Protein kinase A regulates the disposition of Ca^{2+} which enters the cytoplasmic space through store-activated Ca^{2+} channels in rat hepatocytes by diverting inflowing Ca^{2+} to mitochondria

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The roles of a trimeric GTP-binding regulatory protein, protein kinase A and mitochondria in the regulation of store-activated (thapsigargin-stimulated) Ca^{2+} inflow in freshly-isolated rat hepatocytes were investigated. Rates of Ca^{2+} inflow were estimated by measuring the increase in the fluorescence of intracellular fura-2 following the addition of extracellular Ca^{2+} (Ca^{2+}_o) to cells incubated in the absence of added Ca^{2+}_o . Guanosine 5'-[γ -thio]-triphosphate (GTP[S]) and AlF_4^- inhibited the thapsigargin-stimulated Ca^{2+}_o -induced increase in cytoplasmic free Ca^{2+} concentration ($[\text{Ca}^{2+}]_c$) and this inhibition was prevented by the *Rp* diastereoisomer of adenosine 3',5'-(cyclic)-phosphorothioate. cAMP, forskolin and glucagon (half-maximal effect at 10 nM) mimicked inhibition of the thapsigargin-stimulated Ca^{2+}_o -induced increase in $[\text{Ca}^{2+}]_c$ by GTP[S], but had little effect on thapsigargin-induced release of Ca^{2+} from in-

tracellular stores. Azide and carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone inhibited the thapsigargin-stimulated Ca^{2+}_o -induced increase in $[\text{Ca}^{2+}]_c$ in the presence of increased cAMP (induced by glucagon). In contrast, Ruthenium Red markedly enhanced the thapsigargin-stimulated Ca^{2+}_o -induced increase in $[\text{Ca}^{2+}]_c$ in both the presence and absence of increased cAMP (induced by forskolin and dibutyl cAMP). It is concluded that, in hepatocytes, protein kinase A regulates the disposition of Ca^{2+} , which enters the cytoplasmic space through store-activated Ca^{2+} channels, by directing some of this Ca^{2+} to the mitochondria. The idea that caution should be exercised in using observed values of Ca^{2+}_o -induced increase in $[\text{Ca}^{2+}]_c$ as estimates of rates of agonist-stimulated Ca^{2+} inflow is briefly discussed.

INTRODUCTION

Receptor-activated Ca^{2+} channels (RACCs) play a major role in regulation of the functions of 'non-excitable' and some 'excitable' cells by extracellular signals [1,2]. An RACC is activated by the binding of an extracellular signal molecule (e.g. hormone or growth factor) to its receptor. In contrast to voltage-operated Ca^{2+} channels, RACCs are inhibited rather than activated by membrane depolarization, and generally have a different ion selectivity [1]. Although not yet shown conclusively, it is likely that the RACC protein is separate and different from the receptor protein for the extracellular signal [3]. In this respect, and in their ion selectivity, RACCs differ from ligand-gated Ca^{2+} channels. There are several types of RACC distinguished by their ion selectivity and mechanisms of activation [1,3]. In many cells, one or more types of RACCs are activated by the release of Ca^{2+} from the smooth endoplasmic reticulum (SER) induced by $\text{Ins}(1,4,5)\text{P}_3$ [2]. These store-activated Ca^{2+} channels include the Ca^{2+} -specific Ca^{2+} -release-activated Ca^{2+} channels (CRACs) and non-specific Ca^{2+} -release-activated Ca^{2+} channels [1,3]. Store-activated Ca^{2+} channels can be activated artificially by agents which inhibit the $\text{Ca}^{2+} + \text{Mg}^{2+}$ -ATPase of the SER (SERCA), such as thapsigargin, 2,5-di-*t*-butylhydroquinone (DBHQ) and cyclopiazonic

acid (CPA) [1,2]. Other RACCs are thought to be activated by intracellular messengers such as cyclic nucleotides, Ca^{2+} and inositol polyphosphates [1–3]. Notwithstanding their importance in cell function, the structures and mechanisms of activation of RACCs are not well understood [1,3].

Rat hepatocytes possess at least two types of RACC: one type appears to be relatively selective for Ca^{2+} and the other is less selective (reviewed in [4]). Both types are activated by SER Ca^{2+} release [4,5]. *In vivo*, rat hepatocyte RACCs are activated by a variety of extracellular signals including vasopressin, α_1 -adrenergic agonists, angiotensin II and epidermal growth factor [4,5]. Moreover, in the presence of an extracellular signal (e.g. vasopressin), which increases the cytoplasmic free- Ca^{2+} concentration ($[\text{Ca}^{2+}]_c$), hormones such as glucagon, which increase the intracellular concentration of cAMP, synergistically stimulate the net inflow of Ca^{2+} to hepatocytes through RACCs [6–11]. This effect of cAMP is associated with the net uptake of Ca^{2+} by hepatocyte mitochondria [6,7,10,11].

In studies of the mechanism of activation of RACCs in hepatocytes we have obtained evidence which indicates that the trimeric G-protein, G_{12} [12–15], and a monomeric G-protein, most likely one of the Arf proteins [4], are required for the process of store-activated Ca^{2+} inflow. In the course of these studies, we found that guanosine 5'-[γ -thio]triphosphate (GTP-

Abbreviations used: SACC, store-activated Ca^{2+} channel; SER, smooth endoplasmic reticulum; SERCA, smooth endoplasmic reticulum $\text{Ca}^{2+} + \text{Mg}^{2+}$ -ATPase; EGF, epidermal growth factor; PKA, protein kinase A; PKC, protein kinase C; Ca^{2+}_o , extracellular Ca^{2+} ; GTP[S], guanosine 5'-[γ -thio]triphosphate; pp[NH]pG, guanosine 5'-[β , γ -imido]triphosphate; GDP[S], guanosine 5'-[β -thio]diphosphate; ATP[S], adenosine 5'-[γ -thio]triphosphate; pp[NH]pA, adenosine 5'-[β , γ -imido]triphosphate; *Rp* cAMPs, the *Rp* diastereoisomer of adenosine 3',5'-cyclic phosphorothioate; DBHQ, 2,5-di-*t*-butylhydroquinone; CPA, cyclopiazonic acid; FCCP, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone; $[\text{Ca}^{2+}]_c$, cytoplasmic free- Ca^{2+} concentration; RACC, receptor-activated Ca^{2+} channel.

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[S]), an activator of trimeric G-proteins [16], inhibits thapsigargin-stimulated Ca^{2+} inflow [4]. In some other cell types it has been shown that GTP[S]-induced inhibition of thapsigargin-stimulated Ca^{2+} inflow results from the activation (mediated by the appropriate trimeric G-protein and intracellular messenger) of protein kinase C and/or protein kinase A [17,18]. The aim of the present experiments was to investigate the mechanism by which GTP[S] inhibits thapsigargin-stimulated Ca^{2+} inflow in hepatocytes and to test the idea that this involves the activation of a protein kinase. The experiments were performed using the Ca^{2+} add-back protocol, in which the initial rate of increase in $[\text{Ca}^{2+}]_i$ following the addition of extracellular Ca^{2+} (Ca^{2+}_o) to cells previously incubated in the absence of added extracellular Ca^{2+} is taken as a measure of Ca^{2+} inflow across the plasma membrane [19]. Since it was found that initial rates of Ca^{2+}_o -induced increase in $[\text{Ca}^{2+}]_i$ are influenced by the rate of disposition of Ca^{2+} in the cytoplasmic space, the term ' Ca^{2+}_o -induced increase in $[\text{Ca}^{2+}]_i$ ' has been used in preference to ' Ca^{2+} inflow' in describing the parameter measured.

