G-protein regulation of capacitative calcium entry may be mediated by protein kinases A and C in *Xenopus* oocytes

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Inositol 2,4,5-trisphosphate irreversibly activated capacitative calcium entry in *Xenopus* oocytes, whereas guanosine thiotriphosphate (GTP[S]) and AlF_4^- only activated capacitative calcium entry transiently. Both GTP[S] and AlF_4^- inhibited capacitative calcium entry activated by thapsigargin pretreatment, but guanosine thiodiphosphate (GDP[S]), inositol 2,4,5-trisphosphate and dibutyl cyclic GMP did not affect capacitative calcium entry. This suggests the involvement of heterotrimeric GTP-binding proteins in the regulation of capacitative

calcium entry. Activation of protein kinase C or cyclic-AMP-dependent protein kinase had profound effects on capacitative calcium entry, which were consistent with the hypothesis that the effects of GTP[S] and AlF_4^- on capacitative calcium entry may be mediated via heterotrimeric GTP-binding protein stimulation of kinases. Further evidence for this hypothesis was derived from the result that the effects of GTP[S] on calcium entry could be inhibited by the application of the protein kinase inhibitor staurosporine.

INTRODUCTION

After the release of calcium from intracellular stores by elevation of the cytosolic inositol 1,4,5-trisphosphate (InsP_3) concentration, a calcium-influx pathway is activated in many cell types [1–3]. This calcium-influx pathway has been termed capacitative, since it depends on the depletion of the intracellular stores irrespective of how this has been carried out [1].

The molecular mechanism activating this capacitative calcium-entry pathway remains controversial, with suggestions including: a low-molecular-mass diffusible messenger produced at the calcium stores [4,5]; cyclic GMP (cGMP) [6]; a direct protein–protein interaction between a plasma-membrane calcium channel and a regulatory protein situated on the stores [7–9]; or the direct involvement of G-proteins [3,10–12].

Activation of G-proteins can have a profound effect on the capacitative calcium-entry pathway in rat basophilic leukaemia cells [10], mouse lacrimal acinar cells [11] and HL-60 granulocytes [12]. The results from these different studies are not entirely compatible. Although all three papers report inhibition of capacitative calcium entry by the introduction of guanosine 5'-[γ -thio]triphosphate (GTP[S]) into the cell, guanosine 5'-[β -thio]diphosphate (GDP[S]) only inhibited capacitative calcium entry in mouse lacrimal cells, having no effect in the other cell types. Equally, the effects of GTP[S] could be fully mimicked by fluoride activation of G-proteins in HL-60 granulocytes, but only partially in rat basophilic leukaemia cells. Bird and Putney [11] and Fasolato et al. [10] conclude that a small monomeric G-protein is essential for the activation of capacitative calcium entry, whereas Jaconi et al. [12] conclude that a heterotrimeric G-protein is involved. Following on from these papers, we have investigated the G-protein regulation of capacitative calcium entry in *Xenopus* oocytes. Our data suggest that only heterotrimeric G-proteins are involved, evoking their effects on capacitative calcium entry indirectly through activation of phospholipase C and adenylate cyclase.

MATERIALS AND METHODS

Preparation of oocytes

Oocytes were obtained from *Xenopus laevis* and manually defolliculated. The oocytes were maintained at 17 °C in an incubation medium containing (mM): 115 NaCl, 2 KCl, 1 MgCl_2 , 2 CaCl_2 , 2.5 sodium pyruvate, 50 mg/ml gentamicin, 5 Hepes (pH 7.4 adjusted by NaOH). All chemicals (except caged compounds) were obtained from Sigma.

Electrophysiological measurements

In the cell types where it has been directly measured, the capacitative calcium entry current is very small [13,14], and we were unable to detect it directly in *Xenopus* oocytes. However, *Xenopus* oocytes have many calcium-activated chloride channels in the plasma membrane, which we used as an amplification system. Thus a small quantity of calcium entering the cell across the plasma membrane can activate chloride channels giving rise to currents of several microamperes when the cell is voltage-clamped at a suitable potential. The calcium-activated chloride current is very sensitive to changes in calcium concentration near the plasma membrane. Experiments involving calcium injections and flash photolysis of DM-nitrophen [1-(2-nitro-4,5-dimethoxyphenyl)-1,2-diaminoethane-*NNN'*-tetra-acetic acid] show that the peak amplitude of currents evoked has a linear relationship to the amount of calcium released [9,15], and thus the calcium-activated chloride current appears to provide a useful measure of the calcium influx.

