTP[S] activates trimeric G-proteins [32] and many monomeric G-proteins [33–35] and inhibits some functions of the ADP ribosylation factor (Arf) monomeric G-proteins [34,35]. We found that GTP[S] inhibited store-activated Ca^{2+} inflow in isolated rat hepatocytes in a manner that could be prevented by GTP. This observation suggested a requirement for (1) a monomeric G-protein and (2) the hydrolysis of GTP in the mechanism of activation of SACCs in hepatocytes. Because Arf proteins are the only known monomeric G-proteins, the function of which is inhibited by GTP[S] [34,35], we investigated the role of an Arf protein in store-activated Ca^{2+} inflow. Intracellular processes that require the normal functioning of an Arf protein include the activation of cholera toxin and phospholipase D, the addition of coat proteins to Golgi and endosome membranes, vesicle budding and fusion, and endocytosis [36]. As we thought that the molecular events that underlie the enhancement by GTP of $\text{Ins}(1,4,5)\text{P}_3$ -induced release of Ca^{2+} from isolated liver microsomes [30] might also account for the requirement for a monomeric G-protein in the activation of SACCs, we also investigated the role of an Arf protein in the $\text{Ins}(1,4,5)\text{P}_3$ /GTP-induced release of $^{45}\text{Ca}^{2+}$ from microsomes.

Elucidation of the role of an Arf protein in a given intracellular process is difficult because the reagents available for intervention in Arf pathways are somewhat limited [36]. Two reagents that have been employed are Brefeldin A and the peptide human Arf-1-(2–17), which corresponds to the N-terminus of the hArf-1 protein. Brefeldin A inhibits the activity of a GDP:GTP exchange protein that catalyses the exchange of Arf-bound GDP for GTP to form activated Arf·GTP [37,38]. Arf-1-(2–17) is a positively charged α -helical peptide that is thought to inhibit the functions of Arf proteins by competing for the Arf-binding site on effector proteins [39]. In the present study, Brefeldin A and Arf-1-(2–17) were found to inhibit the activation of SACCs in intact rat hepatocytes, and Arf-1-(2–17) inhibited the GTP/ $\text{Ins}(1,4,5)\text{P}_3$ -induced release of $^{45}\text{Ca}^{2+}$ from rat liver microsomes. These results provide evidence for the requirement for an Arf protein in store-activated Ca^{2+} inflow and suggest a possible mechanism involving the Arf protein.

EXPERIMENTAL

Materials

DBHQ, CPA, dithiothreitol, poly(ethylene glycol) (average molecular mass 8000 Da), ATP, $\text{Ins}(1,4,5)\text{P}_3$, oligomycin, Ruthenium Red and Brefeldin A were obtained from Sigma–Aldrich (Castle Hill, N.S.W., Australia); fura 2 and fluo-3 acetoxymethyl ester from Molecular Probes (Eugene, OR, U.S.A.); thapsigargin from Sapphire Bioscience (Alexandria, N.S.W., Australia); ionomycin from Calbiochem (Alexandria, N.S.W., Australia); and GTP[S], GTP, guanosine 5'-[β -imido]triphosphate (p[NH]ppG) and EGF from Boehringer Mannheim (Castle Hill, N.S.W., Australia). All other chemicals were obtained from the sources described previously [18–21]. $^{45}\text{CaCl}_2$ was from Amersham Australia (Castle Hill, N.S.W., Australia), and ReadySafe scintillation fluid came from Beckman Instruments (Fullerton, CA, U.S.A.).

The peptides GNIFANLFKGLFGKKE [Arf-1-(2–17)], RVGLVRGEKARKGK [insulin-like growth factor II (IGF-II) receptor-(2410–2423)], INLGALAALAKKIL (MP14), an analogue of mastoparan, IKNNLKDCGLF [G $_2\alpha$ -(345–355) (G $_2\alpha$ peptide)] and IKNNLKECGLY [G $_3\alpha$ -(344–354) (G $_3\alpha$ peptide)] were synthesized by the Merrifield solid-phase synthesis pro-

cedure with an Applied Biosystems 433A synthesizer, and were analysed by quantitative amino acid analysis and MS as described previously [40]. Succinylated Arf-1-(2–17) was prepared by treating the peptide (10 mg) with a 40-fold molar excess of succinic anhydride in dimethylformamide containing triethylamine (10%, v/v). The reaction was monitored with ninhydrin; the succinylated peptide (succinylated mass 2181.8 Da compared with the native molecular mass of 1782.1 Da) was purified by reverse-phase chromatography.

Methods

The isolation of rat hepatocytes, attachment of hepatocytes to coverslips coated with collagen, loading of hepatocytes with fura 2 by microinjection and measurement of the fluorescence of individual hepatocytes at 30 °C were performed as described previously [18]. Ca^{2+} inflow was measured with the Ca^{2+} add-back protocol [18] in which hepatocytes loaded with fura 2 were initially incubated in the absence of extracellular Ca^{2+} and a SERCA inhibitor or vasopressin was added. After Ca^{2+} had been released from the SER, extracellular Ca^{2+} (1.5 mM) was added and the rate of the subsequent increase in fluorescence ratio was measured. The dilution factor for the microinjection of agents to hepatocytes was estimated to be 1:75 [19]. This factor was used to estimate the intracellular concentration of agents microinjected into hepatocytes. Some experiments with Brefeldin A were performed with hepatocytes loaded with fluo-3 rather than fura 2. In these experiments the excitation filter was a 10 nm bandpass quartz filter centred on 490 nm (Oriel Corporation, Stratford, CT, U.S.A.); the emitted fluorescence was isolated via a DM510 dichroic mirror and a BA520 long-pass barrier filter (Nikon). Freshly isolated hepatocytes attached to glass coverslips were incubated for 2 h in Dulbecco's modified Eagle's medium containing Brefeldin A or vehicle (methanol). For the last 20 min of this period, the acetoxymethyl ester of fluo-3 (20 μM) was also present. Subsequently the cells were washed several times with modified Hanks medium [137 mM NaCl/5.4 mM KCl/0.8 mM MgSO_4 /0.8 mM Na_2HPO_4 /0.4 mM KH_2PO_4 /4.2 mM NaHCO_3 /20 mM Hepes (pH 7.4)/0.002% (w/v) Phenol Red/5.6 mM glucose; incubation medium] [18] before being placed in fresh modified Hanks medium containing Brefeldin A or vehicle for fluorescence measurement. Qualitatively similar results were observed with both fura 2 and fluo-3.

