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Store Depletion by Caffeine/Ryanodine Activates Capacitative Ca^{2+} Entry in Nonexcitable A549 Cells[1]

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Capacitative Ca^{2+} entry is essential for refilling intracellular Ca^{2+} stores and is thought to be regulated primarily by inositol 1,4,5-trisphosphate (IP_3)–sensitive stores in nonexcitable cells. In nonexcitable A549 cells, the application of caffeine or ryanodine induces Ca^{2+} release in the absence of extracellular Ca^{2+} similar to that induced by thapsigargin (Tg), and Ca^{2+} entry occurs upon the readdition of extracellular Ca^{2+}. The channels thus activated are also permeable to Mn^{2+}. The channels responsible for this effect appear to be activated by the depletion of caffeine/ryanodine-sensitive stores *per se*, as evidenced by the activation even in the absence of increased intracellular Ca^{2+} concentration. Tg pretreatment abrogates the response to caffeine/ryanodine, whereas Tg application subsequent to caffeine/ryanodine treatment induces further Ca^{2+} release. The response to caffeine/ryanodine is also abolished by initial ATP application, whereas ATP added subsequent to caffeine/ryanodine induces additional Ca^{2+} release. RT-PCR analyses showed the expression of a type 1 ryanodine receptor, two human homologues of transient receptor potential protein (hTrp1 and hTrp6), as well as all three types of the IP_3 receptor. These results suggest that in A549 cells, (i) capacitative Ca^{2+} entry can also be regulated by caffeine/ryanodine-sensitive stores, and (ii) the RyR-gated stores interact functionally with those sensitive to IP_3, probably *via* Ca^{2+}-induced Ca^{2+} release.

Key words: capacitative Ca^{2+} entry, inositol 1,4,5-trisphosphate receptor, ryanodine receptor, transient receptor potential protein.

In our previous study (*1*), human lung epithelial–like carcinoma-derived A549 cells were found to express multiple Ca^{2+} channels including ATP-gated P2X4 channel, the L-type voltage-dependent Ca^{2+} channel (VDCC), and capacitative Ca^{2+} entry (CCE) channels, with Ca^{2+} entry through P2X4 being the target of inhibition by a macrolide antibiotics, erythromycin. A549 cells also express G protein–coupled P2Y2, P2Y4, and P2Y6 receptors, and ATP and UTP induce Ca^{2+} mobilization from inositol 1,4,5-trisphosphate (IP_3)–sensitive stores by acting on P2Y2 and/or P2Y4. Unexpectedly, caffeine and low concentrations of ryanodine cause an increase in intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$), suggesting the expression of some isoforms of ryanodine receptor (RyR) (unpublished observation). In addition, Ca^{2+} influx through P2X or through the L-type VDCC evokes a global $[Ca^{2+}]_i$ response, suggesting that Ca^{2+}-induced Ca^{2+} release (CICR) from IP_3 receptor (IP_3R)– and/or RyR-gated stores is involved in this $[Ca^{2+}]_i$ response.

In nonexcitable cells, agonist-induced $[Ca^{2+}]_i$ response is a biphasic process consisting of an initial spike and a subsequent plateau (*2*). The spike represents Ca^{2+} release from intracellular Ca^{2+} stores through IP_3Rs, whereas the plateau is ascribed to Ca^{2+} entry from extracellular spaces, *i.e.* CCE, which is believed to be activated by the depletion of IP_3-sensitive stores (*2, 3*). In excitable cells, on the other hand, the increase in $[Ca^{2+}]_i$ is more often accounted for by RyR-mediated Ca^{2+} release from intracellular stores and the stores have been proposed to refill from voltage-dependent Ca^{2+} entry (*4*). Although IP_3Rs have been found to be coexpressed with RyRs in many excitable cells, the role of the CCE pathway in the replenishment of intracellular Ca^{2+} stores in these cells remains controversial. In gonadotrophs, an excitable endocrine cell type, gonadotropin-releasing hormone induces IP_3-mediated Ca^{2+} mobilization, but this is Ca^{2+} entry through VDCCs rather than CCE channels that account for the refilling of the stores (*5, 6*). A role of the CCE pathway has been documented in some other

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Abbreviations: AM, acetoxylmethyl ester; BAPTA, 1,2-bis(2-aminophenoxy)ethane-*N,N,N',N'*-tetraacetic acid; $[Ca^{2+}]_i$, intracellular free Ca^{2+} concentration; Ca^{2+}_o, extracellular Ca^{2+}; CCE: capacitative Ca^{2+} entry; CICR, Ca^{2+}-induced Ca^{2+} release; DMSO, dimethyl sulfoxide; ER, endoplasmic reticulum; HBSS, Hank's balanced salt solution; hTrp, human homologue of Trp; IP_3, inositol 1,4,5-trisphosphate; IP_3R, inositol 1,4,5-trisphosphate receptor; RyR, ryanodine receptor; SERCA: sarcoplasmic and endoplasmic reticulum Ca^{2+}-ATPase; sarcoplasmic reticulum, SR; Tg, thapsigargin; Trp, transient receptor potential protein; VDCC, voltage-dependent Ca^{2+} channel.

excitable cells, but in this case, it remains disputable whether CCE is regulated solely by IP_3-sensitive stores (*7*) or by both IP_3Rs and RyRs (*8*). Recent studies indicate that, at least in some excitable cells such as hippocampal neurons (*9*) and PC12 cells (*10*, *11*), CCE can be triggered by RyRs.