To record the calcium-activated chloride current, the oocyte was impaled in the animal pole by two microelectrodes (2 M KCl-filled, resistance 0.5–2 M Ω) and voltage clamped to –60 mV with a Warner Instruments oocyte clamp amplifier (OC-725B). All experiments were carried out at room temperature (18–22 °C). The perfusion system employed allowed rapid and consistent

Abbreviations used: InsP_3 , inositol 1,4,5-trisphosphate; cAMP, cyclic AMP; cGMP, cyclic GMP; G-protein, GTP-binding protein; GTP[S], guanosine 5'-[γ -thio]triphosphate; GDP[S], guanosine 5'-[β -thio]diphosphate; PKC, protein kinase C; PKA, cAMP-dependent protein kinase; DM-nitrophen, 1-(2-nitro-4,5-dimethoxyphenyl)-1,2-diaminoethane-*NNN'*-tetra-acetic acid; caged InsP_3 , *D*-myo-inositol 1,4,5-trisphosphate $P^{4(6)}$ -1-(2-nitrophenyl)ethyl ester; $\text{Ins}(2,4,5)\text{P}_3$, inositol 2,4,5-trisphosphate; db-cAMP, dibutyl cAMP; db-cGMP, dibutyl cGMP; PMA, phorbol 12-myristate 13-acetate.

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changes of the perfusate in the vicinity of the oocyte, with a half-time of exchange of 0.5 s, as previously described [16]. The perfusates contained (mM): 115 NaCl, 2 KCl, 1 MgCl₂, 5 Hepes (pH 7.4 adjusted by NaOH) with either 2 mM CaCl₂ added to give calcium-containing perfusate or 1 mM EGTA added to give calcium-free perfusate.

Depletion of intracellular stores by thapsigargin

Most of the experiments investigating the regulation of capacitative calcium entry were done on oocytes which had been pretreated with thapsigargin. The oocytes were incubated at 17 °C for over 3 h in a medium containing (mM): 115 NaCl, 2 KCl, 1 MgCl₂, 1 EGTA, 5 Hepes (pH 7.4 adjusted by NaOH) with 1 μ M thapsigargin. DMSO was used to dissolve thapsigargin, and after dilution the final concentration of DMSO was 0.1 %.

Injection of oocytes

While under two-electrode voltage clamp, oocytes were injected with various compounds by using a Drumond Nanoject injector. GDP (Na⁺ salt), GDP[S] (Li⁺ salt), GTP (Li⁺ salt), GTP[S] (Li⁺ salt), AlF₄⁻ (Na⁺ salt) and Ins(2,4,5)P₃ (NH₄⁺ salt) were dissolved in distilled water (pH 6.5–7) and a volume of 23 nl was injected. Injection of the same concentration of Hepes-buffered solution (pH 6.5–7) had no effect on capacitative calcium entry. The volume of a *Xenopus* oocyte is approx. 1 μ l, and all intracellular concentrations after injection are worked out on this assumption.

Flash photolysis of caged compounds

In some experiments oocytes were injected by using the Drumond Nanoject apparatus, with 23 nl of 1 mM caged InsP₃ [D-*myo*-inositol 1,4,5-trisphosphate P⁴⁽⁵⁾-1-(2-nitrophenyl)ethyl ester] or 50 mM DM-nitrophen (90 % loaded with calcium). Caged compounds were obtained from Calbiochem (La Jolla, CA, U.S.A.) and dissolved in distilled water. The oocytes were left to equilibrate for over 30 min before proceeding with the electrophysiological measurements. UV light flashes were produced by a xenon flash lamp system XF-10 (Hi-Tech Scientific Ltd., Salisbury, Wilts., U.K.).