GTP[S], guanine and adenine nucleotides, and other agents indicated in the tables and figures, were introduced into the cytoplasmic space of cells by co-injection with fura 2. Except as indicated below, cells were cultured in Dulbecco's modified Eagle's medium at 37 °C, washed with CO_2 -independent medium (Gibco-BRL), microinjected while in this medium and further incubated for 15 min in CO_2 -independent medium before being transferred to modified Hanks medium just before the measurement of fluorescence. In experiments in which GTP[S] was microinjected into the cells, the microinjection and the subsequent 15 min incubation were conducted in modified Hanks medium.

Rat liver microsomes were isolated and incubated as described by Dawson [41]. Briefly, incubation mixtures (1 ml) contained 150 mM sucrose, 50 mM KCl, 2 mM MgCl_2 , 1 mM dithiothreitol, 5% (w/v) polyethylene glycol, 2 μM Ruthenium Red, 1 $\mu\text{g}/\text{ml}$ oligomycin, 10 mM Hepes, pH 7.0, 0.17 μCi of $^{45}\text{Ca}^{2+}$, 20 μM added CaCl_2 and 3–4 mg of microsomal protein. The total Ca^{2+} concentration of microsomal incubation mixtures (estimated by atomic absorption spectroscopy) was approx. 50 μM and was used to calculate the specific radioactivity of $^{45}\text{Ca}^{2+}$ in the incubation mixtures. Incubations were performed at 30 °C and 5 mM ATP was added to promote $^{45}\text{Ca}^{2+}$ uptake.

The $^{45}\text{Ca}^{2+}$ content of microsomes was determined by rapidly filtering (with $0.45\ \mu\text{m}$ cellulose acetate membrane filters) $50\ \mu\text{l}$ aliquots of the incubation mixtures, washing the filters (twice, with $1\ \text{ml}$ each time) and counting radioactivity on the filters. Data were corrected for non-specific binding of $^{45}\text{Ca}^{2+}$ to pellets and filters. Microsomal protein concentration was determined by the biuret method with BSA as a standard. Samples were pretreated with sodium deoxycholate and trichloroacetic acid to avoid problems of turbidity [42].

RESULTS

Effects of GTP[S] and analogues of GTP in intact hepatocytes

As shown previously [18,19], a low intracellular concentration of GTP[S] (approx. $130\ \mu\text{M}$ in the present study) mimicked the ability of vasopressin to activate Ca^{2+} inflow (Table 1). No

activation of Ca^{2+} inflow was observed when the concentration of GTP[S] was increased to approx. $530\ \mu\text{M}$ or when GTP (approx. $130\ \mu\text{M}$) was injected in place of GTP[S] (results not shown). The higher concentration of GTP[S] (approx. $530\ \mu\text{M}$) inhibited the Ca^{2+} inflow stimulated by thapsigargin (compare Figures 1A and 1B, and Table 1), vasopressin, DBHQ or EGF (results not shown). p[NH]ppG also inhibited thapsigargin-stimulated Ca^{2+} inflow (Table 1). GTP prevented the inhibition of thapsigargin-stimulated Ca^{2+} inflow by GTP[S] (Figure 1C and Table 1) and by p[NH]ppG (Table 1), but did not prevent the activation of Ca^{2+} inflow by the low GTP[S] concentration in the absence of thapsigargin (Table 1).

Effects of Brefeldin A in intact hepatocytes

Arf proteins are the only monomeric G-proteins, the function of which is known to be inhibited by GTP[S] [34,35]. Therefore

Table 1 Inhibition of store-activated Ca^{2+} inflow by analogues of GTP in hepatocytes

Measurement of the fluorescence of hepatocytes loaded with fura-2 was conducted as described in the Experimental section and in the legend to Figure 1. The nucleotides were introduced into the cytoplasmic space by microinjection, as described in the Experimental section. The values shown for the initial rate of Ca^{2+} inflow are the means $\pm$ S.E.M. of the initial rates of extracellular Ca^{2+} -induced increase in fluorescence ratio observed for those cells that showed (1) substantial or partial Ca^{2+} inflow, where analysis of the numbers of cells giving a particular response indicated that the predominant effect of the agent was to activate or cause no change in the rate of Ca^{2+} inflow (compared with the effect observed in the absence of an agent), or (2) partial or little or no Ca^{2+} inflow, where analysis of the numbers of cells giving a particular response indicated that the predominant effect of the agent under test was to inhibit Ca^{2+} inflow.

Agonist	Analogue of GTP	Estimated intracellular concentration (μM)	Ca^{2+} inflow				Initial rate of Ca^{2+} inflow (fluorescence ratio/min)
			Numbers of cells giving a particular response				
			Total tested	Substantial inflow	Partial inflow	Little or no inflow	
None	None		4	0	0	4	0.02 ± 0.004
	GTP[S]	130	14	8	2	4	0.46 ± 0.14
	GTP[S] + GTP	130 530	10	6	1	3	0.35 ± 0.15
Thapsigargin (10 μM)	None		58	39	18	1	0.70 ± 0.08
	GTP[S]	530	22	1	5	16	0.09 ± 0.02
	GTP[S] + GTP	530 530	6	4	1	1	0.62 ± 0.3
	p[NH]ppG	530	6	0	1	5	0.04 ± 0.01
	p[NH]ppG + GTP	530 530	7	4	1	2	0.47 ± 0.17

Figure 1 Inhibition by GTP[S] (A,B) of thapsigargin-stimulated Ca^{2+} inflow to isolated hepatocytes and prevention of this inhibition by GTP (C)

Measurement of the fluorescence of single hepatocytes loaded with fura 2, and the microinjection of GTP[S] and GTP[S] plus GTP were performed as described in the Experimental section. The estimated intracellular concentration of GTP[S] was approx. $530\ \mu\text{M}$. Thapsigargin ($10\ \mu\text{M}$) and Ca^{2+} ($1.5\ \text{mM}$) were added to the incubation dish at the beginning of the periods indicated by the horizontal bars. The traces shown are representative of the results obtained for: 21 cells (22 cells tested) (A); 57 cells (58 cells tested) (B); 21 cells (22 cells tested) (black trace) and 5 cells (6 cells tested) (grey trace) (C).