In this study, we attempted to identify the isoforms of IP_3R and RyR expressed in A549 cells and to examine whether RyR-mediated Ca^{2+} release activates CCE channels in a nonexcitable cell line.

MATERIALS AND METHODS

Cell Culture and $[Ca^{2+}]_i$ Measurement—A549 cells were obtained from American Type Culture Collection, and maintained in DMEM (Life Technologies, Rockville, MD, USA) supplemented with 10% fetal bovine serum and 10 mg/ml penicillin–0.1 mg/ml streptomycin, at 37°C in a humidified atmosphere of 5% CO_2/95% air.

A549 cells were loaded with 5 μM fura-2/acetoxylmethyl ester (AM) as described in our previous study (*1*). The pair of fluorescence intensities emitted (at 510 nm) by excitation at 340 and 380 nm were recorded at 20 s intervals, and the ratios of the 340- to 380-nm fluorescence intensity were used to represent the changes in $[Ca^{2+}]_i$. In some experiments, raw fluorescence with excitation at 340 nm, a wavelength that is sensitive to changes in $[Ca^{2+}]_i$ and quenching by Mn^{2+} (*12*), was recorded to monitor changes in $[Ca^{2+}]_i$ and Mn^{2+} entry. Hank's balanced salt solution (HBSS) containing 1.2 mM $CaCl_2$ was used as the standard extracellular medium. For measurement of $[Ca^{2+}]_i$ in the absence of extracellular Ca^{2+} (Ca^{2+}_o), $CaCl_2$ in HBSS was replaced by 1 mM EGTA, and the cells were perfused before agent addition with the Ca^{2+}-free HBSS for 2–3 min at a flow rate of about 1 ml/min.

RT-PCR—Total RNA was prepared from subconfluent A549 cells using Isogen (Wako Pure Chemical Industries, Osaka) according to the manufacturer's instructions. Poly-$(A)^+$ RNA was isolated from DNase I–treated total RNA with Oligotex-dT30 (Roche Diagnostics, Tokyo). First-strand cDNA was synthesized from 0.5 μg poly$(A)^+$ RNA by an oligo(dT)-primed reaction with a Superscript preamplification system (Life Technologies).

The ratio PCR analysis was used to determine IP_3R isoforms and the relative amounts of their mRNAs, based on the simultaneous amplification of two or more targets that have identical primer-template sequences (*13*, *14*). The amplified products were discriminated using restriction enzymes with specific cleavage sites in the divergent areas of the targets. Primers used were identical to those described by Morgan *et al.* (*14*). One primer pair matched sequences of the human type 1 and type 2 IP_3Rs, and was expected to amplify a 256 bp fragment. The other primer pair, which matched sequences of the human type 1 and type 3 IP_3Rs, was to amplify a 243 bp fragment. PCR amplification was carried out by the hot-start method using AmpliWax PCR Gem 100 (Perkin-Elmer, Foster City, CA, USA). Briefly, 25 pmol of primers were mixed with 10 nmol each of dNTP to give a lower mixture of 20 μl. One pilule of wax Gem was added to each reaction, and the mixture was incubated at 75°C for 7 min, followed by further incubation at 25°C for 3 min. Then, 30 μl of the upper mixture containing 1 μl of first-strand cDNA, 2.5 μl of dimethyl sulfoxide (DMSO) and 2.5 units of *Taq* plus *Pwo* polymerase (Roche Diagnostics) was layered on the solidified wax phase. PCR was started by incubation at 95°C for 2 min, followed by a 35-cycle (95°C for 1 min, 50°C for 1 min, and 72°C for 2 min) amplification, and a further extension at 72°C for 7 min. After phenol extraction and ethanol precipitation, the PCR products were subjected to digestion with restriction enzymes, and the fragments were separated on 5% polyacylamide gels.

RyR isoforms expressed in A549 cells were determined by two approaches. One was the simultaneous amplification of all three isoforms with a pair of primers that completely matched the 3'-region of these isoforms (*15*), followed by restriction enzyme analysis. The other was the use of selective primers for amplification of the respective isoforms. The common and selective primers used were the same as those described by Sei *et al.* (*15*). RyRs isoforms were amplified by the hot-start method using the same quantity of each component as described for the identification of IP_3Rs. PCR conditions were 95°C for 3 min, followed by amplification for 10 cycles (95°C for 45 s, 45°C for 1.5 min, and 72°C for 3 min) and 30 cycles (95°C for 45 s, 50°C for 1.5 min, and 72°C for 3 min), and a further 10-min extension at 72°C.

For amplification of hTrp1, hTrp3, and hTrp4, the selective primer sets for each described by Groschner *et al.* (*16*) were used, producing 504, 448, and 387 bp products, respectively. For identification of Trp5, primers based on sequences of the murine homologue were used as reported by Garcia and Schilling (*17*), generating a 340 bp product. Primers used to identify hTrp6, 5'-GACATCTTCAAGTTCATGGTC and 5'-ATCAGCGTCATCCTCAATTTC were designed based on the reported cDNA sequence (GenBank Accession No. AF080394), and were expected to amplify a 321 bp product. PCR conditions were the same as those described for the identification of IP_3R isoforms.