RESULTS

Ins(2,4,5)P₃, GTP[S] and AlF₄⁻ can activate calcium entry

Injection of *Xenopus* oocytes with Ins(2,4,5)P₃ [a poorly metabolizable analogue of Ins(1,4,5)P₃] to an intracellular concentration of 2 μ M in the absence of extracellular calcium evoked an early oscillatory response in the chloride current, due to the pulsatile release of calcium from intracellular InsP₃-sensitive calcium stores (Figure 1a; *n* = 8). When 2 mM calcium is applied extracellularly for a 20 s period before the injection of Ins(2,4,5)P₃, no currents are evoked. However, after the injection of Ins(2,4,5)P₃ the pulses of calcium applied at 2 min intervals evoked chloride currents that gradually increase over the duration of the experiment. The activation of the calcium-influx pathway presumably occurs due to the emptying of the intracellular stores. The calcium-influx-evoked currents showed marked inactivation over the period of a 20 s application of calcium. Previous reports of experiments in *Xenopus* oocytes, using very similar protocols to deplete the internal stores, have shown nearly identical results [9,17,18].

Injection of oocytes with 2 mM GTP[S] again evoked oscillations in the chloride current, presumably due to the production of InsP₃ mediated by the irreversible activation of heterotrimeric

G-proteins linked to the phosphoinositide signalling pathway (Figure 1b; *n* = 14). Calcium influx was also activated, as shown by the currents evoked by extracellular application of 2 mM calcium. However, there are two obvious differences between calcium influx evoked by InsP₃ and that evoked by GTP[S]. Firstly, the calcium-influx-evoked currents in the presence of GTP[S] did not show inactivation during the 20 s calcium pulse, and secondly, calcium influx was only activated transiently by GTP[S]. The calcium-entry-evoked currents observed after injection of GTP[S] were often markedly smaller than after injection of Ins(2,4,5)P₃, presumably due to the additional inhibitory actions of G-protein stimulation.

Injection of AlF₄⁻ to an intracellular concentration of 50 μ M had very similar effects to those observed with injection of GTP[S]. Again, small-amplitude oscillations were recorded in the calcium-activated chloride current after injection of AlF₄⁻, and 20 s pulses of calcium revealed transient activation of the influx pathway (Figure 1c; *n* = 7). Again, the inactivation kinetics of the calcium-influx-evoked currents observed in Ins(2,4,5)P₃-injected oocytes (Figure 1a) were blocked by AlF₄⁻, as seen in Figure 1(c) by the gradual change in shape of the calcium-influx-evoked currents.

Effects of G-protein activation on capacitative calcium entry

The previous experiments indicated that GTP[S] and AlF₄⁻ are capable of transiently activating capacitative calcium entry. The next question that we considered was how G-protein activation might influence calcium entry that had already been activated by depleting calcium pools with thapsigargin. In many cell types, the specific endoplasmic reticulum Ca²⁺-ATPase inhibitor thapsigargin is able to activate capacitative calcium entry without increasing the concentration of inositol phosphates [1,19]. In *Xenopus* oocytes, capacitative calcium entry can be activated by incubating the cells in calcium-free medium with 1 μ M thapsigargin for over 3 h [9,17]. The capacitative calcium entry pathway was monitored by administering repeated extracellular pulses of 2 mM calcium.

When GTP[S] was injected into thapsigargin-treated oocytes to an intracellular concentration of 200 μ M, the calcium-influx-evoked currents were markedly inhibited (Figure 2a; *n* = 8), with a half-time of inhibition of 11.9 ± 1.0 min (*n* = 8, mean $\pm$ S.E.M.). This inhibition can be partially and transiently reversed by 2 mM GTP (Figure 2a, *n* = 3), showing the effect of GTP[S] to be evoked by G-protein stimulation, as opposed to a non-specific effect. GTP[S] (200 μ M) also affected the kinetics of the currents evoked by the calcium pulses. The immediate effect of GTP[S] was to block the inactivation kinetics of the calcium-entry-evoked currents (compare Figure 2a, i, ii and iii). This block of the inactivation resulted in a transient potentiation of the peak calcium-influx-evoked currents in 6 out of 8 oocytes by 7 ± 2 % (*n* = 6). Later, the calcium entry was inhibited (Figure 2a, iv).