Figure 2 Inhibition by Brefeldin A (BFA) of thapsigargin-stimulated (A) and vasopressin-stimulated (B) Ca^{2+} inflow to isolated hepatocytes

Cells were preincubated for 2 h in the presence (grey traces) or absence (black traces) of 18 μM Brefeldin A (which was also present during measurement) before the measurement of fluorescence. Measurement of the fluorescence of single hepatocytes loaded with fura 2 or fluo-3, and the microinjection of fura 2 or loading with fluo-3 acetoxymethyl ester, were performed as described in the Experimental section. Thapsigargin (10 μM), vasopressin (10 nM) and Ca^{2+} (1.5 mM) were added to the incubation dish at the beginning of the periods indicated by the horizontal bars. The traces shown are representative of the results obtained for: 57 cells (58 cells tested) (black trace) and 11 cells (14 cells tested) (grey trace) (A); 30 cells (30 cells tested) (black trace) and 4 cells (4 cells tested, delayed release of Ca^{2+} from stores was observed in 3 out of the 4 cells tested) (grey trace) (B).

Brefeldin A and the peptide Arf-1-(2–17), inhibitors of Arf function [36–39], were tested for their effects on store-activated Ca^{2+} inflow. Brefeldin A inhibited thapsigargin-stimulated Ca^{2+} inflow (Figure 2A and Table 2) and altered the shape of the thapsigargin-induced Ca^{2+} release peak (Table 3). In the presence of Brefeldin A, the ratio of peak width at half peak height to peak height for the thapsigargin-induced increase in fluorescence was doubled (Table 3). Methanol (the solvent for Brefeldin A) had only a small effect on thapsigargin-stimulated Ca^{2+} inflow and no effect on thapsigargin-induced Ca^{2+} release from intracellular stores (Tables 2 and 3). Maximal effect of Brefeldin A on thapsigargin-stimulated Ca^{2+} inflow was observed after 2 h of preincubation (Table 4). Brefeldin A also inhibited Ca^{2+} inflow stimulated by DBHQ, CPA (Table 2) or vasopressin (Figure 2B and Table 2), with little effect on the Ca^{2+} release induced by these agents. In some experiments with vasopressin, Brefeldin A delayed the vasopressin-induced release of Ca^{2+} (Table 2).

Effects of peptide Arf-1-(2–17) in intact hepatocytes

Arf-1-(2–17) (approx. 20 μM intracellular) inhibited thapsigargin-, DBHQ-, CPA- and vasopressin-stimulated Ca^{2+} inflow (Figures 3A and 3C, and Table 2). These changes were generally associated with a decrease in the amount of Ca^{2+} released from intracellular stores (Figure 3A and 3C, and Table 2) and a change in the profile of the thapsigargin- and vasopressin-induced Ca^{2+} release peaks (Table 3). In the absence of SERCA inhibitor or vasopressin, cells injected with Arf-1-(2–17) exhibited no Ca^{2+} inflow on the addition of extracellular Ca^{2+} (results not shown). When the time elapsed between the injection of Arf-1-(2–17) (20 μM) and the addition of an agonist was reduced from approx. 15 to approx. 7 min, the degree of inhibition of thapsigargin-stimulated Ca^{2+} inflow was also decreased by approx. 50% (results not shown). Succinylated Arf-1-(2–17) had little effect on thapsigargin- or vasopressin-stimulated Ca^{2+} inflow or release (Figures 3B and 3D, and Table 2). A second peptide used as a control for Arf-1-(2–17), IGF-II receptor-(2410–2423), the G-protein-activating sequence (without an attached membrane-spanning region) of the cytoplasmic domain of the IGF II

receptor [43], also had little or no effect on vasopressin- or thapsigargin-stimulated Ca^{2+} inflow or on Ca^{2+} release induced by these agents (Table 2). In contrast, peptide MP14, an analogue of mastoparan in which Lys-4 is replaced by a glycine residue, (approx. 5–20 μM intracellular) killed 90% of the injected cells within 5 min. In those cells that survived, MP14 did not stimulate Ca^{2+} inflow in the absence of an agonist (results not shown).

Surprisingly, lower concentrations of Arf-1-(2–17) (approx. 4 or 10 μM) enhanced vasopressin-stimulated Ca^{2+} inflow by approx. 70–150% with no detectable effect on vasopressin-induced Ca^{2+} release (results not shown). There was little effect of 4–10 μM Arf-1-(2–17) on thapsigargin-stimulated Ca^{2+} inflow. The equivalent concentration of succinylated Arf-1-(2–17) caused no enhancement of vasopressin-stimulated Ca^{2+} inflow (Figure 3D and Table 2).