Materials—Fura-2/AM and bradykinin were obtained from Wako Pure Chemical Industries, and 1,2-bis(2-aminophenoxy)ethane-*N,N,N',N'*-tetraacetic acid (BAPTA)/AM, ATP, ionomycin, thapsigargin, ryanodine and caffeine were from Sigma (St. Louis, MO, USA). Fura-2/AM, BAPTA/AM, and thapsigargin were dissolved in DMSO just before use.

RESULTS AND DISCUSSION

Ca^{2+} Entry Induced by Store Depletion with Tg—Capacitative Ca^{2+} entry is a ubiquitous phenomenon in "non-excitable" cells. In agreement with our previous studies (*1*), robust Ca^{2+} entry was induced in A549 cells when Ca^{2+} was added after the stores were passively depleted by the application of thapsigargin (Tg), an irreversible inhibitor of sarcoplasmic and endoplasmic reticulum Ca^{2+}-ATPases (SERCAs) (Fig. 1A). Without Tg treatment, little increase in $[Ca^{2+}]_i$ was induced by the Ca^{2+} addition (Fig. 1B). The channels thus activated were also permeable to Mn^{2+} (Fig. 1C). At an excitation wavelength of 340 nm, the raw fluorescence of fura-2 increased with increasing $[Ca^{2+}]_i$ (Fig. 1C), whereas the fluorescence at 360 nm excitation was insensitive to changes in $[Ca^{2+}]_i$. However, the fluorescence at both excitation wavelengths has been shown to be quenched by Mn^{2+} (*12*). In addition, when 0.1% DMSO (the vehicle of Tg) was added to the cells instead of Tg, the fluorescence quenching occurred only very slowly, if at all, upon

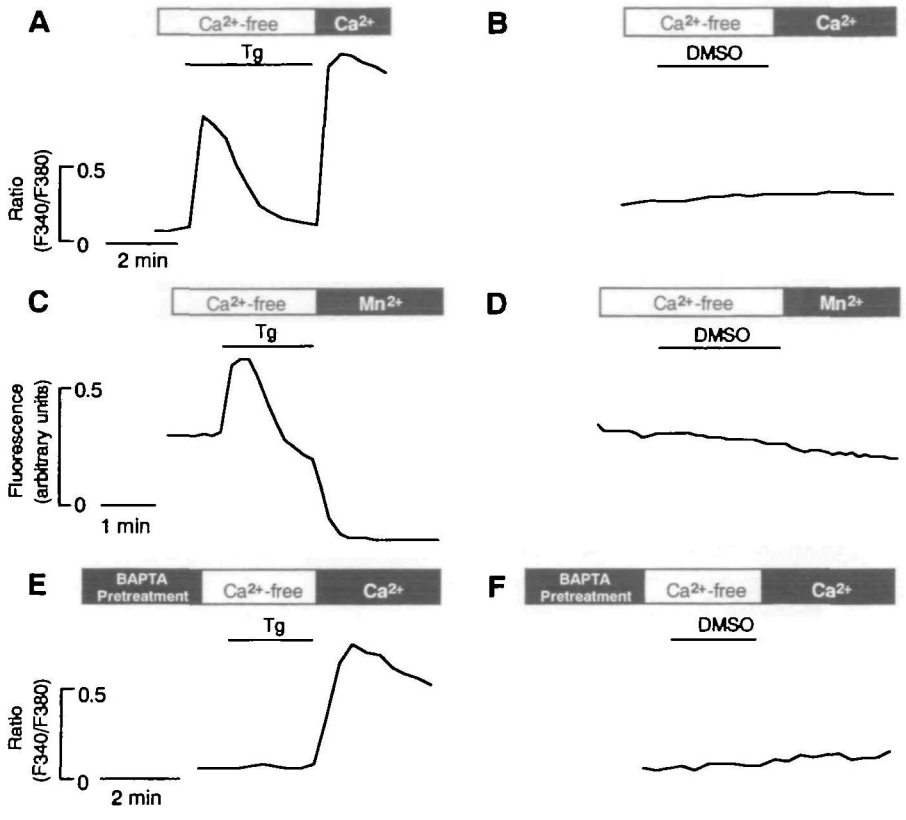

Fig. 1. **Ca^{2+} and Mn^{2+} entry induced by store depletion with Tg.** Fura-2 loaded A549 cells were perfused with Ca^{2+}-free HBSS for 2–3 min before the addition of agents (1 μM Tg in 0.1% DMSO in A, C, and E; 0.1% DMSO in B, D, and F as indicated above each panel). In E and F, cells were preincubated with 25 μM BAPTA/AM for 10 min before the medium was changed to Ca^{2+}-free HBSS. In C and D, raw fluorescence at 340 nm excitation was recorded to monitor both the increase in $[Ca^{2+}]_i$ and the quenching of fura-2 fluorescence due to Mn^{2+} entry. In other experiments (A, B, E, and F), the ratio of the fluorescence intensity at excitation wavelengths of 340 nm (F340) and 380 nm (F380) was recorded, and $[Ca^{2+}]_i$ is shown as the F340/F380 ratio. In each experiment, changes in $[Ca^{2+}]_i$ in 7–14 cells were recorded at 20-s intervals. Recordings, each from a single cell, are shown as representatives of 3 independent experiments.

the subsequent addition of Mn^{2+} (Fig. 1D). These results suggest that the store depletion by Tg also activates the entry of Mn^{2+} into cells.

