0026-895X/95/010021-08$03.00/0

MOLECULAR PHARMACOLOGY, 47:21-28, 1995.

A Ryanodine Receptor-like Ca^{2+} Channel Is Expressed in Nonexcitable Cells

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Received April 7, 1994; Accepted October 5, 1994

SUMMARY

In this study we investigated the effects of 4-chloro-3-ethylphenol on Ca^{2+} homeostasis, and we report that this compound induces Ca^{2+} release from a ruthenium red-sensitive Ca^{2+} release channel present in skeletal muscle terminal cisternae. When tested with bovine cerebellar microsomes, the Ca^{2+}-releasing activity of 4-chloro-3-ethylphenol was reduced by ruthenium red and unaffected by heparin. In PC-12 cells, HL-60 cells, human fibroblasts, the human hepatoma cell line PLC/PRF/5, and Jurkat cells, 4-chloro-3-ethylphenol released Ca^{2+} from intracellular thapsigargin-sensitive stores. Although decreased, its effect was retained after treatment of intact/permeabilized cells with inositol-1,4,5-trisphosphate (IP_3)-mobilizing agonists/IP_3, whereas pretreatment of permeabilized cells with ruthenium red reduced the Ca^{2+}-releasing activity of 4-chloro-3-ethylphenol. These results provide functional evidence for the presence of a Ca^{2+} channel distinct from the IP_3 receptor, having pharmacological similarities to the ryanodine receptor, in the intracellular Ca^{2+} stores of a variety of nonexcitable cells. We also demonstrate that a monoclonal anti-ryanodine receptor antibody recognizes a protein in human fibroblasts with similar apparent molecular mass the ryanodine receptor. Thus, the intracellular Ca^{2+} stores of mammalian cells appear to be endowed with two distinct intracellular Ca^{2+} channels.

Stimulation of a variety of cell surface receptors with inositol phosphate-mobilizing agonists leads to a rapid increase in $[Ca^{2+}]_i$ (for review, see Refs. 1–3). It is well documented that IP_3 that is generated binds to an intracellular receptor, the IP_3R, causing Ca^{2+} release from intracellular stores. The cDNA encoding the IP_3R has been cloned, sequenced, and expressed (4, 5); Northern blot analysis confirms its widespread tissue distribution, whereas reconstitution experiments demonstrate that it assembles in a tetrameric structure that functions as a Ca^{2+} channel (4–6).

In skeletal and cardiac muscle on the other hand, Ca^{2+} release from the SR is mediated by a caffeine-sensitive Ca^{2+} channel, termed the RYR (for review, see Refs. 7 and 8). The cDNA encoding the RYR has also been cloned, and at least three distinct isoforms have been identified, one specific for skeletal muscle, one present in heart and in cerebellum, and a third present in brain (9–12).

This work was supported by grants from Telethon Italy and Consiglio Nazionale delle Ricerche (93.00450.CT04). Part of this work was also supported by Ministero Università Ricerca Scientifica e tecnologica, 40% and 60% to Prof. G. Benassi and Prof P. Melandri (Institute of General Pathology, University of Ferrara, Ferrara, Italy), respectively. F.L. and P.M. are recipients of Telethon and Banca Popolare Veneta scholarships, respectively.

To date, structural evidence supporting the existence of a RYR in nonexcitable tissues has been scant (13, 14); however, functional studies indicate that some cells, particularly those present in the central nervous system, are endowed with two types of intracellular Ca^{2+} channels (3, 15–17). In this case a RYR-like Ca^{2+} channel has been postulated to be involved in Ca^{2+} oscillations, via a Ca^{2+}-induced Ca^{2+} release mechanism (3, 15–17). Indirect evidence supporting the existence of multiple types of intracellular Ca^{2+} channels/Ca^{2+} pools has been recently obtained in pancreatic acinar cells, pituitary cells, and Jurkat cells (18–20).

Our interests have focused on intracellular Ca^{2+} channels, in particular on whether all cells possess only an IP_3-gated channel or are endowed with other intracellular Ca^{2+} channels. As a preliminary step in this direction, we have identified a molecular probe that directly activates the skeletal muscle RYR Ca^{2+} channel. Here we show that 4-chloro-3-ethylphenol acts by releasing Ca^{2+} from a thapsigargin-sensitive intracellular pool in a variety of cell types. Furthermore, its Ca^{2+}-releasing activity is retained after pretreatment of cells with IP_3-mobilizing agonists. We also show that in permeabilized cells the Ca^{2+}-releasing activity of 4-chloro-3-ethylphenol is diminished by