Effects of inhibitors and activators of G-protein function on GTP and Ins(1,4,5) P_3 -induced Ca^{2+} release from isolated microsomes

As shown previously [30,41], the addition of Ins(1,4,5) P_3 to isolated rat liver microsomes that had been allowed to accumulate $^{45}\text{Ca}^{2+}$ in the presence of 5 mM ATP, 2 μM Ruthenium Red and oligomycin (1 $\mu\text{g}/\text{ml}$) did not cause a substantial release of $^{45}\text{Ca}^{2+}$. The addition of GTP alone released approx. 40% of $^{45}\text{Ca}^{2+}$ (results not shown). However, the combination of Ins(1,4,5) P_3 and GTP caused a greater release of $^{45}\text{Ca}^{2+}$, generally amounting to approx. 70% of the microsomal $^{45}\text{Ca}^{2+}$ content (Figure 4A). GTP/Ins(1,4,5) P_3 -induced $^{45}\text{Ca}^{2+}$ release was inhibited by GTP[S] (200 μM) (Figure 4A), as shown previously [30]. Inhibition of Ca^{2+} release was also observed at a lower concentration of GTP[S] (5 μM) (results not shown). The inhibition caused by 5 μM GTP[S] was largely prevented by raising the GTP concentration to 500 μM (results not shown). The effect of AlF_4^- , an activator of trimeric but not monomeric G-proteins [44], on the GTP/Ins(1,4,5) P_3 -induced $^{45}\text{Ca}^{2+}$ release was also investigated. The experiments were conducted in the presence of GDP to facilitate the formation of the GDP- AlF_4^- complex, which activates trimeric G-proteins [45]. When 20 μM GDP, 5 mM NaF and 30 μM AlCl_3 were added to microsomes pre-

Table 2 Effects of Brefeldin A and Arf-1 (2–17) on store-activated Ca^{2+} inflow and the release of Ca^{2+} from intracellular stores in hepatocytes

Measurement of the fluorescence of hepatocytes loaded with fura 2 or fluo-3 (some experiments with vasopressin, no other additions and vasopressin, methanol) was conducted as described in the Experimental section and in the legends to Figures 1 and 2. The values shown for the initial rate of Ca^{2+} inflow were calculated as described in the caption to Table 1. The values shown for the maximum increase in fluorescence are the means $\pm$ S.E.M. of the differences between the maximum value of fluorescence ratio observed after the addition of agonist and the basal value of fluorescence ratio observed before addition of the agonist. Where analysis of the numbers of cells giving a particular response indicated that the predominant effect of the agent under test was to give little or no change in the fluorescence ratio increase, the values shown are the means $\pm$ S.E.M. of the fluorescence ratio increase for all cells that gave 'substantial release'. Where analysis of the numbers of cells giving a particular response indicated that the predominant effect of the agent under test was to reduce the fluorescence ratio increase markedly, the values shown are the means $\pm$ S.E.M. of fluorescence ratio increase for all cells that gave 'decreased release'.

Agonist	Agent*	Estimated concentration* (μM)	Total number of cells tested	Ca ²⁺ inflow			Initial rate of Ca ²⁺ inflow (fluorescence ratio/min)	Release of Ca ²⁺ from intracellular stores		
				Number of cells giving a particular response				Number of cells giving a particular response		Maximum increase in fluorescence units
				Substantial inflow	Partial inflow	Little or no inflow		Substantial release	Reduced release	
Thapsigargin (10 μM)	None		58	39	18	1	0.70 $\pm$ 0.08	50	8	0.28 $\pm$ 0.02
	Brefeldin A	18	14	3	2	9	0.06 $\pm$ 0.01	8	6	0.27 $\pm$ 0.05
	Methanol		6	4	1	1	0.42 $\pm$ 0.10	6	0	0.25 $\pm$ 0.05
	Arf-1 (2–17)	20	22	5	7	10	0.13 $\pm$ 0.03	15	7	0.22 $\pm$ 0.03
	Succinylated Arf-1 (2–17)	20	4	3	1	0	0.61 $\pm$ 0.08	2	2	0.20 $\pm$ 0.04
		50	10	7	3	0	0.53 $\pm$ 0.06	7	3	0.21 $\pm$ 0.03
	IGF-II (2410–2423)	50	8	7	1	0	0.66 $\pm$ 0.35	6	2	0.16 $\pm$ 0.01
DBHQ (25 μM)	None		12	8	3	1	0.53 $\pm$ 0.09	9	3	0.24 $\pm$ 0.03
	Brefeldin A	18	7	0	1	6	0.11 $\pm$ 0.03	4	3	0.21 $\pm$ 0.07
	Arf-1 (2–17)	20	10	2	4	4	0.23 $\pm$ 0.03	8	2	0.22 $\pm$ 0.06
CPA (50 μM)	None		6	5	1	0	0.42 $\pm$ 0.07	5	1	0.29 $\pm$ 0.08
	Brefeldin A	18	4	0	3	1	0.22 $\pm$ 0.06	4	0	0.29 $\pm$ 0.09
	Arf-1 (2–17)	20	7	1	3	3	0.15 $\pm$ 0.05	6	1	0.24 $\pm$ 0.03
Vasopressin (10 nM)	None		30	24	6	0	0.63 $\pm$ 0.08	28	2	0.56 $\pm$ 0.04
	Brefeldin A	18	11	6	5	0	0.49 $\pm$ 0.09	11†	0	0.41 $\pm$ 0.04
	Methanol		13	11	1	1	0.70 $\pm$ 0.02	12	1	0.62 $\pm$ 0.08
	Arf-1 (2–17)	20	10	0	4	6	0.13 $\pm$ 0.03	7†	3†	0.28 $\pm$ 0.06
	Succinylated Arf-1 (2–17)	10	4	2	2	0	0.44 $\pm$ 0.17	3	1	0.44 $\pm$ 0.09
		20	4	3	1	0	0.54 $\pm$ 0.16	3	1	0.45 $\pm$ 0.12
	IGF-II (2410–2423)	10	5	4	1	0	0.51 $\pm$ 0.15	5	0	0.60 $\pm$ 0.04

* The peptides ARF-1 (2–17), succinylated Arf-1 (2–17), and IGF-II (2410–2423) were injected into cells, and the concentrations given are intracellular concentrations. Hepatocytes were preincubated for 2 h with the indicated extracellular concentration of Brefeldin A in methanol (0.1%, v/v) or with methanol alone.