The results have shown that the inhibition by GTP[S] of the thapsigargin-stimulated Ca^{2+}_o -induced increase in $[\text{Ca}^{2+}]_i$ is due, at least in part, to the activation of G_s , the formation of cAMP and the activation of protein kinase A. Experiments conducted with Ruthenium Red indicate that activated protein kinase A, in turn, inhibits the Ca^{2+}_o -induced increase in $[\text{Ca}^{2+}]_i$ by directing, in some way, Ca^{2+} which enters the cytoplasmic space to the mitochondria. Thus it appears that, while it has been observed by others that cAMP stimulates the net inflow of Ca^{2+} to hepatocytes [6–11], this effect of cAMP on RACCs is associated with, and may possibly be due to, the enhanced uptake of Ca^{2+} by mitochondria. The results also indicate that in some instances caution should be exercised in equating observed rates of agonist-stimulated Ca^{2+}_o -induced increase in $[\text{Ca}^{2+}]_i$ with rates of Ca^{2+} inflow.

MATERIALS AND METHODS

Materials

AlCl_3 was obtained from BDH Chemicals Australia, Adelaide, Australia; NaF, staurosporin, DBHQ, CPA, genistein, PMA ($\equiv$ TPA), glucagon, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone (FCCP), sodium azide, Ruthenium Red and dibutyl cAMP from Sigma-Aldrich, Castle Hill, N.S.W., Australia; thapsigargin from Sapphire Bioscience, Alexandria, N.S.W., Australia; desferrioxamine mesylate (desferoxamine) from Ciba-Geigy Australia, Lane Cove, N.S.W., Australia; GTP[S], adenosine 5'-[γ -thio]triphosphate (ATP[S]), GTP, guanosine 5'-[β , γ -imido]-triphosphate (pp[NH]pG), guanosine 5'-[β -thio]diphosphate (GDP[S]), epidermal growth factor (EGF), adenosine 5'-[β , γ -imido]-triphosphate (pp[NH]pA), cAMP and cGMP were from Boehringer Mannheim, Castle Hill, N.S.W., Australia; and adenosine 3',5'-cyclic phosphorothioate-Rp (Rp cAMPS) from Calbiochem, Alexandria, N.S.W., Australia. All other chemicals were obtained from the sources given previously [4,5,12–15].

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 33 °C were performed as described previously [12]. Rates of agonist-stimulated Ca^{2+}_o -induced increase in $[\text{Ca}^{2+}]_i$ were estimated using the Ca^{2+} add-back protocol [19], in which hepatocytes loaded with fura-2 were initially

incubated in the absence of extracellular Ca^{2+}_o and a SERCA inhibitor, vasopressin or other agonist was added. After Ca^{2+} had been released from the SER, Ca^{2+}_o (1.5 mM) was added and the rate of the subsequent increase in the fluorescence ratio was measured. Initial rates of Ca^{2+}_o -induced increase in $[\text{Ca}^{2+}]_i$ were calculated as described previously [12]. For a given experimental condition, data for Ca^{2+}_o -induced increase in $[\text{Ca}^{2+}]_i$ or Ca^{2+} release from intracellular stores obtained for individual cells were classified according to the magnitude of the response observed using the descriptors 'substantial', 'partial' and 'little or none'. Where the majority of cells gave a 'substantial' response, the means $\pm$ S.E.M. of the initial rate of Ca^{2+}_o -induced increase in $[\text{Ca}^{2+}]_i$, maximum increase in fluorescence induced by Ca^{2+}_o and maximum increase in fluorescence induced by agonist were determined using the data for these cells. Where the majority of cells gave a 'partial' or 'little or none' response, the means $\pm$ S.E.M. were determined using the data for these cells. The means $\pm$ S.E.M. together with the number of cells giving the response and the total number of cells tested are given in the Tables.

GTP[S], guanine and adenine nucleotides, and other agents indicated in the Tables and Figures were introduced to the cytoplasmic space of cells by co-injection with fura-2 as described previously [12]. In experiments in which GTP[S] or ATP[S] was microinjected into the cells, the microinjection and the subsequent 15 min incubation were conducted in modified Hanks medium [4]. The dilution factor for the microinjection of agents into hepatocytes was estimated to be 75-fold [13]. This factor was used to estimate the intracellular concentration of agents microinjected into hepatocytes. Ruthenium Red, which was co-injected with fura-2, was mixed with the fura-2 immediately before the microinjection in order to prevent the adsorption of Ruthenium Red to the plastic and glass surfaces of the mixing tube and micropipette.

RESULTS

Effects of GTP[S], analogues of GTP and AlF_4^-

As shown previously [4], GTP[S] (approx. 530 μM), an activator of trimeric G-proteins [16], introduced into hepatocytes by microinjection, inhibited the initial rate of thapsigargin-stimulated Ca^{2+}_o -induced increase in $[\text{Ca}^{2+}]_i$ (Table 1). GTP[S] also inhibited the initial rate of Ca^{2+}_o -induced increase in $[\text{Ca}^{2+}]_i$ when this was stimulated by vasopressin, DBHQ or EGF (Table 1). Substantial inhibition of the initial rate of thapsigargin-stimulated Ca^{2+}_o -induced increase in $[\text{Ca}^{2+}]_i$ was also observed at approx. 270 and 400 μM GTP[S] (Table 1). In GTP[S]-loaded cells incubated in the absence of extracellular Ca^{2+} , the addition of vasopressin or thapsigargin did not induce an increase in fluorescence (results not shown), indicating that pre-incubation with GTP[S] released Ca^{2+} from the intracellular stores [4]. The ability of GTP[S] to inhibit the initial rate of thapsigargin-stimulated Ca^{2+}_o -induced increase in $[\text{Ca}^{2+}]_i$ was shared by GMP-PNP, ATP[S], AMP-PNP (approx. intracellular concentrations 530 μM) and, as shown previously [12], by GDP[S] (Table 1). ATP[S] also inhibited the initial rate of vasopressin-stimulated Ca^{2+}_o -induced increase in $[\text{Ca}^{2+}]_i$ (Table 1).