An important concern with the use of Tg is that the depletion of stores is invariably accompanied by an increase in $[Ca^{2+}]_i$. Indeed, Ca^{2+}-activated non-selective cation channels have been identified in human neutrophils (*18*) and other animal cells (*19*, *20*). Activation of these channels, either by direct binding of Ca^{2+} to a site on the channel protein or *via* a Ca^{2+}-binding regulatory protein such as calmodulin (*21*), could contribute to the Ca^{2+} influx. To distinguish between the channels activated by store depletion and those activated by an increase in $[Ca^{2+}]_i$, we used a fast Ca^{2+} chelator, BAPTA (*22*), to buffer the increase in $[Ca^{2+}]_i$ caused by Tg application. The acetoxylmethyl ester of BAPTA (BAPTA/AM) does not interfere with the fluorescence spectrum of fura-2 when it is loaded into cells with the Ca^{2+} indicator (*23*). When A549 cells were preincubated with 25 μM BAPTA/AM for 10 min, the increase in $[Ca^{2+}]_i$ caused by Tg application was effectively suppressed (Fig. 1E). After the release phase was over, as judged from control cells (such as in Fig. 1A), extracellular Ca^{2+} was added back to the medium. As shown in Fig. 1E, Ca^{2+} influx occurred and overwhelmed the buffering capacity of the preloaded BAPTA, resulting in an increase in $[Ca^{2+}]_i$. When 0.1% DMSO was added to BAPTA-preloaded cells in place of Tg, $[Ca^{2+}]_i$ did not change upon the subsequent addition of Ca^{2+}_o (Fig. 1F). These results suggest that the Ca^{2+} channels responsible for Tg-activated Ca^{2+} entry are *bona fide* CCE channels, rather than Ca^{2+}-activated non-selective cation channels.

Ca^{2+} Entry Induced by Store Depletion with Caffeine or Ryanodine—Ca^{2+} influx activated by 3′-*O*-(4-benzoyl)benzoyl ATP or KCl produced a global increase in $[Ca^{2+}]_i$ in A549 cells (*1*), suggesting a role of CICR in the amplification of the Ca^{2+} signal. A candidate intracellular Ca^{2+} channel responsible for the CICR is IP_3R(s), whose presence in A549 cells has already been suggested by the UTP-induced and U73122-sensitive increase in $[Ca^{2+}]_i$ (*1*). Ca^{2+} is also a physiological activator of RyR, and caffeine is known to activate RyR by increasing its sensitivity to Ca^{2+} (*24*). In the present study, the application of caffeine to A549 cells induced a remarkable increase in $[Ca^{2+}]_i$ in the absence of Ca^{2+}_o, indicating Ca^{2+} release through RyR (Fig. 2, A and C). As in the case of Tg, the addition of Ca^{2+}_o and Mn^{2+} after the released Ca^{2+} was pumped out led to the excitation and quenching of fura-2 fluorescence, respectively. We also tested the effect of ryanodine, which is highly specific for RyR. At low concentrations, ryanodine is known to maintain the RyR channel in a persistently open state, and thus to induce depletion of the store where the channel is expressed (*24*, *25*). A549 cells responded to 1 μM ryanodine as in the case of caffeine application, *i.e.*, the release of Ca^{2+} through RyR was followed by the activation of Ca^{2+} entry channels that were permeable to both Ca^{2+} and Mn^{2+} (Fig. 2, B and D). Furthermore, the Ca^{2+} entry channels were activated even when the caffeine/ryanodine-induced increase in $[Ca^{2+}]_i$ was suppressed with preloaded BAPTA (Fig. 2, E and F). Taken together, these results suggest that there are caffeine/ryanodine-sensitive stores in A549 cells and that their depletion by either caffeine or ryanodine activates Ca^{2+} entry through CCE channels. It is not yet clear whether the Ca^{2+} entry channels activated by the depletion of Tg-sensitive stores and those activated by the