† Seven cells showed a delay in Ca^{2+} release.

loaded with $^{45}\text{Ca}^{2+}$, the addition of GTP (0.7 mM) plus Ins(1,4,5) P_3 (1 μM) 30 s after GDP/NaF/ AlCl_3 addition released an amount of $^{45}\text{Ca}^{2+}$ similar to that observed in experiments conducted in the absence of GDP, NaF and AlF_4^- (results not shown). However, if microsomes were preincubated with GDP/NaF/ AlCl_3 for 10 min before the initiation of $^{45}\text{Ca}^{2+}$ uptake (compare with [46]), the subsequent addition of GTP (20 μM) plus Ins(1,4,5) P_3 (1 μM) did not release significant amounts of $^{45}\text{Ca}^{2+}$ (results not shown). These results indicate that a short exposure to AlF_4^- did not inhibit GTP/Ins(1,4,5) P_3 -induced $^{45}\text{Ca}^{2+}$ release, whereas a longer exposure to AlF_4^- caused substantial inhibition.

Arf-1-(2–17) (50 μM) inhibited GTP/Ins(1,4,5) P_3 -induced $^{45}\text{Ca}^{2+}$ release (Figure 4A) but succinylated Arf-1-(2–17), $\text{G}_i3\alpha$ peptide (part of the C-terminal region of $\text{G}_i3\alpha$) (Figure 4B) or $\text{G}_22\alpha$ peptide (part of the C-terminal region of $\text{G}_22\alpha$) had no effect (results not shown). The concentration of Arf-1-(2–17) that gave half-maximal inhibition was approx. 10–15 μM (Figure 5). L-Lysine (150 μM), equimolar with the concentration of the lysine residues in 50 μM Arf-1-(2–17), did not inhibit either the uptake or release of $^{45}\text{Ca}^{2+}$ by microsomes (results not shown). The mastoparan analogue MP14 (30 μM) caused some inhibition of

GTP/Ins(1,4,5) P_3 -induced $^{45}\text{Ca}^{2+}$ release (Figure 4C) but, in contrast with Arf-1-(2–17), maximal concentrations of MP14 caused only partial inhibition of GTP/Ins(1,4,5) P_3 -induced $^{45}\text{Ca}^{2+}$ release (Figures 4C and 5). A half-maximal effect was given by approx. 10 μM MP14 (Figure 5). Arf-1-(2–17) (50 μM) did not inhibit the release of $^{45}\text{Ca}^{2+}$ induced by either thapsigargin/Ins(1,4,5) P_3 or ionomycin. Thus the amount of $^{45}\text{Ca}^{2+}$ released from $^{45}\text{Ca}^{2+}$ -loaded microsomes during the 5 min after the addition of thapsigargin (5 μM) plus Ins(1,4,5) P_3 (1 μM) was 37 % of the maximal $^{45}\text{Ca}^{2+}$ accumulated in either the presence or absence of Arf-1-(2–17). Likewise, the amount of $^{45}\text{Ca}^{2+}$ released within 1 min of the addition of ionomycin (10 μM) was 97 % of the maximal $^{45}\text{Ca}^{2+}$ accumulated in either the presence or absence of Arf-1-(2–17).

DISCUSSION

The results obtained for the effects of GTP analogues on Ca^{2+} inflow in intact rat hepatocytes have shown that (1) a low concentration of GTP[S] activates Ca^{2+} inflow in the absence of an agonist and (2) high concentrations of GTP[S] and p[NH]ppG inhibit store-activated Ca^{2+} inflow. Inhibition by the high con-

Table 3 Effects of Brefeldin A Arf-1 (2–17) on the shape of increased fluorescence caused by the release of Ca^{2+} from intracellular stores in hepatocytes

Measurements of the fluorescence of hepatocytes loaded with fura 2 or fluo-3 and the release of Ca^{2+} from intracellular stores were performed as described in the Experimental section. Arf-1 (2–17) was microinjected into the cells at the same time as fura 2. Brefeldin A [in 0.1% (v/v) methanol] was added to the extracellular medium 2 h before the microinjection of fura 2 or the completion of loading with fluo-3 acetoxymethyl ester, and was present during the measurement of fluorescence. Values are means $\pm$ S.E.M. for the numbers of cells given in parenthesis. The degrees of significance for a comparison of the results obtained in the presence and absence of Brefeldin A or Arf-1 (2–17), determined with Student's *t* test for unpaired samples, were: **P* $\leq$ 0.05; ***P* $\leq$ 0.01; and ****P* $\leq$ 0.005.

Agonist	Agent	Ratio of peak width at half peak height: peak height (min per fluorescence units)
Thapsigargin	None	9 $\pm$ 1 (10)
	Methanol	13 $\pm$ 2 (6)
	Brefeldin A (18 μM)	25 $\pm$ 4 (13)***
Thapsigargin	None	11 $\pm$ 1 (12)
	Peptide Arf-1 (2–17) (20 μM)	58 $\pm$ 19 (15)*
Vasopressin	None	2.9 $\pm$ 0.4 (7)
	Peptide Arf-1 (2–17) (20 μM)	16.5 $\pm$ 4.8 (6)**

Table 4 Effect of time of preincubation with Brefeldin A on thapsigargin-stimulated Ca^{2+} inflow in hepatocytes

Measurements of the fluorescence of hepatocytes loaded with fura 2 or fluo-3 and estimation of the rates of thapsigargin-stimulated Ca^{2+} inflow were performed as described in the Experimental section and in the legend to Figure 2. Brefeldin A was added to the incubation dish at the times indicated before the measurement of fluorescence, and was present during the measurement of fluorescence. Values are means $\pm$ S.E.M. for the number of cells indicated in parenthesis.