The effects of another activator of trimeric G-proteins, AlF_4^- [20], were also investigated. The microinjection of AlCl_3 plus NaF, which form AlF_4^- *in situ* [20], inhibited both the thapsigargin- and vasopressin-stimulated Ca^{2+}_o -induced increase in $[\text{Ca}^{2+}]_i$ [Figure 1B (AlCl_3 + NaF), cf. Figure 1A; and Table 1]. NaF, which can combine with endogenous Al^{3+} to form AlF_4^- [20,21], partially inhibited the thapsigargin-stimulated Ca^{2+}_o -induced increase in $[\text{Ca}^{2+}]_i$ [Table 1, Figure 1B (NaF)], whereas

Table 1 Inhibition of agonist-stimulated Ca^{2+}_o -induced increase in $[\text{Ca}^{2+}]_c$ by analogues of GTP and ATP, and by AlF_4^-

Experiments were performed as described in the Materials and methods section and in the legends to the Figures. The nucleotides, AlCl_3 and NaF were microinjected into hepatocytes. When present, deferoxamine was added to the extracellular medium after the microinjection of fura-2 to give a concentration of $500 \mu\text{M}$. The values for the initial rate of the Ca^{2+}_o -induced increase in $[\text{Ca}^{2+}]_c$ are the means $\pm$ S.E.M. of those obtained for the number of cells indicated. The degrees of significance, determined using Student's *t*-test for unpaired samples, for a comparison of the initial rate of Ca^{2+}_o -induced increase in $[\text{Ca}^{2+}]_c$ in the presence of a given analogue of GTP or ATP with the rate in the absence of the analogue of GTP or ATP, are: * $P \leq 0.05$; ** $P \leq 0.005$ and *** $P \leq 0.0005$.

Agonist	Analogue of GTP or ATP	Estimated intracellular concentration (μM)	Number of cells (number giving the response/total number tested)	Initial rate of Ca^{2+}_o -induced increase in $[\text{Ca}^{2+}]_c$ (ratio units/min)
Vasopressin (10 nM)	None		24/30	0.63 ± 0.08
	GTP[S]	530	6/6	$0.02 \pm 0.004^{**}$
	ATP[S]	530	5/7	$0.2 \pm 0.02^*$
	AlCl_3 + NaF	2	5/5	$0.09 \pm 0.04^*$
		530		
Thapsigargin (10 μM)	None		57/58	0.70 ± 0.08
	GTP[S]	270	5/8	$0.15 \pm 0.05^*$
		400	4/5	$0.20 \pm 0.07^*$
		530	21/22	$0.09 \pm 0.02^{***}$
	pp[NH]pG	530	6/6	$0.04 \pm 0.01^*$
	ATP[S]	530	6/7	$0.10 \pm 0.03^*$
	pp[NH]pA	100	4/4	0.65 ± 0.05
		530	5/5	$0.14 \pm 0.04^*$
	GDP[S]	1100	4/4	0.25 ± 0.08
	AlCl_3 + NaF	2	12/15	$0.10 \pm 0.02^{**}$
DBHQ (25 μM)	None		8/12	0.53 ± 0.09
	GTP[S]	530	4/5	$0.04 \pm 0.01^*$
EGF (30 nM)	None		4/5	0.93 ± 0.24
	GTP[S]	530	4/4	$0.03 \pm 0.02^{**}$

NaCl (approximate intracellular concentration $530 \mu\text{M}$) plus AlCl_3 (approx. $2 \mu\text{M}$) had no effect (results not shown). The effects of NaF were reversed by deferoxamine, a chelator of Al^{3+} [22] [Figure 1B (NaF + deferoxamine), cf. Figure 1A and Table 1]. The observations that (i) AlCl_3 greatly increased inhibition of the thapsigargin-stimulated Ca^{2+}_o -induced increase in $[\text{Ca}^{2+}]_c$ by NaF and (ii) deferoxamine reversed the inhibition by NaF indicate that it is AlF_4^- rather than NaF that induces inhibition of the thapsigargin-stimulated Ca^{2+}_o -induced increase in $[\text{Ca}^{2+}]_c$. In the absence of thapsigargin or vasopressin, AlCl_3 plus NaF caused no stimulation of the Ca^{2+}_o -induced increase in $[\text{Ca}^{2+}]_c$ (results not shown). In cells loaded with AlCl_3 plus NaF, and in the absence of extracellular Ca^{2+} , the addition of vasopressin (results not shown) or thapsigargin (Figure 1B) caused no increase in fluorescence. This result suggests that, as in the case of GTP[S], AlCl_3 plus NaF caused the release of Ca^{2+} from intracellular stores during the pre-incubation period.

Effects of protein kinase inhibitors and activators

The knowledge that GTP[S] and AlF_4^- are activators of trimeric G-proteins [16,20] suggested that a protein kinase is involved in the GTP[S]- and AlF_4^- -induced inhibition of the store-activated Ca^{2+}_o -induced increase in $[\text{Ca}^{2+}]_c$ [17,18]. This possibility was investigated by testing (i) the abilities of protein kinase inhibitors to prevent GTP[S]-induced inhibition of the store-activated Ca^{2+}_o -induced increase in $[\text{Ca}^{2+}]_c$ and (ii) the abilities of protein kinase activators to mimic the actions of GTP[S] and AlF_4^- .

Neither staurosporin nor genistein, inhibitors of protein kinase C [23] and protein-tyrosine kinases [24] respectively, prevented the GTP[S]-induced inhibition of the thapsigargin- and vasopressin-stimulated Ca^{2+}_o -induced increase in $[\text{Ca}^{2+}]_c$ (Table 2). In the absence of GTP[S], genistein inhibited the thapsigargin-stimulated Ca^{2+}_o -induced increase in $[\text{Ca}^{2+}]_c$ with little or no effect on the thapsigargin-induced release of Ca^{2+} from intracellular stores (Table 2). Staurosporin did not inhibit either the thapsigargin- or the vasopressin-stimulated Ca^{2+}_o -induced increase in $[\text{Ca}^{2+}]_c$ in the absence of GTP[S] (results not shown).

The observation that staurosporin did not prevent the inhibition of store-activated Ca^{2+}_o -induced increase in $[\text{Ca}^{2+}]_c$ by GTP[S] was somewhat unexpected, since it has been shown that

Figure 1 Inhibition by AlF_4^- of thapsigargin-stimulated Ca^{2+}_o -induced increase in $[\text{Ca}^{2+}]_c$

(A) Representative cell incubated in the absence of AlCl_3 , NaF or deferoxamine and (B) representative cells treated with AlCl_3 plus NaF, NaF alone, or NaF plus deferoxamine. Measurement of the fluorescence of single hepatocytes loaded with fura-2, and the microinjection of AlCl_3 and NaF, were performed as described in the Materials and methods section. The estimated intracellular concentrations of AlCl_3 and NaF were approx. 2 and $530 \mu\text{M}$ respectively, and the concentration of deferoxamine (extracellular medium) was 0.5 mM. Thapsigargin ($10 \mu\text{M}$) 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) (A); 12 cells (15 cells tested) (B), AlCl_3 plus NaF; 5 cells (6 cells tested) (B), NaF plus deferoxamine and 5 cells (9 cells tested) (B), NaF.