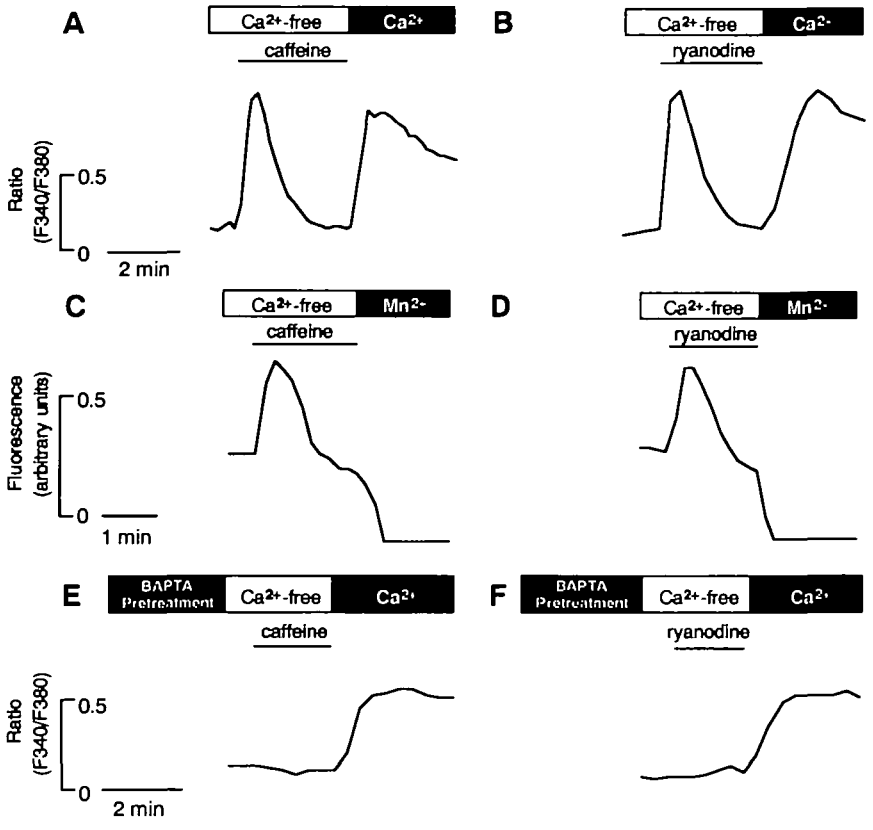

Fig. 2. **Ca^{2+} and Mn^{2+} entry induced by store depletion with caffeine or ryanodine.** Fura-2 loaded A549 cells were perfused with Ca^{2+}-free HBSS for 2–3 min before the addition of agents (25 mM caffeine in A, C, and E; 1 μM ryanodine in B, D, and F as indicated above each panel). In E and F, cells were preincubated with 25 μM BAPTA/AM for 10 min before the medium was changed to Ca^{2+}-free HBSS. In C and D, raw fluorescence at an excitation of 340 nm was recorded to monitor both the increase in $[Ca^{2+}]_i$ and the quenching of fura-2 fluorescence due to Mn^{2+} entry. In other experiments (A, B, E, and F), $[Ca^{2+}]_i$ is shown as the F340/F380 ratio. In each experiment, changes in $[Ca^{2+}]_i$ in 7–14 cells were recorded at 20-s intervals. Recordings, each from a single cell, are shown as representatives of 3 independent experiments.

depletion of the caffeine/ryanodine-sensitive stores are the same, but they share some common properties: permeability to both Ca^{2+} and Mn^{2+} and activation by store depletion rather than increase in $[Ca^{2+}]_i$. To date, the activation of CCE by the depletion of caffeine/ryanodine-sensitive stores has been observed only in excitable cells (*8–11*), but our findings showed that nonexcitable cells may also be equipped with a caffeine/ryanodine-sensitive store-derived Ca^{2+}-entry signal.

Functional Interaction of Intracellular Ca^{2+} Stores—Intracellular stores are believed to be portions of the lumenal space enclosed by the membranes of the endoplasmic reticulum (ER) or sarcoplasmic reticulum (SR). It appears that there are no structural boundaries in the ER lumenal space (*26*), although a functional heterogeneity of Ca^{2+} stores has frequently been observed in a variety of cells (*27*). The pretreatment of cells with SERCA inhibitors such as Tg is known to result in an abolition of Ca^{2+} release by agonists coupled to IP_3 generation. As shown in Fig. 3A, Tg pretreatment of A549 cells in the absence of Ca^{2+}_o prevents the $[Ca^{2+}]_i$ response to IP_3-generating agonists, ATP (*1*) and bradykinin (*28*), indicating that IP_3 induced Ca^{2+} mobilization from Tg-sensitive stores. Residual Ca^{2+} release caused by ionomycin may be explained by the existence of Tg-resistant SERCA (*29*), or by non-SR/ER Ca^{2+} stores such as mitochondria (*30*) or other organelles (*31*). Similarly, the pretreatment of A549 cells with Tg abolished the response to both caffeine and ryanodine, suggesting that these agents also induce Ca^{2+} release from Tg-sensitive stores (Fig. 3, B and C). In contrast, the application of Tg after caffeine or ryanodine caused further Ca^{2+} release (Fig. 3, D and E). These agents, especially caffeine, may have been only partially effective in depleting intracellular Ca^{2+} stores, because caffeine has been reported to release Ca^{2+} in a dose-dependent fashion (*32*). However, Ca^{2+} release experiments and RyR single-channel recordings (*24*) have shown that ryanodine at micromolar concentrations locks the sarcoplasmic RyR channel partially open, a state with subnormal conductance (about 40 to 60% of the conductance of the open channel). Such a modified state of the RyR channel is characterized by a remarkable (more than 20-fold) increase in open time with a decreased closed time. In this case, 1 μM ryanodine may keep the RyR channel in a persistently open state and lead to the depletion of caffeine/ryanodine-sensitive stores, especially in the absence of extracellular Ca^{2+}. Although the effects of ryanodine could vary among cells, type 1 RyR (the skeletal muscle type) was found to be expressed in A549 cells as described below. It is therefore interesting to assume that the Tg-induced further release of Ca^{2+} after caffeine or ryanodine application can be accounted for, at lease in part, by the presence of a caffeine/ryanodine-insensitive portion in the Tg-sensitive stores. A store that can be released only by ionomycin was also observed in all these cases (Fig. 3, B–E).