Concentration of Brefeldin A (μM)	Preincubation time (h)	Rate of thapsigargin-stimulated Ca^{2+} inflow (fluorescence ratio/min)
0	—	0.70 $\pm$ 0.08 (58)
18	1	0.43 $\pm$ 0.31 (3)
18	1.5	0.46 $\pm$ 0.23 (3)
18	2.0	0.07 $\pm$ 0.02 (3)

centration of GTP[S] was prevented by GTP, whereas GTP did not prevent the activation of Ca^{2+} inflow by the low GTP[S] concentration. As discussed previously [13,18,19], the activation of SACCs by the low GTP[S] concentration is most probably due to the binding of GTP[S] to $\text{G}_{\alpha/11}$, the activation of phospholipase $\text{C}\beta$, the formation of $\text{Ins}(1,4,5)\text{P}_3$ and the release of Ca^{2+} from the SER, which in turn leads to an activation of SACCs via a process that requires G_i2 . The binding of GTP[S] to G_i2 [21], the activation of G_i2 and the subsequent activation of SACCs might also account for the effect of the low concentration of GTP[S].

In cells loaded with the high concentration of GTP[S], thapsigargin or vasopressin induced little release of Ca^{2+} from intracellular stores. One explanation for this observation is that the high GTP[S] concentration (1) induces the release of Ca^{2+} from intracellular stores during the preincubation period so that no further Ca^{2+} is available in the SER for thapsigargin-induced Ca^{2+} release and (2) inhibits the coupling between the Ca^{2+} -depleted SER and RACCs located in the plasma membrane. An alternative explanation is that the high GTP[S] concentration

inhibits the ability of thapsigargin and vasopressin [acting via $\text{Ins}(1,4,5)\text{P}_3$] to release Ca^{2+} from the SER by preventing the binding of $\text{Ins}(1,4,5)\text{P}_3$ and thapsigargin to their respective target proteins. Several observations indicate that it is unlikely that GTP[S] inhibits the ability of thapsigargin and $\text{Ins}(1,4,5)\text{P}_3$ to release Ca^{2+} from the SER. (1) Thapsigargin and $\text{Ins}(1,4,5)\text{P}_3$ act by different mechanisms and it is unlikely that GTP[S] inhibits both of these. (2) 'Caged' GTP[S] has previously been shown to induce Ca^{2+} release from the SER and stimulate Ca^{2+} inflow in hepatocytes [19]. (3) In the presence of GTP, the high concentration of GTP[S] did not inhibit thapsigargin-stimulated Ca^{2+} inflow. Although the addition of extracellular Ca^{2+} to cells loaded with the high GTP[S] concentration and incubated in the absence of a SERCA inhibitor or vasopressin does not result in Ca^{2+} inflow (Ca^{2+} inflow would be expected if the only effect of GTP[S] were to release Ca^{2+} from the SER), the absence of Ca^{2+} inflow is likely to be due to the high GTP[S] concentration's inhibiting coupling between the Ca^{2+} -depleted SER and RACCs in the plasma membrane.

The observations that GTP[S] inhibits store-activated Ca^{2+} inflow and that this inhibition can be prevented by GTP indicate that GTP hydrolysis is required for the activation of Ca^{2+} inflow initiated by the depletion of Ca^{2+} from the SER. Because some monomeric G-proteins require GTP hydrolysis in order to transmit a signal downstream [47], but this is not known to be a requirement for any trimeric G-proteins, the results suggest that a monomeric G-protein is required for store-activated Ca^{2+} inflow in rat hepatocytes.

Because the Arf proteins are the only monomeric G-proteins, the function of which is known to be inhibited by GTP[S] [34,35], and this inhibition of Arf function is prevented by GTP [34], the possibility that the putative monomeric G-protein required for store-activated Ca^{2+} inflow is a member of the Arf family was investigated with Brefeldin A and Arf-1-(2–17). Brefeldin A has been shown to inhibit the activity of the Arf GDP:GTP exchange protein [37,38] with consequent inhibition of Arf function. Arf-1-(2–17) also inhibits the function of Arf proteins [39] but has been reported to have other effects, including the non-specific destruction of membranes, attributed to its amphipathic helical structure [48].

In intact hepatocytes Brefeldin A and Arf-1-(2–17) inhibited store-activated Ca^{2+} inflow initiated by SERCA inhibitors and vasopressin, and altered the pattern of Ca^{2+} release from intracellular stores induced by these agents. Methanol, the solvent for Brefeldin A, did not affect Ca^{2+} inflow or release of Ca^{2+} from intracellular stores. The time course for the effect of Brefeldin A reported here and the effective concentration are similar to those that inhibit other Brefeldin A-sensitive processes [36–39]. It is therefore concluded that the present results provide reasonable evidence that Brefeldin A inhibits one of the processes involved in coupling Ca^{2+} release from the SER to the activation of SACCs.

The results obtained for the effect of Arf-1-(2–17) on intact hepatocytes are more difficult to interpret because it is known that this peptide affects some other processes in addition to the inhibition of the function of Arf proteins [48]. However, in the present study, succinylated Arf-1-(2–17) and an unrelated peptide (part of the cytoplasmic domain of the IGF II receptor) had little or no effect on store-activated Ca^{2+} inflow. Succinylation of α -amino and ϵ -amino groups on Arf-1-(2–17) replaces the positive charges on the peptide with negative charges but would not be expected to substantially alter the three-dimensional structure of the peptide. The mastoparan derivative MP14, which is amphipathic and likely to have an α -helical secondary structure [48], caused very rapid cell death when present at concentrations

Figure 3 Inhibition by peptide Arf-1-(2-17) of thapsigargin- and vasopressin-stimulated Ca^{2+} inflow to isolated hepatocytes

Measurement of the fluorescence of single hepatocytes loaded with fura 2, and the microinjection of fura 2, Arf-1-(2-17) (A,C), and succinylated Arf-1-(2-17) (B,D) were performed as described in the Experimental section. Thapsigargin (10 μM) (A,B), vasopressin (10 nM) (C,D) and Ca^{2+} (1.5 mM) were added to the incubation dish at the beginning of the periods indicated by the horizontal bars. The intracellular concentration of Arf-1-(2-17) was approx. 20 μM (A,C; grey traces) and the intracellular concentration of succinylated Arf-1-(2-17) was approx. 50 μM (B) or 10 μM (D). The black traces in (A) and (C) represent cells injected with fura 2 alone (control). The traces shown are representative of the results obtained for: 57 cells (58 cells tested) (black trace) and 14 cells (18 cells tested) (grey trace) (A); 10 cells (10 cells tested) (B); 30 cells (30 cells tested) (black trace) and 7 cells (7 cells tested) (grey trace) (C); and 4 cells (4 cells tested) (D).