ABBREVIATIONS: $[Ca^{2+}]_i$, intracellular Ca^{2+} concentration; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; MOPS, 3-(*N*-morpholino)propanesulfonic acid; IP_3, inositol-1,4,5-trisphosphate; IP_3R, inositol trisphosphate receptor; RYR, ryanodine receptor; TC, terminal cisternae; LSR, longitudinal sarcoplasmic reticulum; SERCA, sarco/endoplasmic reticulum Ca^{2+}-ATPase; $[Ca^{2+}]_{med}$, Ca^{2+} concentration in the medium; FA, free acid; AM, acetoxymethyl ester; SR, sarcoplasmic reticulum.

pretreatment with ruthenium red, a known antagonist of Ca^{2+} release mediated by the skeletal and cardiac muscle RYR (21). The existence of a RYR-like receptor in nonexcitable cells is also supported by Western blot analysis.

Experimental Procedures

Materials. The experiments presented here were carried out on the parental PC-12 line subcultured in our laboratory as described previously (22). The human hepatoma cell line PLC/PRF/5, HL-60 cells, and Jurkat cells were obtained from the Institute of Microbiology, University of Ferrara (Ferrara, Italy), and cultured according to American Type Culture Collection recommendations. Human fibroblasts were a kind gift from the Institute of Medical Genetics, University of Ferrara. Ruthenium red, peroxidase-conjugated Protein A, and antipyrylazo III were from Fluka (Buchs, Switzerland); 4-chloro-3-ethylphenol was from Aldrich; ATP, NADH, creatine phosphate, creatine phosphate kinase, phosphoenolpyruvate, pyruvate kinase, lactate dehydrogenase, and the enhanced chemiluminescence kit were from Boehringer Mannheim; bradykinin, saponin, formyl-methionine-leucine-phenylalanine, heparin, and IP_3 were from Sigma Chemical Co. (St. Louis, MO); ionomycin, A23187, and thapsigargin were from Calbiochem (San Diego, CA); fura-2/AM/FA and fluo-3 FA were from Molecular Probes (Eugene, OR). OKT3 monoclonal antibodies and concanavalin A were a kind gift from the Institute of Medical Genetics, University of Ferrara. All other materials were analytical grade or the highest available grade.

Subcellular fractionation. SR was isolated from white muscles of New Zeland White rabbits, fractionated into LSR and TC as described by Saito *et al.* (22), and stored in liquid nitrogen.

Bovine cerebella were obtained from the local slaughterhouse, from freshly slaughtered animals, and the microsomal fraction was prepared and stored as described previously (23). Protein concentration was determined according to the method of Bradford (24), using bovine serum albumin as the standard.

Ca^{2+}-dependent ATPase. ATPase rate was measured by a spectrophotometric enzyme-coupled assay (25). The absorbance change at 340 nm (i.e., NADH oxidation) was continuously monitored with a Beckman DU7400 diode-array spectrophotometer. The assay was carried out at 37° in a medium containing, in a final volume of 1 ml, 20 mM histidine, pH 7.2, 0.1 M KCl, 5 mM $MgCl_2$, 2 mM ATP, 0.15 mM NADH, 0.5 mM phosphoenolpyruvate, 2 units of pyruvate kinase, 2 units of lactate dehydrogenase, and 5–10 μg of LSR. Basal ATPase activity was measured in the presence of 200 μM EGTA, and the total ATPase rate was measured after the addition of 200 μM $CaCl_2$ (estimated Ca^{2+} concentration, 10 μM). The maximal Ca^{2+}-dependent ATPase rate was obtained in the presence of the Ca^{2+} ionophore A23187 (2 μg/ml).

$[Ca^{2+}]_i$ measurements. Loading with fura-2/AM (final concentration, 5 μM) and $[Ca^{2+}]_i$ measurements were carried out in 140 mM NaCl, 5 mM KCl, 1 mM $MgSO_4$, 20 mM HEPES, 1 mM NaH_2PO_4, 5 mM glucose, pH 7.4, as described (23, 26).

Ca^{2+} release from membrane fractions. Ca^{2+} release from isolated SR fractions was measured with a Beckman DU7400 diode-array spectrophotometer by monitoring the $A_{710-790}$ of the Ca^{2+} indicator antipyrylazo III, as described by Palade (21) and detailed by us (27). Pulses of 25 nmol of Ca^{2+} were administered to load the SR fractions with approximately 2.3 μmol of Ca^{2+}/mg of protein. When steady state was reached, Ca^{2+} release was triggered by the addition of different compounds.