In a caffeine-sensitive clone derived from PC12 cells (*25*), IP_3 and caffeine/ryanodine were shown to elicit Ca^{2+} discharge from the same Ca^{2+} store that was sensitive to SERCA inhibitors. On the other hand, in astrocytes and artery myocytes, caffeine/ryanodine-sensitive Ca^{2+} stores appear to be spatially distinct from those sensitive to SERCA inhibitors and IP_3-generating agonists (*30, 33*). To explore the relationship between the IP_3- and caffeine/ryanodine-sensitive stores in A549 cells, we applied ATP and caffeine/ryanodine in a different order before the cells were

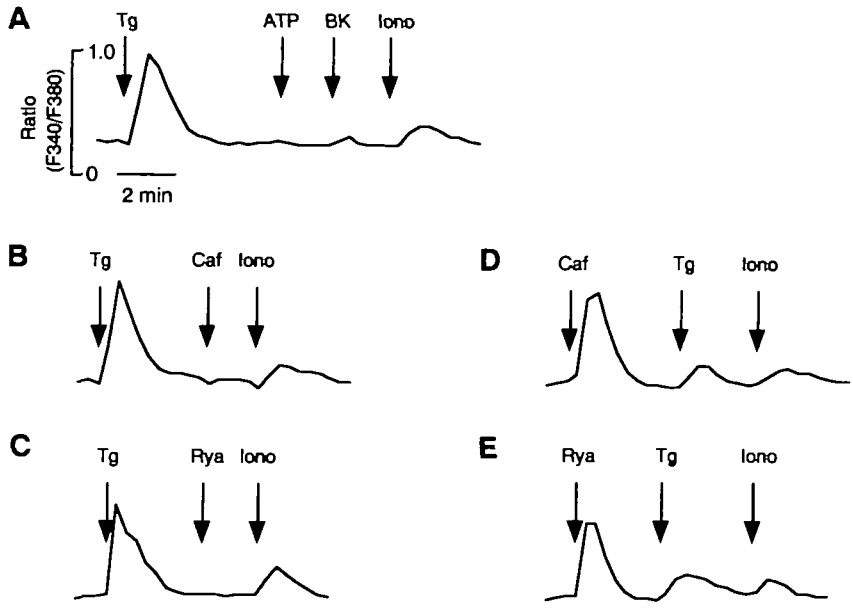

Fig. 3. **Effects on Ca^{2+} release of sequential additions of various agents in different orders.** Experimental details are described under "MATERIALS AND METHODS" and in the legend to Fig. 1. All experiments were carried out in the absence of Ca^{2+}_o. The arrows indicate the times of addition of Tg (1 μM), ATP (1 mM), bradykinin (BK, 5 μM), caffeine (Caf, 25 mM), ryanodine (Rya, 1 μM), and ionomycin (Iono, 5 μM). Each agent was washed out at the time the next was added. Recordings, each from a single cell, are shown as representatives of 3 independent experiments.

exposed to Tg and ionomycin. As shown in Fig. 4, A and B, the initial application of ATP abolished the response to caffeine or ryanodine. Although a single application of ATP, even at a supramaximal concentration (100 μM), was insufficient to produce a complete depletion of the IP_3-sensitive stores, the absence of a $[Ca^{2+}]_i$ response to caffeine and ryanodine suggests that caffeine/ryanodine-sensitive stores can be completely released by IP_3. In contrast, when caffeine or ryanodine was applied first, the subsequent addition of ATP caused an apparent increase in $[Ca^{2+}]_i$ (Fig. 4, C and D). Again, caffeine and ryanodine could be only partially effective in depleting intracellular Ca^{2+} stores, but the above results are also compatible with the possibility that some portions of the intracellular stores in A549 cells express both IP_3Rs and RyRs, whereas others express IP_3Rs alone. Indeed, immunocytochemical studies with cultured mouse hippocampal neurons have shown that clusters of IP_3R are more diffusely distributed than those of RyR (*34*). IP_3R and RyR channels can also be functionally coupled *via* CICR. Because the range of action of spatially localized Ca^{2+} has been shown to be much narrower than that of IP_3 (*35*), it can be envisaged that IP_3 may induce Ca^{2+} release through both IP_3R and RyR channels, whereas the CICR elicited by RyR activation is limited to the vicinity of the channel.