Figure 4 Effects of GTP[S], Arf-1-(2-17), succinylated Arf-1-(2-17), MP14 and $\text{G}_3\alpha$ peptide on the synergistic action of $\text{Ins}(1,4,5)\text{P}_3$ and GTP in releasing $^{45}\text{Ca}^{2+}$ from isolated liver microsomes

The incubation of microsomes and measurement of the amount of $^{45}\text{Ca}^{2+}$ associated with the microsomes were performed as described in the Experimental section. The uptake of $^{45}\text{Ca}^{2+}$ was initiated by the addition of 5 mM ATP added at 0 min. At the times indicated, aliquots (50 μl) of the incubation mixtures were removed for analysis of the microsomal $^{45}\text{Ca}^{2+}$ content. GTP (20 μM) and $\text{Ins}(1,4,5)\text{P}_3$ (1 μM) (filled symbols) or vehicle (open symbols) were added after 5 min (indicated by the arrow) to initiate $^{45}\text{Ca}^{2+}$ release. Other additions (see below) were made just before ATP. (A) Effects of GTP[S] and Arf-1-(2-17). Symbols: $\square$, no additions; $\blacksquare$, GTP plus $\text{Ins}(1,4,5)\text{P}_3$ (arrow); $\diamond$, GTP[S] (200 μM); $\blacklozenge$, GTP[S] (200 μM) and GTP plus $\text{Ins}(1,4,5)\text{P}_3$ (arrow); $\circ$, Arf-1-(2-17) (50 μM); $\bullet$, Arf-1-(2-17) (50 μM) and GTP plus $\text{Ins}(1,4,5)\text{P}_3$ (arrow). (B) Effects of succinylated Arf-1-(2-17) and $\text{G}_3\alpha$ peptide. Symbols: $\square$, no addition; $\blacksquare$, GTP plus $\text{Ins}(1,4,5)\text{P}_3$ (arrow); ∇ , succinylated Arf-1-(2-17) (50 μM); $\blacktriangledown$, succinylated Arf-1-(2-17) (50 μM) and GTP plus $\text{Ins}(1,4,5)\text{P}_3$ (arrow); $\triangle$, $\text{G}_3\alpha$ peptide (50 μM); $\blacktriangle$, $\text{G}_3\alpha$ peptide (50 μM) and GTP plus $\text{Ins}(1,4,5)\text{P}_3$ (arrow). (C) Effect of MP14 peptide. Symbols: $\square$, no addition; $\blacksquare$, GTP plus $\text{Ins}(1,4,5)\text{P}_3$ (arrow); $\triangle$, MP14 (30 μM); $\blacktriangle$, MP14 (30 μM) and GTP plus $\text{Ins}(1,4,5)\text{P}_3$ (arrow). Each trace is representative of the results obtained from at least two experiments.

Figure 5 Dose-response curves for the inhibition by Arf-1-(2-17) and MP14 of the synergistic action of Ins(1,4,5) P_3 and GTP in inducing the release of $^{45}\text{Ca}^{2+}$ from isolated rat microsomes

The incubation of microsomes and measurement of the amount of $^{45}\text{Ca}^{2+}$ associated with the microsomes were performed as described in the Experimental section and the legend to Figure 4. Arf-1-(2-17) (●) and MP14 (▲) were added to the incubation mixtures before ATP (added at 0 min to initiate $^{45}\text{Ca}^{2+}$ uptake); 5 min after ATP addition, 20 μM GTP and 1 μM Ins(1,4,5) P_3 were added together, and after a further 5 min of incubation aliquots (50 μl) were taken for analysis of the $^{45}\text{Ca}^{2+}$ content of the microsomes. The amount of $^{45}\text{Ca}^{2+}$ associated with the microsomes at 5 min after the addition of GTP/Ins(1,4,5) P_3 is expressed as a percentage of the maximal amount of $^{45}\text{Ca}^{2+}$ accumulated. The results shown are representative of those obtained for at least two experiments.

comparable to those of Arf-1-(2-17). Because this effect was not observed with Arf-1-(2-17), it is considered unlikely that the inhibition of store-activated Ca^{2+} inflow by Arf-1-(2-17) in isolated hepatocytes is due to non-specific disruption of the SER and/or plasma membrane by Arf-1-(2-17).

Arf-1-(2-17) not only inhibited the ability of thapsigargin or vasopressin [acting via Ins(1,4,5) P_3] to stimulate Ca^{2+} inflow but also decreased the ability of these agents to release Ca^{2+} from the SER. It is considered unlikely that Arf-1-(2-17) induces the release of Ca^{2+} from the SER in intact cells because succinylated Arf-1-(2-17) did not affect the ability of thapsigargin or vasopressin to release Ca^{2+} , and Arf-1-(2-17) alone did not release Ca^{2+} from isolated microsomes. The inhibition of Ca^{2+} inflow caused by Arf-1-(2-17) could be due to inhibition of the release of Ca^{2+} from the SER (or that region of the SER responsible for activating RACCs) and/or to inhibition by Arf-1-(2-17) of the coupling between the Ca^{2+} -depleted SER and RACCs in the plasma membrane. The results obtained with succinylated Arf-1-(2-17), which did not affect the release of Ca^{2+} from intracellular stores or Ca^{2+} inflow, indicate that it is unlikely that Arf-1-(2-17) acts by blocking the ability of thapsigargin or vasopressin [Ins(1,4,5) P_3] to release Ca^{2+} from the SER. In view of the results obtained with Brefeldin A in intact hepatocytes, and the effects of Arf-1-(2-17) on isolated microsomes, it is considered (as discussed below) that the most likely mechanism by which Arf-1-(2-17) inhibits thapsigargin- and vasopressin-stimulated Ca^{2+} inflow is the prevention (or modification) of the release of Ca^{2+} from the SER. As also discussed below, the process involved is thought to be independent of the direct effects of Arf-1-(2-17) on the Ins(1,4,5) P_3 receptor and the SERCA.