The injection of GTP[S] to give an intracellular concentration of 2 mM into thapsigargin-treated oocytes completely inhibited the peak currents evoked by calcium pulses, with a half-time of 3.2 ± 1.1 min (*n* = 9). Injection of GTP to an intracellular concentration of 2 mM weakly inhibited calcium-entry-evoked currents in thapsigargin-treated oocytes by 28 ± 4 % (*n* = 3), presumably due to heterotrimeric G-protein stimulation. GDP[S] (*n* = 5) or GDP (*n* = 3) had very little effect at 2 mM on the capacitative calcium-entry-evoked currents in thapsigargin-treated oocytes.

Injection of AlF₄⁻ to an intracellular concentration of 50 μ M inhibited calcium entry-evoked currents (Figure 2b; *n* = 8). AlF₄⁻

Figure 1 Ins(2,4,5)P₃, GTP[S] and AIF₄⁻ activate calcium entry

(a) Injection of Ins(2,4,5)P₃ into *Xenopus* oocytes to an intracellular concentration of 2 μM evoked calcium oscillations detected in the calcium-activated chloride current under voltage clamp at -60 mV. Pulsed extracellular applications of 2 mM calcium gradually evoked increasing calcium-influx-evoked chloride currents, each response showing marked inactivation kinetics. (b) Injection of GTP[S] to an intracellular concentration of 2 mM similarly caused oscillations and the activation of calcium entry. However, the kinetics of the calcium-entry-evoked currents during the calcium pulse did not show inactivation kinetics, and the calcium entry was only activated transiently. (c) Injection of AIF₄⁻ to an intracellular concentration of 50 μM evoked small-amplitude calcium oscillations and activated calcium entry. Again, calcium entry is only activated transiently and the inactivation kinetics of the calcium-influx-evoked currents were gradually inhibited.

Figure 2 GTP[S] and AIF₄⁻ inhibit capacitative calcium entry

(a) Introduction of 200 μM GTP[S] into thapsigargin-treated oocytes inhibited calcium entry. This inhibition was partially and transiently reversed by injection of 2 mM GTP. The calcium-entry-evoked currents showed inactivation kinetics in the pre-injected situation (i), and gradually changed upon injection of GTP[S] to have no inactivation kinetics (ii, iii). Later the peak amplitude of the calcium entry-evoked currents was inhibited (iv). (b) Introduction of 50 μM AIF₄⁻ into thapsigargin-treated oocytes also showed inhibition of calcium-entry-evoked currents and a block of the inactivation kinetics.

Figure 3 The effects of GTP[S] on capacitative calcium entry may be mediated by PKA and PKC

(a) Application of 1 μ M db-cAMP inhibited calcium-entry-evoked currents in thapsigargin-treated oocytes; (b) 1 mM db-cGMP had no effect on calcium-entry-evoked currents; (c) application of 100 nM PMA caused biphasic effects on the calcium-entry-evoked currents. First the currents were potentiated and the inactivation kinetics blocked. Later the calcium-entry-evoked currents were inhibited.

had similar effects to GTP[S] on the kinetics of the calcium-entry-evoked currents, in that the inactivation was also blocked.

Effects of protein kinases on capacitative calcium entry

The fact that both GTP[S] and AIF_4^- exerted an inhibitory effect suggested the possibility that G-protein activation may affect capacitative calcium entry indirectly, by stimulating phospholipase C and adenylate cyclase. The second-messenger pathways resulting from these effectors were studied in oocytes which had been treated with thapsigargin to activate the capacitative entry pathway.

Activation of cyclic AMP (cAMP)-dependent protein kinase (PKA) had a biphasic effect on the amplitude of calcium-entry-evoked currents, without changing the activation or inactivation kinetics. Application of dibutyryl cAMP (db-cAMP) at 1–10 μ M to thapsigargin-treated oocytes inhibited the currents evoked by pulsed application of 2 mM calcium (Figure 3a) by $34 \pm 8\%$ ($n = 4$), whereas application of 1–10 mM db-cAMP potentiated calcium-entry-evoked currents by $44 \pm 15\%$ ($n = 9$). The maximal inhibition after application of low doses of db-cAMP observed was 85%, and the maximal potentiation at higher doses was 100%. Application of 1–10 nM forskolin showed a similar biphasic effect on the calcium-entry-evoked currents. Initially, forskolin application inhibits calcium-entry-evoked currents by $10 \pm 3\%$ ($n = 6$) and then potentiates by $36 \pm 14\%$ ($n = 6$). Both the inhibitory and the potentiating effects on the calcium-entry-evoked currents could also be observed upon application of 10 mM caffeine ($n = 4$), theophylline ($n = 3$) or 3-isobutyl-1-methylxanthine ($n = 3$) to thapsigargin-treated

oocytes, which are known to inhibit cAMP phosphodiesterase, thus elevating the cytosolic cAMP concentration.