Table 2 Effects of inhibitors and activators of protein kinases on thapsigargin- and vasopressin-stimulated Ca^{2+}_o -induced increase in $[\text{Ca}^{2+}]_i$ and the release of Ca^{2+} from intracellular stores in the presence and absence of GTP[S]

Experiments were performed as described in the Materials and methods section and in the legends to the Figures. GTP[S], cAMP, Rp cAMPS, AlCl_3 and NaF were microinjected into hepatocytes (estimated intracellular concentrations indicated). For treatment with genistein, hepatocytes were pre-incubated with genistein for 2.25 h before the injection of fura-2 and the inhibitor was also present during the measurement of fluorescence. Staurosporin and forskolin were added to the incubation dish 1.5 min before addition of thapsigargin or vasopressin. The concentrations of genistein, staurosporin and forskolin listed are extracellular concentrations. Data obtained for thapsigargin and vasopressin in the absence of GTP[S] or AlF_4^- and for thapsigargin plus GTP[S] and thapsigargin plus AlCl_3 and NaF are taken from Table 1. The values for the initial rate of Ca^{2+}_o -induced increase in $[\text{Ca}^{2+}]_i$ and the maximum increase in fluorescence induced by Ca^{2+}_o (used as a measure of Ca^{2+} inflow) and the maximum increase in fluorescence induced by agonist (Ca^{2+} release from intracellular Ca^{2+} stores) are the means $\pm$ S.E.M. of those obtained for the number of cells indicated. The degree of significance, determined using Student's *t*-test for unpaired samples, for a comparison of results obtained in the presence of a given combination of agonist (thapsigargin or vasopressin), GTP[S] or AlCl_3 plus NaF, and inhibitor or activator of protein kinase with the value in the presence of agonist alone, are: * $P \leq 0.05$; ** $P \leq 0.005$ and *** $P \leq 0.0005$.

							Ca ²⁺ release from intracellular stores	
Ca ²⁺ _o -induced increase in [Ca ²⁺] _i								
Agonist	GTP[S] or AlF ₄ [−]	Inhibitor or activator of protein kinase	Concentration of inhibitor or activator (μM)	Number of cells (number giving the response/total number tested)	Initial rate of Ca ²⁺ _o -induced increase in [Ca ²⁺] _i (fluorescence ratio units/min)	Maximum increase in fluorescence induced by Ca ²⁺ _o (fluorescence ratio units)	Number of cells (number giving a response/total number tested)	Maximum increase in fluorescence induced by agonist (fluorescence ratio units)
Thapsigargin (10 μM)	None	None	—	57/58	0.70 ± 0.08	0.50 ± 0.03	50/58	0.28 ± 0.02
		Genistein	100	6/6	0.16 ± 0.05*	0.27 ± 0.06*	6/6	0.23 ± 0.05
		cAMP	50	6/9	0.39 ± 0.07	0.60 ± 0.04	7/9	0.13 ± 0.04
		Forskolin	10	8/11	0.52 ± 0.07	0.33 ± 0.05***	10/11	0.22 ± 0.02
			80	7/10	0.16 ± 0.05*	0.14 ± 0.04***	7/10	0.30 ± 0.04
	GTP[S] (530 μM)	None	—	21/22	0.09 ± 0.01**	0.08 ± 0.01***	22/22	0.0
		Staurosporin	0.1	5/5	0.11 ± 0.04*	0.11 ± 0.02***	5/5	0.0
		Genistein	100	5/5	0.15 ± 0.06*	0.10 ± 0.05***	5/5	0.0
		Rp cAMPS	20	11/11	1.18 ± 0.25*	0.28 ± 0.05*	11/11	0.0
	AlCl ₃ (2 μM) + NaF (530 μM)	None	—	12/15	0.10 ± 0.02**	0.11 ± 0.04***	15/15	0.0
AlCl ₃ (2 μM) + NaF (530 μM)	Rp cAMPS	20	8/10	0.56 ± 0.19	0.20 ± 0.04**	10/10	0.0	
Vasopressin (10 nM)	None	None	—	30/30	0.63 ± 0.08	0.45 ± 0.03	28/30	0.56 ± 0.04
		Genistein	100	3/4	0.16 ± 0.03*	0.27 ± 0.09*	3/4	0.43 ± 0.08
		cAMP	50	4/5	0.19 ± 0.05*	0.21 ± 0.02**	4/5	0.41 ± 0.06
		Forskolin	80	6/7	0.21 ± 0.06*	0.25 ± 0.05**	6/7	0.41 ± 0.06
	None	None	—	7/7	0.86 ± 0.16	0.40 ± 0.08	6/7	0.37 ± 0.07
		Glucagon	1	6/8	0.65 ± 0.06	0.53 ± 0.13	4/8	0.53 ± 0.13

Figure 2 Rp cAMPS prevents the inhibition by GTP[S] of thapsigargin-stimulated Ca^{2+}_o -induced increase in $[\text{Ca}^{2+}]_i$

(A) Representative cell incubated in the absence of GTP[S] and Rp cAMPS and (B) representative cells incubated in the presence of GTP[S] alone or GTP[S] plus Rp cAMPS. Measurement of the fluorescence of single hepatocytes loaded with fura-2, and the microinjection of fura-2, GTP[S] and Rp cAMPS were performed as described in the Materials and methods section. The estimated intracellular concentrations of GTP[S] and Rp cAMPS were approx. 530 μM and 20 μM respectively. Thapsigargin (10 μM) 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) (A); 21 cells (22 cells tested) [(B), GTP[S]] and 11 cells (11 cells tested) [(B), GTP[S] plus Rp cAMPS].

Figure 3 Inhibition of thapsigargin-stimulated Ca^{2+} -induced increase in $[\text{Ca}^{2+}]_i$ by cAMP and forskolin

(A) Effect of cAMP on thapsigargin-stimulated Ca^{2+} -induced increase in $[\text{Ca}^{2+}]_i$ in a single cell, (B) concentration–response curve for the effect of cAMP on thapsigargin-stimulated Ca^{2+} inflow and (C) the effect of forskolin on thapsigargin-stimulated Ca^{2+} -induced increase in $[\text{Ca}^{2+}]_i$ in a single cell. Measurement of the fluorescence of single hepatocytes loaded with fura-2, the microinjection of fura-2 and cAMP, estimation of the intracellular concentrations of cAMP, and estimation of the rate of thapsigargin-stimulated Ca^{2+} -induced increase in $[\text{Ca}^{2+}]_i$ were performed as described in the Materials and methods section. The estimated intracellular concentration of cAMP in (A) was approx. 530 μM. In (C), forskolin (extracellular concentration 80 μM) was added to the incubation dish 1.5 min before thapsigargin. In (A) and (C) thapsigargin (10 μM) 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 in (A) and (C) are representative of the results obtained for 57 cells (58 cells tested) [(A) and (C), control]; 6 cells (9 cells tested) [(A), cAMP]; and 7 cells (10 cells tested) [(C), forskolin]. In (B), the results shown are the means $\pm$ S.E.M. of those obtained from 7–9 cells (cAMP present) and 57 cells (no cAMP present).

protein kinase C, which can be activated indirectly by GTP[S] (through activation of phospholipase C and the generation of diacylglycerol [17]), inhibits store-activated Ca^{2+} inflow in some other cell types [17,25]. In order to verify that staurosporin does inhibit protein kinase C under the conditions of the present experiments, the effect of staurosporin on the protein kinase C-mediated inhibition of the adrenaline (epinephrine)-stimulated increase in $[\text{Ca}^{2+}]_i$ [26] was tested. Staurosporin reversed inhibition of the adrenaline-stimulated Ca^{2+} -induced increase in $[\text{Ca}^{2+}]_i$ by PMA (an activator of protein kinase C [26]). The rates of Ca^{2+} -induced increase in $[\text{Ca}^{2+}]_i$ in hepatocytes treated with 10 μM adrenaline, adrenaline plus 450 nM PMA, and adrenaline plus PMA plus 100 nM staurosporin were 0.75 ± 0.23 (5), 0.01 ± 0.002 (4) and 0.86 ± 0.1 (4) fluorescence ratio units per min respectively (numbers of cells are given in parentheses). Thus the inability of staurosporin to reverse the GTP[S]-induced inhibition of the thapsigargin-stimulated Ca^{2+} -induced increase in $[\text{Ca}^{2+}]_i$ is unlikely to be due to the failure of this agent to inhibit protein kinase C.