The synergistic action of GTP and Ins(1,4,5) P_3 in inducing the release of $^{45}\text{Ca}^{2+}$ from liver microsomes [30,41,49], and this

paper) was inhibited by GTP[S] as shown by others [30], but not by a short exposure of the microsomes to AlF_4^- . The effect of GTP[S] could be due either to the activation of a trimeric G-protein that in turn initiates the inhibition of GTP/Ins(1,4,5) P_3 -induced $^{45}\text{Ca}^{2+}$ release, or to the inhibition by GTP[S] of a monomeric G-protein required to allow GTP/Ins(1,4,5) P_3 -induced $^{45}\text{Ca}^{2+}$ release. Because a short exposure to AlF_4^- would be expected to activate trimeric G-proteins [50], it is concluded that the observed inhibition of $^{45}\text{Ca}^{2+}$ release by GTP[S] is not due to the activation of a trimeric G-protein. This conclusion is also consistent with the observation that GTP prevented the inhibition of GTP/Ins(1,4,5) P_3 -induced $^{45}\text{Ca}^{2+}$ release by GTP[S]. This last observation suggests that, as proposed previously [30], a cycle of GTP hydrolysis is required to allow the enhanced $^{45}\text{Ca}^{2+}$ release from microsomes.

The present results obtained with a short exposure to AlF_4^- differ from those reported here for a longer preincubation with AlF_4^- and those reported previously by Comerford and Dawson [46]. Those authors showed that when isolated liver microsomes are exposed to AlF_4^- for a prolonged period in the presence of GDP, AlF_4^- inhibits the GTP-induced membrane fusion that is associated with the release of $^{45}\text{Ca}^{2+}$ [30]. This result was interpreted in terms of the binding of AlF_4^- to a small G-protein with consequent inhibition of GTP-dependent processes.

The results indicate that Arf-1-(2-17) mimicked the effect of GTP[S] in inhibiting the GTP/Ins(1,4,5) P_3 -induced release of $^{45}\text{Ca}^{2+}$ from isolated microsomes. Several observations provide evidence that this action of Arf-1-(2-17) is specific for this peptide and for the action of GTP. Succinylated Arf-1-(2-17), L-lysine, which like Arf-1-(2-17) is positively charged, and several unrelated peptides (regions of the C-terminus of $G_{12\alpha}$ and $G_{13\alpha}$), each had no effect on GTP/Ins(1,4,5) P_3 -induced $^{45}\text{Ca}^{2+}$ release. Moreover, Arf-1-(2-17) did not inhibit $^{45}\text{Ca}^{2+}$ release induced by thapsigargin plus Ins(1,4,5) P_3 or ionomycin. The observation that Arf-1-(2-17) did not inhibit the uptake of $^{45}\text{Ca}^{2+}$ by microsomes or induce the release of $^{45}\text{Ca}^{2+}$ in the absence of GTP and Ins(1,4,5) P_3 indicates that the actions of Arf-1-(2-17) reported here are not due to the non-specific destruction of microsomal membranes. The latter is sometimes observed and has been attributed to the amphipathic helical structure of Arf-1-(2-17) [48]. The results obtained with thapsigargin plus Ins(1,4,5) P_3 and with ionomycin indicate that Arf-1-(2-17) did not inhibit the activity of the $(\text{Ca}^{2+} + \text{Mg}^{2+})$ -ATPase that pumps Ca^{2+} into microsomes nor did it block Ca^{2+} flow through the Ins(1,4,5) P_3 receptor. Inhibition of the GTP/Ins(1,4,5) P_3 -induced release of $^{45}\text{Ca}^{2+}$ was not entirely specific for Arf-1-(2-17) because the mastoparan analogue MP14 also caused some inhibition. However, maximal inhibition by Arf-1-(2-17) was close to 100%, whereas with MP14 the maximal inhibition was only 60%, suggesting that the mechanisms of action of the two peptides are different.

Although there are some reservations about the interpretation of the results obtained with the Arf-1-(2-17) peptide, it is considered that the following observations provide reasonable evidence that an Arf protein is involved in the activation of SACCs: (1) GTP[S], Brefeldin A and Arf-1-(2-17) inhibit the activation of store-activated Ca^{2+} inflow in intact hepatocytes, (2) GTP can prevent inhibition by GTP[S] and (3) Arf proteins are the only known monomeric G-proteins, the function of which is inhibited by GTP[S] [34,35]. Moreover, the observations that GTP[S] (but not a short exposure to AlF_4^-) and Arf-1-(2-17) inhibit the GTP/Ins(1,4,5) P_3 -induced release of $^{45}\text{Ca}^{2+}$ from isolated microsomes is also considered to provide reasonable evidence for the role of an Arf protein in the synergistic action of GTP and Ins(1,4,5) P_3 in releasing $^{45}\text{Ca}^{2+}$ from isolated micro-

somes. Furthermore other workers have shown that several members of the Arf family of proteins are present in rat liver [51–53], and Arf proteins have been detected in microsomes isolated by a method similar to that employed here [53,54].

Arf proteins are required for vesicle membrane fusion [36], and evidence has been obtained that the synergistic action of $\text{GTP/Ins}(1,4,5)\text{P}_3$ in releasing $^{45}\text{Ca}^{2+}$ from isolated rat liver microsomes involves the enhancement by GTP of the fusion of microsomal vesicles [30]. Moreover, Brefeldin A and Arf-1-(2–17) each altered the profile of Ca^{2+} release from the SER. Thus it is possible that, in the intact cell, an Arf protein is involved in the release of Ca^{2+} from the SER, which precedes the activation of SACCs. Another possibility is that the monomeric G-protein is responsible for maintaining a region of the SER in the correct intracellular location so that it can interact with SACC proteins in the plasma membrane [12,13,15,16,55].

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