Application of 0.1 mM or 1 mM (Figure 3b) dibutyryl cGMP (db-cGMP) had no effect on the capacitative calcium entry ($n = 3$). This observation is in agreement with a previous report on capacitative calcium entry in *Xenopus* oocytes [5] and rat basophilic leukaemia cells [10], but disagrees with the prominent role for cGMP suggested in pancreatic acinar cells [6]. It thus appears that cGMP does not have a significant role in general for capacitative calcium entry.

Activation of protein kinase C (PKC) had biphasic effects on the calcium-entry evoked currents (Figure 3c; $n = 5$) as previously described [9]. Application of 100 nM phorbol 12-myristate 13-acetate (PMA) initially potentiated the calcium entry-evoked currents and blocked the inactivation kinetics observed in response to calcium pulses before addition of PMA. Further stimulation with PMA inhibited calcium entry. In contrast with these marked effects of stimulating PKC, activation of the other limb of the bifurcating phosphoinositide signalling pathway had no effect. Injection of $\text{Ins}(2,4,5)P_3$ to an intracellular concentration of 2 μ M had no significant effect on the calcium-entry-evoked currents in thapsigargin-pretreated oocytes ($n = 7$), as previously described [9].

If GTP[S] exerts its effects on capacitative calcium entry indirectly through protein kinases, then one would expect to be able to inhibit its action by using the non-specific protein kinase inhibitor staurosporine. In oocytes which had been incubated in 100 nM staurosporine for 3 h, injection of GTP[S] to an intracellular concentration of 2 mM inhibited calcium-entry-evoked currents with a half-time of 9.5 ± 1.2 min ($n = 3$) (compared with the control half-time of 3.2 ± 1.1 min).

Application of PMA, forskolin or db-cGMP does not affect the chloride current or release of calcium by InsP_3

By flash photolysis of oocytes injected with DM-nitrophen, we monitored the effects of application of 100 nM PMA, 10 nM forskolin and 1 mM db-cGMP on the calcium-activated chloride current. No effects were observed on the amplitude of the currents evoked over a period of 15 min ($n = 3$ for each compound). Similar experiments were performed on oocytes injected with caged InsP_3 , and again no effect was observed on the amplitude of the chloride currents evoked ($n = 3$ for each compound). PKC and PKA thus seem to affect specifically the capacitative entry component of calcium signals in *Xenopus* oocytes, since they affected neither the chloride current nor the release of calcium from InsP_3 -sensitive stores.

DISCUSSION

Activation of G-proteins can have profound effects on capacitative calcium entry induced by the depletion of intracellular stores. Agonist stimulation results in the formation of InsP_3 , which releases calcium from intracellular stores. As these stores empty, a signal is transmitted to the plasma membrane to activate the inward calcium current through the calcium release-activated channel (I_{CRAC}) [13]. There are two ways in which G-proteins might influence this signalling pathway responsible for controlling I_{CRAC} . Firstly, heterotrimeric G-proteins activate phospholipase C, generating the InsP_3 necessary to deplete the intracellular calcium stores. Secondly, either heterotrimeric or small monomeric G-proteins may play a more direct role in regulating the transmission of information from the empty stores to the plasma membrane [10–12]. The evidence for a direct role of G-proteins in the regulation of capacitative calcium entry is based on the introduction of GTP[S], GDP[S] and AlF_4^- into the cytoplasm, which has given conflicting results depending on cell type. In rat basophilic leukaemia cells (RBL-2H3), GTP[S] blocked the activation of I_{CRAC} induced by store depletion using either ionomycin or InsP_3 [10]. Likewise, thapsigargin-induced calcium entry into lacrimal cells [11] and HL-60 granulocytes [12] was also inhibited by GTP[S]. Since capacitative calcium entry in lacrimal cells could also be inhibited by GDP[S] [11], it was proposed that the signal was mediated via small monomeric G-proteins. On the other hand, Jaconi et al. [12] argued that heterotrimeric G-proteins were involved, because the inhibitory effects of GTP[S] could be duplicated by treating cells with fluoride, which only activates heterotrimeric G-proteins [20]. Indeed, AlF_4^- was also found to have a profound effect on RBL-2H3 cells, initially evoking a rapid activation of I_{CRAC} (presumably through the depletion of intracellular stores) and then inhibition of calcium entry [10]. Previous experiments have therefore proposed a role for both heterotrimeric and small G-proteins operating downstream of the agonist-induced formation of InsP_3 .