In contrast to the actions of staurosporin and genistein, Rp cAMPS, an inhibitor of protein kinase A [27], prevented GTP[S]-induced inhibition of the thapsigargin-stimulated Ca^{2+} -induced increase in $[\text{Ca}^{2+}]_i$ [Figure 2B (broken trace), cf. Figure 2A, and Table 2] and partially prevented the inhibition induced by AlCl_3 plus NaF (Table 2). In the absence of GTP[S], Rp cAMPS had little effect on the thapsigargin-stimulated Ca^{2+} -induced increase in $[\text{Ca}^{2+}]_i$ or on Ca^{2+} release, and did not stimulate the Ca^{2+} -induced increase in $[\text{Ca}^{2+}]_i$ in the absence of thapsigargin or vasopressin (results not shown). These results suggested that inhibition of the store-activated Ca^{2+} -induced increase in $[\text{Ca}^{2+}]_i$ by GTP[S] may be mediated by protein kinase A.

The involvement of protein kinase A was tested further by investigating the effects of intracellular cAMP. The microinjection of cAMP caused a substantial inhibition of the thapsigargin- and vasopressin-stimulated Ca^{2+} -induced increase in $[\text{Ca}^{2+}]_i$ (Figure 3A and Table 2). cAMP had little effect on the thapsigargin- and vasopressin-induced release of Ca^{2+} from intracellular stores (Table 2) and no effect on GTP[S]-inhibited thapsigargin-stimulated Ca^{2+} -induced increase in $[\text{Ca}^{2+}]_i$ (results not shown). The intracellular concentration of cAMP which

gave half maximal inhibition of the thapsigargin-stimulated initial rate of Ca^{2+} -induced increase in $[\text{Ca}^{2+}]_i$ was approximately 80 μM (Figure 3B). Forskolin, an activator of adenylate cyclase [28], also inhibited thapsigargin- and vasopressin-stimulated Ca^{2+} -induced increase in $[\text{Ca}^{2+}]_i$ with little effect on the release of Ca^{2+} from intracellular stores (Figure 3C and Table 2). In contrast to the actions of cAMP, cGMP did not mimic the inhibitory effects of GTP[S] on the thapsigargin-stimulated Ca^{2+} -induced increase in $[\text{Ca}^{2+}]_i$ and had no effect on the increase in $[\text{Ca}^{2+}]_i$ induced by the addition of Ca^{2+} in the absence of thapsigargin or vasopressin (results not shown).

Effects of glucagon

In order to assess the physiological role of the inhibition by cAMP and protein kinase A of an agonist-stimulated Ca^{2+} -induced increase in $[\text{Ca}^{2+}]_i$, the effects of glucagon were tested. In the absence of vasopressin or a SERCA inhibitor, and in the absence of added Ca^{2+} , glucagon (1 μM) itself caused a small release of Ca^{2+} from intracellular stores (observed as an increase in the fluorescence ratio) in approximately 30% of the cells tested (results not shown). Glucagon inhibited the thapsigargin-stimulated initial rate of Ca^{2+} -induced increase in $[\text{Ca}^{2+}]_i$, but did not greatly affect either the maximum increase in fluorescence induced by Ca^{2+} or the thapsigargin-induced release of Ca^{2+} from stores (Figure 4A and Table 3). The concentration of glucagon that gave half-maximal inhibition of the initial rate of thapsigargin-stimulated Ca^{2+} -induced increase in $[\text{Ca}^{2+}]_i$ was approximately 10 nM (Figure 4B). Glucagon caused a small inhibition of the vasopressin-stimulated initial rate of Ca^{2+} -induced increase in $[\text{Ca}^{2+}]_i$ and some reduction in the vasopressin-induced release of Ca^{2+} from intracellular stores (Table 2).

Evaluation of the role of mitochondria in the protein kinase A-mediated inhibition of the agonist-stimulated Ca^{2+} -induced increase in $[\text{Ca}^{2+}]_i$

The results described above indicate that protein kinase A inhibits the thapsigargin- and vasopressin-stimulated Ca^{2+} -

Figure 4 Inhibition of thapsigargin-stimulated Ca^{2+} -induced increase in $[\text{Ca}^{2+}]_i$ by glucagon

(A) Effect of glucagon on thapsigargin-induced release of Ca^{2+} from intracellular stores and Ca^{2+} -induced increase in $[\text{Ca}^{2+}]_i$ in single cells. (B) Concentration–response curve for the inhibition by glucagon of thapsigargin-stimulated Ca^{2+} -induced increase in $[\text{Ca}^{2+}]_i$. Measurement of the fluorescence of single hepatocytes loaded with fura-2, the microinjection of fura-2 and calculation of rates of thapsigargin-stimulated Ca^{2+} -induced increase in $[\text{Ca}^{2+}]_i$ were performed as described in the Materials and methods section. In (A) glucagon ($1 \mu\text{M}$) (broken trace) was added to the incubation dish at the beginning of the period indicated by the horizontal bar. The traces shown in (A) are representative of the results obtained for 23 cells (26 cells tested) (solid trace, glucagon absent) and for 15 cells (18 cells tested) (broken trace, glucagon present). In (B), rates of thapsigargin-stimulated Ca^{2+} -induced increase in $[\text{Ca}^{2+}]_i$ obtained in the presence of glucagon are expressed as a percentage of those obtained in the absence of hormone. The data shown are the means $\pm$ S.E.M. of those obtained for 6–7 individual cells in two separate experiments.

Table 3 Effects of inhibitors of mitochondrial Ca^{2+} uptake on thapsigargin-stimulated Ca^{2+} -induced increase in $[\text{Ca}^{2+}]_i$

Experiments were performed as described in the Materials and methods section and in the legends of the Figures. Thapsigargin ($10 \mu\text{M}$) was present in all experiments. Azide, forskolin and dibutylryl cAMP were added to the extracellular medium (extracellular concentrations indicated). Ruthenium Red was introduced by microinjection (estimated intracellular concentration indicated). The values for the initial rate of Ca^{2+} -induced increase in $[\text{Ca}^{2+}]_i$ and the maximum increase in fluorescence induced by Ca^{2+} (Ca^{2+} inflow) and the maximum increase in fluorescence induced by agonist (Ca^{2+} release from intracellular Ca^{2+} stores) are the means $\pm$ S.E.M. for the number of cells indicated. The degrees of significance, determined using Student's *t*-test for unpaired samples, for a comparison of the results obtained with a given concentration of activator of cAMP-dependent protein kinase and inhibitor of mitochondrial Ca^{2+} uptake with those obtained in the absence of an activator of cAMP-dependent protein kinase and inhibitor of mitochondrial Ca^{2+} uptake are: * $P \leq 0.05$; ** $P \leq 0.005$; *** $P \leq 0.0005$ and **** $P \leq 0.00005$.