Identification of IP_3R, RyR Isoforms, and hTrp Homologues—To substantiate the involvement of IP_3- and caffeine/ryanodine-sensitive stores in Ca^{2+} homeostasis in A549 cells on a molecular basis, we amplified mRNAs for IP_3R and RyR by means of RT-PCR. Using the ratio PCR analysis (*13*, *14*), we first attempted to identify IP_3R isoforms expressed in this cell line. The two primer pair sets designed to co-amplify the type 1 and type 2, or type 1 and type 3 IP_3R isoforms indeed amplified the type 1 and type 2 IP_3Rs (256 bp) and the type 1 and type 3 IP_3Rs (243 bp), respectively, and with the same efficiency (Fig. 5, A and B). The 256 bp product was digested with *Pst*I and *Taq*I, which specifically cut type 1 and type 2 isoforms, respectively, yielding digestion fragments of the expected size. The combined use of the two restriction enzymes resulted in complete digestion, confirming that both type 1 and type 2 isoforms were expressed. Similarly, the 243 bp product was digested to fragments of the expected size with *Pml*I and *Rsa*I, which specifically cut type 1 and type 3 isoforms, respectively, and a combination of *Pml*I and *Rsa*I led to complete digestion. The type 3 isoform appeared to be more abundant than the type 1, because type 1–specific *Pml*I digested only a small portion of the 243 bp product, whereas type 3–specific *Rsa*I cut most of it. Although different restriction enzymes may have not worked with identical efficiencies, the incubations with the restriction enzymes were carried out until complete digestion was achieved to compare reasonably the relative abundance of each isoform. Taken together, these results indicate that all three isoforms of IP_3R are expressed in A549 cells, and that type 3 is the most abundant isoform. The expression of multiple isoforms of IP_3R has been observed in a variety of vertebrate tissues and cell lines (*36*), and there is evidence that different isoforms of IP_3R subunits assemble to form heterotetramer channels (*37*). Significant differences in the reactivity of each isoform to agonists (IP_3 and Ca^{2+}) provide further functional diversity to IP_3R channels (*36*, *38*, *39*). Type 3 IP_3R is least sensitive to IP_3 (*35–37*) and its channel open probability increases monotonically with increased $[Ca^{2+}]_i$ in the presence of IP_3 (*40*). In our previous study, we showed that in A549 cells both P2X4-mediated Ca^{2+} influx and P2Y2/P2Y4-mediated Ca^{2+} mobilization from IP_3-sensitive stores are activated simultaneously by extracellular ATP and contribute to the shaping of the ATP-induced $[Ca^{2+}]_i$ spike (*1*). The expression pattern of IP_3R isoforms in A549 cells, type 3 > type 2 ≒ type 1 as judged from the ratio RT-PCR analysis, may provide a means by which the cells integrate the input of Ca^{2+} stimuli into a global $[Ca^{2+}]_i$ response.

Ratio PCR analysis was also used to identify which type(s) of RyR is expressed in A549 cells. A primer pair recognizing all the RyR isoforms amplified a ~1,200 bp product (Fig. 5C). This product was completely digested to 740- and 420-bp fragments by type 1 RyR-specific *Nco*I, but was resistant to type 2–specific *Tth111*I and type 3–specific

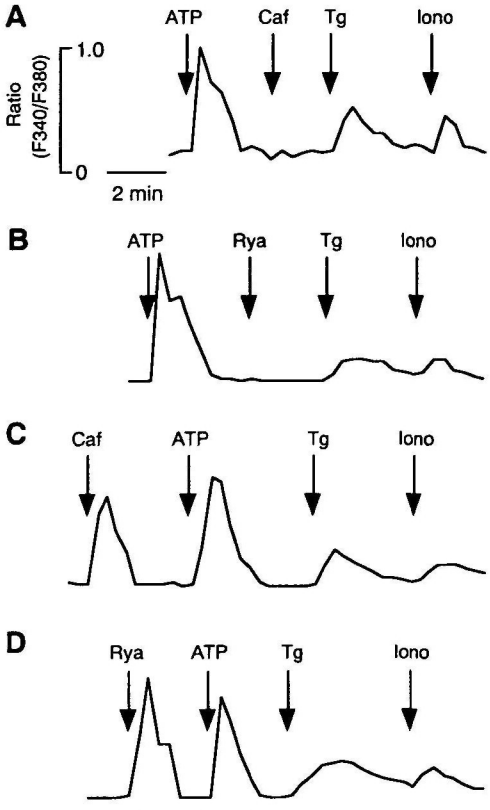

Fig. 4. **Functional interaction between IP_3-sensitive and caffeine/ryanodine-sensitive stores.** All experiments were carried out in the absence of Ca^{2+}_o. Experimental details are given under "MATERIALS AND METHODS" and in the legend to Fig. 1. The arrows indicate the times of addition of Tg (1 μM), ATP (1 mM), caffeine (Caf, 25 mM), ryanodine (Rya, 1 μM), and ionomycin (Iono, 5 μM). Each agent was washed out at the time the next was added. Recordings, each from a single cell, are shown as representatives of 3 independent experiments.