In the case of *Xenopus* oocytes, all the evidence that we have obtained is consistent with a G-protein having an effect early in the signalling cascade, i.e. at the initial signal-transduction steps where phospholipase C and adenylate cyclase are activated to generate InsP_3 , diacylglycerol and cAMP. The data show that both GTP[S] and AlF_4^- can activate calcium influx in control oocytes, presumably through phospholipase C hydrolysis producing InsP_3 , which depletes the intracellular calcium stores. However, the calcium influx is only activated transiently, and the calcium-influx-evoked currents show no inactivation, unlike oocytes with $\text{Ins}(2,4,5)\text{P}_3$ -depleted stores. The transient nature of the activation of calcium entry by GTP[S] may well be due to the observed inhibitory effects of GTP[S] on capacitative calcium entry in thapsigargin-treated oocytes, which also blocks the

inactivation kinetics of the calcium-entry-induced currents. This inhibition can be mimicked by AlF_4^- , but not by GDP[S], suggesting that GTP[S] inhibits calcium influx via heterotrimeric G-proteins rather than small monomeric G-proteins [20].

Effects of stimulation of heterotrimeric G-proteins include activation of phospholipase C (producing InsP_3 and diacylglycerol) and adenylate cyclase (elevating the cAMP level). $\text{Ins}(2,4,5)\text{P}_3$ has no profound effect on the capacitative calcium entry pathway activated by thapsigargin pretreatment. On the other hand, activation of PKC by PMA first potentiates the calcium influx-evoked currents and then inhibits them, while also blocking the inactivation kinetics. Thus, increased activity of PKC by GTP[S] activation of phospholipase C can account for part of the observed effects of G-proteins on calcium entry. Both show inhibition of calcium entry and a block of the inactivation kinetics of the evoked currents. However, low-level PKC activity additionally potentiates calcium entry to a much greater extent than does GTP[S]. This difference could be accounted for by production of cAMP evoked by G-protein stimulation of adenylate cyclase, which at low concentrations inhibits calcium entry. Further evidence that GTP[S] may mediate its effects via PKC and PKA is revealed through the increase in half-time of inhibition of calcium entry by 2 mM GTP[S] in the presence of the protein kinase inhibitor staurosporine to 9.5 ± 1.2 min from the control value of 3.2 ± 1.1 min.

It is possible that similar mechanisms may account for the effects of GTP[S] on calcium entry observed in other cell types. The slight stimulatory effect of GDP[S] on calcium entry observed in both RBL-2H3 [10] and HL-60 [12] cells has been interpreted to be a result of the removal of tonic inhibition from 'basal trimeric G protein activity, possibly through kinase phosphorylation' [10]. PKC stimulation has been shown to inhibit capacitative calcium entry in HL-60 granulocytes [12], neutrophils [21] and thyroid cells [22]. It is thus likely that at least part of the inhibition of capacitative calcium entry by G-proteins in RBL-2H3 and HL-60 cells is mediated via protein kinases indirectly activated by heterotrimeric G-proteins.

Although we cannot exclude the possibility of a direct effect of G-proteins on capacitative calcium entry, as suggested by previous studies [10–12], the simplest interpretation of our data would suggest that GTP[S] stimulates the production of diacylglycerol and cAMP, which together mediate the observed effects of GTP[S] on capacitative calcium entry. The specific modulation of capacitative calcium entry by PKC and PKA, without altering InsP_3 -mediated release of stored calcium, gives the cell additional ability to regulate calcium signalling.

We are grateful to Dr. C. W. Taylor for loan of the flash photolysis unit, and also to the Wellcome Trust for funding the research.

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Received 19 October 1994/19 December 1994; accepted 5 January 1995