Activator of cAMP-dependent protein kinase	Inhibitor of mitochondrial Ca^{2+} uptake	Thapsigargin-stimulated Ca^{2+} -induced increase in $[\text{Ca}^{2+}]_i$				Ca ²⁺ release from intracellular stores
		Number of cells (number giving the response/total number tested)	Initial rate of Ca^{2+} -induced increase in $[\text{Ca}^{2+}]_i$ (fluorescence ratio units/min)	Maximum increase in fluorescence induced by Ca^{2+} (fluorescence ratio units)	Number of cells (number giving the response/total number tested)	Maximum increase in fluorescence induced by agonist (fluorescence ratio units)
None	None	23/26	0.91 ± 0.09	0.36 ± 0.04	18/26	0.28 ± 0.04
	Azide (5 mM)	6/8	0.64 ± 0.11	0.44 ± 0.04	7/8	0.21 ± 0.04
	FCCP ($1 \mu\text{M}$)	4/4	0.02 ± 0.01 ****	0.03 ± 0.01	4/4	0.38 ± 0.15
	Ruthenium Red ($20 \mu\text{M}$)	8/8	1.13 ± 0.12	0.51 ± 0.03 **	8/8	0.31 ± 0.13
Glucagon ($1 \mu\text{M}$)	None	14/18	0.22 ± 0.03 ***	0.42 ± 0.04	13/18	0.42 ± 0.04
	Azide (5 mM)	7/12	0.15 ± 0.06 ***	0.17 ± 0.04 ***	7/12	0.17 ± 0.04 *
	FCCP ($1 \mu\text{M}$)	3/3	0.04 ± 0.01 **	0.05 ± 0.01	3/3	0.46 ± 0.14
	None	3/4	0.44 ± 0.06	0.48 ± 0.06	4/4	0.49 ± 0.19
Forskolin ($150 \mu\text{M}$) + dibutylryl cAMP ($50 \mu\text{M}$)	None	3/4	0.44 ± 0.06	0.48 ± 0.06	4/4	0.49 ± 0.19
	Ruthenium Red ($20 \mu\text{M}$)	4/4	0.68 ± 0.27	1.0 ± 0.08 ****	4/4	0.14 ± 0.03

induced increase in $[\text{Ca}^{2+}]_i$, suggesting that this protein kinase inhibits thapsigargin- and vasopressin-stimulated Ca^{2+} inflow. However, it has previously been shown that, in the presence of a hormone that increases $[\text{Ca}^{2+}]_i$, or a SERCA inhibitor, glucagon or cAMP synergistically increases net Ca^{2+} inflow to hepatocytes [6–11] and the net accumulation of Ca^{2+} in liver mitochondria [6,7,10,11]. In addition, it has been shown that in some other cell types, mitochondrial Ca^{2+} uptake can diminish increases in $[\text{Ca}^{2+}]_i$ induced by plasma membrane Ca^{2+} inflow or SER Ca^{2+} release [29–32]. These observations raised the possibility that, in

the present experiments, the observed inhibition of the hormone or SERCA inhibitor-stimulated Ca^{2+} -induced increase in $[\text{Ca}^{2+}]_i$ by protein kinase A is due to enhanced mitochondrial Ca^{2+} uptake. This possibility was investigated by testing the effect of agents which inhibit mitochondrial Ca^{2+} uptake on the thapsigargin-stimulated Ca^{2+} -induced increase in $[\text{Ca}^{2+}]_i$. The agents employed were azide, which inhibits the terminal step in the electron-transport chain [11], FCCP, which renders the mitochondrial inner membrane permeable to protons and uncouples electron transport from ATP synthesis [29], and Ruthenium Red,

Figure 5 Enhancement by Ruthenium Red of thapsigargin-stimulated Ca^{2+} -induced increase in $[\text{Ca}^{2+}]_c$ in cells incubated in the presence of forskolin and dibutyryl cAMP

Measurement of the fluorescence of single hepatocytes loaded with fura-2 and the microinjection of fura-2 and Ruthenium Red were performed as described in the Materials and methods section. Thapsigargin ($10 \mu\text{M}$) and Ca^{2+} (1.5 mM) were added to the incubation dish at the beginning of the periods indicated by the horizontal bars. Forskolin ($80 \mu\text{M}$) and dibutyryl cAMP ($50 \mu\text{M}$) were added to the incubation dish 1.5 min before the addition of thapsigargin. The solid (Ruthenium Red absent) and broken (Ruthenium Red present) traces are representative of the results obtained for 4 cells (6 cells tested) and 3 cells (4 cells tested) respectively.

a potent inhibitor of mitochondrial Ca^{2+} uptake [33]. If the activation of protein kinase A increases mitochondrial Ca^{2+} uptake, the observed rate of the thapsigargin-stimulated Ca^{2+} -induced increases in $[\text{Ca}^{2+}]_c$ would be expected to be greater in the presence of azide, FCCP or Ruthenium Red than the rate observed in the absence of these agents.

The addition of azide or FCCP to fura-2-loaded hepatocytes, incubated in the absence of thapsigargin and in the absence of Ca^{2+} , caused a small increase in fluorescence (results not shown). This is presumably due to the release of Ca^{2+} from mitochondria. Azide and FCCP inhibited the thapsigargin-stimulated rate of Ca^{2+} -induced increase in $[\text{Ca}^{2+}]_c$ but had no effect on the amount of Ca^{2+} released by thapsigargin (Table 3). In hepatocytes incubated with glucagon, both azide and FCCP further inhibited (beyond inhibition caused by glucagon) the initial rate and extent (maximum increase in fluorescence) of the thapsigargin-stimulated Ca^{2+} -induced increase in $[\text{Ca}^{2+}]_c$ (Table 3). Results similar to those shown for FCCP alone were obtained when oligomycin was added with FCCP (not shown). On the other hand, Ruthenium Red, introduced into the cytoplasmic space by co-injection with fura-2, caused a substantial increase in the initial rate of the thapsigargin-stimulated Ca^{2+} -induced increase in $[\text{Ca}^{2+}]_c$ in cells incubated in the absence of agents that increase cAMP (Table 3) and in cells incubated in the presence of forskolin and dibutyryl cAMP (Figure 5 and Table 3). The effect of Ruthenium Red in the presence of dibutyryl cAMP and forskolin was most clearly evident when the magnitude of the plateau of the Ca^{2+} -induced increase in fluorescence was measured (Figure 4 and Table 3). Ruthenium Red did not substantially alter the amount of Ca^{2+} released from intracellular stores by thapsigargin (Table 3).