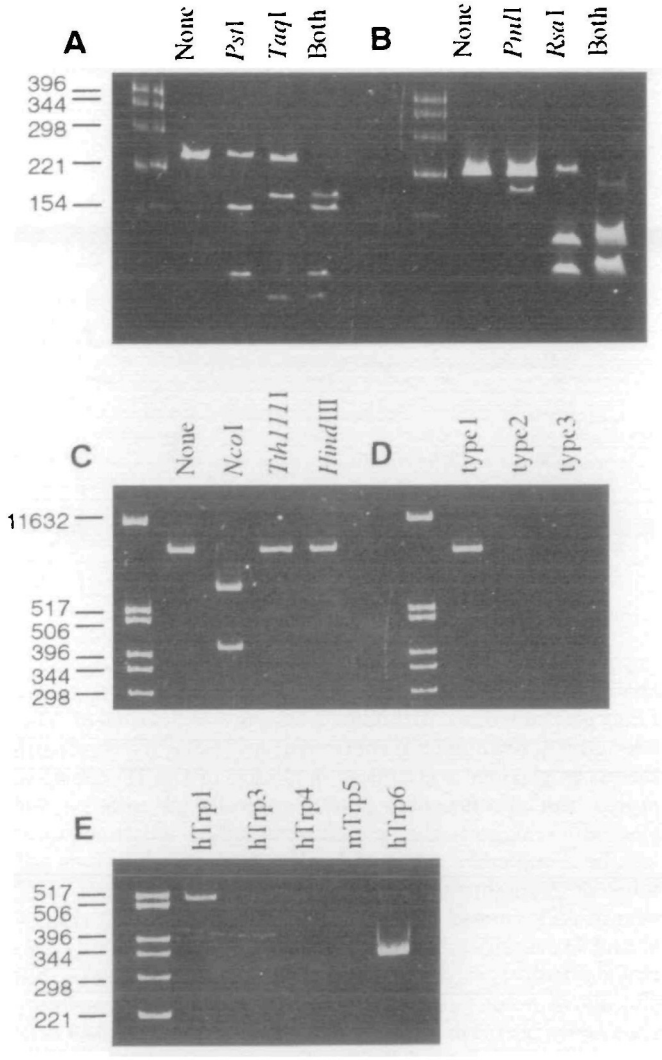

Fig. 5. **Identification of IP_3R, RyR isoforms, and Trp homologues expressed in A549 cells.** Poly(A)$^+$ RNA was reverse-transcribed and PCR-amplified as described in "MATERIALS AND METHODS". A and B: Restriction enzyme analyses of expressed IP_3R isoforms. A: Simultaneous amplification of type 1 and type 2 IP_3Rs. Lane "None" shows the 256 bp product not treated with restriction enzymes. *Pst*I specifically cuts the type 1 isoform to 168- and 88-bp fragments, and *Taq*I cuts only the type 2 isoform to 182- and 74-bp fragments. "Both" indicates digestion with both *Pst*I and *Taq*I. B: Simultaneous amplification of type 1 and type 3 IP_3Rs. "None" shows the 243-bp product not digested with restriction enzymes. *Pml*I specifically cuts the type 1 isoform to 205- and 38-bp fragments, and *Rsa*I specifically cuts the type 3 isoform to 136- and 107-bp fragments. "Both" indicates digestion with both *Pml*I and *Rsa*I. In B, the 38-bp fragment formed from type 1 IP_3R by *Pml*I digestion can not be seen due to its small size and low quantity. C and D: Expression of RyR isoforms. C: Simultaneous amplification of three isoforms using a common primer pair. "None" shows the ~1,200-bp product not digested with restriction enzymes. *Nco*I specifically cuts the type 1 isoform to 740- and 420-bp fragments. *Tth111*I (type 2-specific) and *Hind*III (type 3-specific) are expected to cut the ~1,200-bp product to 640- and 490-bp fragments and 560- and 550-bp fragments, respectively, if the two isoforms are expressed. All the digestions with restriction enzymes were carried out for 2 h. D: Selective amplification of RyR isoforms with specific primer pairs. E: Amplification of Trps with primer pairs specific to the respective homologues. The *Hin*fI digest of pBR322 was used as a size marker. Numbers at left, bp.

*Hin*dIII. In addition, in the PCR amplification with isoform-specific primer pairs, a product of the expected size (~1,200 bp) was obtained only with the type 1–specific primer pair (Fig. 5D). These results suggest that only type 1 RyR is expressed and is the channel that is sensitive to caffeine and ryanodine in nonexcitable A549 cells. This RyR isoform, as well as IP_3Rs, may be involved in the propagation *via* CICR of the Ca^{2+} signal, such as the Ca^{2+} influx through ATP-gated P2X channel or functional L-type VDCC (*1*). Type 1 RyR, the predominant RyR isoform in skeletal muscle, has been shown to be in a reciprocal interaction with the L-type VDCC during excitation–contraction coupling (*41*). If the coexpression of type 1 RyR and the L-type VDCC is not the result of a coincidence, their functional coupling and physiological roles are of interest.

As for CCE channels, neither the activation mechanism nor the molecular identity has been fully elucidated. Transient receptor potential protein (Trp) in *Drosophila* photoreceptor cells was identified as a Ca^{2+} channel activated by store depletion (*42*, *43*). Several mammalian homologues of Trp have been cloned and expression of some trp cDNAs, such as human trp1 (*44*) and bovine trp4 (*45*), result in increased Ca^{2+} entry in response to pretreatment with Tg. Therefore, we then amplified mRNAs of Trp homologues.

As shown in Fig. 5E, hTrp1 and hTrp6 were expressed in A549 cells, whereas hTrp3, hTrp4, and mTrp5 were not detectable even after a 35-cycle amplification. Although Trp6 appeared to be associated with second messenger-regulated Ca^{2+} influx (*46*), Trp1 has been strongly suggested to be a candidate protein for the CCE mechanism (*44*, *47*). Birnbaumer *et al.* (*48*) proposed that different Trps may form homomultimeric or heteromultimeric CCE channels, and thus each subunit would convey functional heterogeneity to the channels. However, whether Trp1 and Trp6 are actually responsible for the CCE observed in A549 cells is left to future study.

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