DISCUSSION

Role of protein kinase A in mediating GTP[S]-induced inhibition of Ca^{2+} -induced increase in $[\text{Ca}^{2+}]_c$

The results indicate that GTP[S] (approximately $530 \mu\text{M}$ intracellular), as well as pp[NH]pG, ATP[S], pp[NH]pA, GDP[S] and AlF_4^- , inhibited the actions of vasopressin or SERCA inhibitors in stimulating the Ca^{2+} -induced increase in $[\text{Ca}^{2+}]_c$ in hepatocytes. It is considered unlikely that the inhibition of the vasopressin- or thapsigargin-stimulated Ca^{2+} -induced increase in $[\text{Ca}^{2+}]_c$ by GTP[S], pp[NH]pG or AlF_4^- is due to inhibition by these agents of $G_{q/11}$ (which activates phospholipase $C\beta$, leading to the formation of $\text{Ins}(1,4,5)\text{P}_3$ and SER Ca^{2+} release; reviewed in [1]), G_{12} (which is required for store-activated Ca^{2+} inflow in hepatocytes [12–15]) or the ability of thapsigargin to interact with the SERCA ($\text{Ca}^{2+} + \text{Mg}^{2+}$)ATP-ase and induce the release of Ca^{2+} from the SER. Thus the addition of thapsigargin or vasopressin to cells previously loaded with the high GTP[S] concentration or treated with AlF_4^- did not cause an increase in fura-2 fluorescence (i.e. release Ca^{2+} from intracellular stores). Moreover, previous studies have shown that the photolysis of 'caged GTP[S]' induces the release of Ca^{2+} from intracellular stores [13]. Thus it can be concluded that upon introduction to the cytoplasmic space, GTP[S] and AlF_4^- released Ca^{2+} from intracellular stores. This indicates that GTP[S] and AlF_4^- each activated $G_{q/11}$. Furthermore, we are not aware of any reports of trimeric G-proteins that are inhibited by GTP[S] or AlF_4^- . Inhibition of the SERCA inhibitor-initiated activation of RACCs by GDP[S], an inhibitor of trimeric G-proteins [34], is thought to be due to the inhibition of G_{12} by GDP[S] [12].

The observations that GTP[S] and AlF_4^- inhibit the stimulation of the Ca^{2+} -induced increase in $[\text{Ca}^{2+}]_c$ induced by SERCA inhibitors suggest that GTP[S] and AlF_4^- activate a trimeric G-protein which initiates a pathway that inhibits the activation of RACCs [17,18,25]. The finding that ATP[S], which is known to cause constitutive activation of protein kinases [35,36], also inhibited the thapsigargin-stimulated Ca^{2+} -induced increase in $[\text{Ca}^{2+}]_c$, together with the results of others which indicate that protein kinases C and A may inhibit store-activated Ca^{2+} channels in other cell types [17,18,25], suggested that a protein kinase might be involved in the GTP[S]-initiated inhibitory pathway.

Genistein and staurosporin, inhibitors of protein-tyrosine kinases [24] and protein kinase C [23] respectively, did not reverse the GTP[S]-induced inhibition of the thapsigargin-stimulated Ca^{2+} -induced increase in $[\text{Ca}^{2+}]_c$, and cGMP, an activator of cGMP-dependent protein kinase, did not inhibit the thapsigargin-stimulated Ca^{2+} -induced increase in $[\text{Ca}^{2+}]_c$ in the absence of GTP[S]. Thus it may be concluded that protein kinase C, cGMP-activated protein kinase and a protein-tyrosine kinase are unlikely to be responsible for the GTP[S]-induced inhibition of the thapsigargin-stimulated Ca^{2+} -induced increase in $[\text{Ca}^{2+}]_c$. The observation that genistein inhibited the thapsigargin-stimulated Ca^{2+} -induced increase in $[\text{Ca}^{2+}]_c$ at concentrations used by others [37] suggests that the action of a protein-tyrosine kinase is required in some way for the activation of RACCs in hepatocytes, as proposed for some other cell types [1,37]. This result also provides evidence that the inability of genistein to prevent the GTP[S] inhibition of the SERCA inhibitor-stimulated Ca^{2+} -induced increase in $[\text{Ca}^{2+}]_c$ is not due to a failure of genistein to inhibit protein-tyrosine kinases.

The observations that (i) Rp cAMPS, an inhibitor of protein kinase A [27], prevented the inhibition of thapsigargin-stimulated Ca^{2+} -induced increase in $[\text{Ca}^{2+}]_c$ by GTP[S] and (ii) cAMP, forskolin and glucagon mimicked the GTP[S] inhibition of the thapsigargin-stimulated Ca^{2+} -induced increase in $[\text{Ca}^{2+}]_c$ indi-

cate that in rat hepatocytes protein kinase A can inhibit the Ca^{2+}_o -induced increase in $[\text{Ca}^{2+}]_i$. Therefore, the inhibition by GTP[S] and AlF_4^- of the thapsigargin-stimulated Ca^{2+}_o -induced increase in $[\text{Ca}^{2+}]_i$ is likely to be due to the activation of G_s , the formation of cAMP and the activation of protein kinase A, which, in turn, inhibits the thapsigargin-stimulated Ca^{2+}_o -induced increase in $[\text{Ca}^{2+}]_i$.

It is considered likely that the inhibition of the thapsigargin-stimulated Ca^{2+}_o -induced increase in $[\text{Ca}^{2+}]_i$ by protein kinase A represents a physiological function of protein kinase A. Thus, glucagon inhibited the thapsigargin-stimulated Ca^{2+}_o -induced increase in $[\text{Ca}^{2+}]_i$ at concentrations within the physiological range [38], the concentrations of intracellular cAMP microinjected into the cells were in the range of cAMP concentrations measured in hepatocytes [38], the actions of microinjected cAMP were mimicked by the application of forskolin, and the concentrations of forskolin and Rp cAMPS used in these experiments are within the ranges used by others [27,39]. Hepatocytes appear to be similar to platelets [40] and *Xenopus* oocytes [17], where it has been shown that protein kinase A regulates store-activated Ca^{2+} inflow.

While ATP[S] , which has been shown to be utilized by protein kinases [35,36], would have been expected to inhibit thapsigargin-stimulated Ca^{2+}_o -induced increase in $[\text{Ca}^{2+}]_i$, pp[NH]pA , which is not known to activate or be utilized by protein kinases [41], would not be expected to inhibit thapsigargin-stimulated Ca^{2+}_o -induced increase in $[\text{Ca}^{2+}]_i$. This observation suggests that the action of pp[NH]pA , and possibly also that of ATP[S] , reflects intervention by these nucleotides at another site in the pathway of RACC activation. Possibilities for another site include the SERCA or plasma membrane ($\text{Ca}^{2+} + \text{Mg}^{2+}$) ATP-ases [42] and the utilization of pp[NH]pA as a substrate for adenylate cyclase [43].

The present results, together with our previous study, which provided evidence that a monomeric G-protein (possibly the Arf protein) is required for store-activated Ca^{2+} inflow in hepatocytes [4], indicate that the observed inhibition of store-activated Ca^{2+}_o -induced increase in $[\text{Ca}^{2+}]_i$ by GTP[S] is due to the action of GTP[S] on at least two sites. These are: (i) inhibition of action of an Arf protein (or other monomeric G-protein) [4], and (ii) the activation of trimeric protein G_s , which leads to the formation of cAMP and activation of protein kinase A (present results).

The role of mitochondria in the protein kinase A-induced inhibition of Ca^{2+}_o -induced increase in $[\text{Ca}^{2+}]_i$

As discussed in the Introduction, agonist- or SERCA inhibitor-stimulated Ca^{2+}_o -induced increase in $[\text{Ca}^{2+}]_i$ in the Ca^{2+} add-back protocol is normally taken as a measure of the stimulation of Ca^{2+} inflow through RACCs. Therefore the observation that protein kinase A inhibits the thapsigargin-stimulated Ca^{2+}_o -induced increase in $[\text{Ca}^{2+}]_i$ was unexpected in view of results reported by others. These have clearly shown that, in conjunction with the actions of either a hormone, which increases $[\text{Ca}^{2+}]_i$, or a SERCA inhibitor, an increase in intracellular cAMP causes a substantial increase in net Ca^{2+} uptake by hepatocytes [6,7] and hepatocyte mitochondria [6,7,10,11]. As far as we are aware, a synergistic effect of cAMP in increasing $[\text{Ca}^{2+}]_i$ has not previously been clearly demonstrated, although there is at least one report which suggests that, in the presence of vasopressin, cAMP induces an increase in $[\text{Ca}^{2+}]_i$ [44]. Thus there is an apparent discrepancy between the pronounced cAMP-induced enhancement of net Ca^{2+} uptake by hepatocytes [6,7] on the one hand and the cAMP-induced inhibition of the agonist-stimulated Ca^{2+}_o -induced increase in $[\text{Ca}^{2+}]_i$ (present results) on the other.

One explanation for this discrepancy is that the effect of cAMP (protein kinase A) is to enhance the movement of Ca^{2+} from the cytoplasmic space to mitochondria and/or to make more mitochondria available for the removal of Ca^{2+} from the cytoplasmic space. This would reduce the increase in $[\text{Ca}^{2+}]_i$ caused by the addition of Ca^{2+}_o and would promote the net movement of Ca^{2+} from the extracellular medium to the mitochondria. This action of the mitochondria may also alleviate the feedback inhibition of RACCs by Ca^{2+} [45] by lowering $[\text{Ca}^{2+}]_i$ in the vicinity of the plasma membrane RACC proteins and hence allowing greater Ca^{2+} flux through open channels.

The idea that protein kinase A causes inhibition of the agonist-stimulated Ca^{2+}_o -induced increase in $[\text{Ca}^{2+}]_i$ by enhancing the uptake of Ca^{2+} to the mitochondria was tested using inhibitors of mitochondrial Ca^{2+} uptake to block the availability of mitochondria as a sink for Ca^{2+} entering the cytoplasmic space. Neither azide nor FCCP restored the agonist-stimulated Ca^{2+}_o -induced increase in $[\text{Ca}^{2+}]_i$ to the value observed in the absence of protein kinase A activation. Indeed, as shown previously [46], FCCP caused further inhibition of agonist-stimulated Ca^{2+}_o -induced increase in $[\text{Ca}^{2+}]_i$. However, interpretation of the results obtained with azide and FCCP is complicated by the likelihood that these agents cause a decrease and an increase respectively in the intracellular concentrations of ATP and ADP, which, in turn, may inhibit store-activated Ca^{2+} inflow in hepatocytes by an unknown mechanism [47,48].

In contrast to the results obtained with FCCP and azide, the microinjection of Ruthenium Red, a potent inhibitor of mitochondrial Ca^{2+} uptake [33], substantially increased the rate of the thapsigargin-stimulated Ca^{2+}_o -induced increase in $[\text{Ca}^{2+}]_i$ in cells incubated in both the presence and absence of forskolin and dibutyryl cAMP. The idea that the protein kinase A-mediated inhibition of the thapsigargin-stimulated Ca^{2+}_o -induced increase in $[\text{Ca}^{2+}]_i$ is due to the action of protein kinase A in enhancing mitochondrial Ca^{2+} uptake, or in making available more mitochondria for Ca^{2+} uptake, is consistent with the results obtained with Ruthenium Red. As mentioned previously, others have clearly shown an enhancement by cAMP of plasma membrane Ca^{2+} inflow (through RACCs) in the presence of a hormone which increases $[\text{Ca}^{2+}]_i$ [6–11]. Together with these results of others, the results reported here suggest that the site of action of protein kinase A in modulating Ca^{2+} inflow through hepatocyte RACCs is on, or is associated with, the mitochondria, rather than a site which leads to an increase in the probability of opening of RACC proteins. However, the possibility that protein kinase A enhances the release of Ca^{2+} from a region of the SER which cannot be detected in the present experiments and this, in turn, enhances the opening of RACCs cannot be eliminated.

The physiological reason for the action of protein kinase A in enhancing Ca^{2+} uptake by hepatocyte mitochondria is not known. One possibility is that enhanced inflow of Ca^{2+} to mitochondria activates Ca^{2+} -sensitive enzymes in these organelles [49]. In some other cell types, mitochondria have been shown to attenuate changes in $[\text{Ca}^{2+}]_i$ induced by either the release of Ca^{2+} from intracellular stores or by the inflow of Ca^{2+} across the plasma membrane [29–32]. It has been proposed that the flow of Ca^{2+} to strategically-located mitochondria activates (or regulates) mitochondrial metabolic and/or biosynthetic pathways in synchrony with other cellular processes activated by the increase in $[\text{Ca}^{2+}]_i$ [29–32].

The present results indicate that caution should be exercised in using the initial rate and extent of the Ca^{2+}_o -induced increase in $[\text{Ca}^{2+}]_i$ (estimated, for example, by measuring the fluorescence of an intracellular fluorescent dye such as fura-2) as a measure of agonist-stimulated Ca^{2+} inflow through RACCs. As pointed out

previously [19], the increase in $[\text{Ca}^{2+}]_c$ which follows the addition of Ca^{2+}_o to cells incubated in the presence of an agonist and in the absence of added Ca^{2+}_o will be determined by both the rate of Ca^{2+} inflow to the cytoplasmic space through RACCs and the rate at which Ca^{2+} is removed from the cytoplasmic space. Processes responsible for Ca^{2+} removal from the cytoplasmic space include the SERCAs, plasma membrane $(\text{Ca}^{2+} + \text{Mg}^{2+})$ -ATP-ases, Ca^{2+} uptake by mitochondria, Ca^{2+} uptake by other organelles (e.g. secretory granules) and the binding of Ca^{2+} to proteins and metabolites [1,2,7–11]. While the effects of some of these removal pathways might be minimized by measuring the initial rate of the Ca^{2+}_o -induced increase in $[\text{Ca}^{2+}]_c$ (rather than the shape of the curve or the extent of the increase), the present results suggest that this may not be the case for Ca^{2+} inflow to mitochondria, which appears to influence both the initial rate and extent of the Ca^{2+}_o -induced increase in $[\text{Ca}^{2+}]_c$. This also seems to be the case for an endothelial cell line where the location of mitochondria near RACCs in the plasma membrane appears to result in the preferential uptake by mitochondria of Ca^{2+} that enters the cells through RACCs [30]. The spatial arrangement of mitochondria in hepatocytes may also explain why it appears that, in these cells, mitochondrial Ca^{2+} uptake has a greater effect on the observed profile of the Ca^{2+}_o -induced increase in $[\text{Ca}^{2+}]_c$ (measured using a Ca^{2+} -sensitive fluorescent dye) than on the observed profile for the release of Ca^{2+} from intracellular stores [44] (cf. the results obtained with endothelial cells [30]).

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