Ca^{2+} release from bovine cerebellar microsomes was measured with the Ca^{2+} indicator fluo-3 FA (final concentration, 200 nM) in a thermostatted Perkin Elmer LS50 spectrofluorimeter equipped with magnetic stirring, as described previously (23). Because the addition of 10 μM ruthenium red quenched fluo-3 fluorescence by approximately 40%, when the latter compound was added 600 nM fluo-3 was used to measure Ca^{2+} release.

Ca^{2+} release from permeabilized cells. Cells were permeabilized as described by Guse *et al.* (28). Briefly, cells (1×10^8 cells/ml) were incubated in 20 mM HEPES, 110 mM KCl, 10 mM NaCl, 2 mM $MgCl_2$, 5 mM KH_2PO_4, 2 mM EGTA, 1 mM dithiothreitol, pH 7.2, in the presence of saponin (30 μg/ml), for 5 min at 37°. Cells were then washed three times with 20 mM HEPES, 110 mM KCl, 10 mM NaCl, 2 mM $MgCl_2$, 5 mM KH_2PO_4, pH 7.2, and resuspended in 18.5 mM MOPS, pH 7.0, 7.5 mM potassium pyrophosphate, pH 7.0, 100 mM KCl, with 5 mM NaN_3 to inhibit mitochondria, at a final concentration of $3–5 \times 10^6$ cells/ml. Ca^{2+} release from intracellular stores was measured as described for the cerebellar microsomes. Qualitatively identical results were obtained when fura-2 FA was used to measure changes in $[Ca^{2+}]_{med}$, except that 10 μM ruthenium red quenched >50% of the fluorescent signal when the latter indicator was used.

Monoclonal antibodies and Western blotting. Monoclonal antibodies were prepared against a RYR fusion protein (27) encompassing a sequence shared by all three RYR isoforms, as described previously (29). For Western blotting, membrane fractions and total cell extracts were loaded on a 5% acrylamide gel. Slab gel electrophoresis, blotting onto nitrocellulose membranes, and indirect immunoenzymatic staining were carried out as described previously (27), except that peroxidase-conjugated Protein A was used instead of a secondary antiserum and the reactive immunopositive bands were visualized by enhanced chemiluminescence, according to the manufacturer's recommendations.

Results

Effect of 4-chloro-3-ethylphenol on isolated membrane fractions. Fig. 1 shows the effect of 4-chloro-3-ethylphenol on the initial rate of Ca^{2+} release from actively loaded TC and LSR vesicles. Addition of 100 μM 4-chloro-3-ethylphenol caused a rapid release of Ca^{2+} from the TC fraction (Fig. 1A). Maximal Ca^{2+}-releasing activity was observed at a concentration of 250 μM 4-chloro-3-ethylphenol (4.38 ± 0.44 μmol Ca^{2+}/min/mg of protein, four experiments) (data not shown). Pretreatment of TC with 10 μM ruthenium red, a known inhibitor of the RYR, completely abolished 4-chloro-3-ethylphenol-induced Ca^{2+} release (Fig. 1B), whereas 4-chloro-3-ethylphenol did not release Ca^{2+} from LSR (Fig. 1C), indicating that it was not damaging the vesicles or acting by inhibiting the SERCA. The latter argument was further substantiated by the lack of effect of 4-chloro-3-ethylphenol on SERCA activity. As shown in Fig. 2, SERCA activity was unaffected by pretreatment of LSR vesicles with 300 μM 4-chloro-3-ethylphenol. Fig. 2, shows that, if anything, the presence of the compound slightly increased the Ca^{2+}-activated ATPase activity. This result is in accordance with the ability of 4-chloro-3-ethylphenol to activate Ca^{2+} efflux from TC and therefore to stimulate the Ca^{2+}-dependent ATPase rate by removing Ca^{2+} back-inhibition of the Ca^{2+} pump. Maximal SERCA activity (obtained in the presence of 2 μg/ml A23187) was identical in the presence and in the absence of 300 μM 4-chloro-3-ethylphenol.

These experiments on isolated TC fractions did not exclude the possibility that 4-chloro-3-ethylphenol acts via the IP_3R; thus, we tested the effect of the compound on bovine cerebellar microsomes. The net increase in $[Ca^{2+}]_{med}$ induced by 100, 300, and 500 μM 4-chloro-3-ethylphenol was 99.7 ± 19.2 (three experiments), 242.0 ± 15.1 (three experiments), and 238.2 ± 6.7 nM (seven experiments), respectively. Thus, maximal Ca^{2+}-releasing activity was obtained at a concentration of 300 μM 4-chloro-3-ethylphenol. Concentrations greater than 500 μM were not investigated. The addition of 4-chloro-3-ethylphenol caused Ca^{2+} release from actively loaded cerebellar microsomes, even

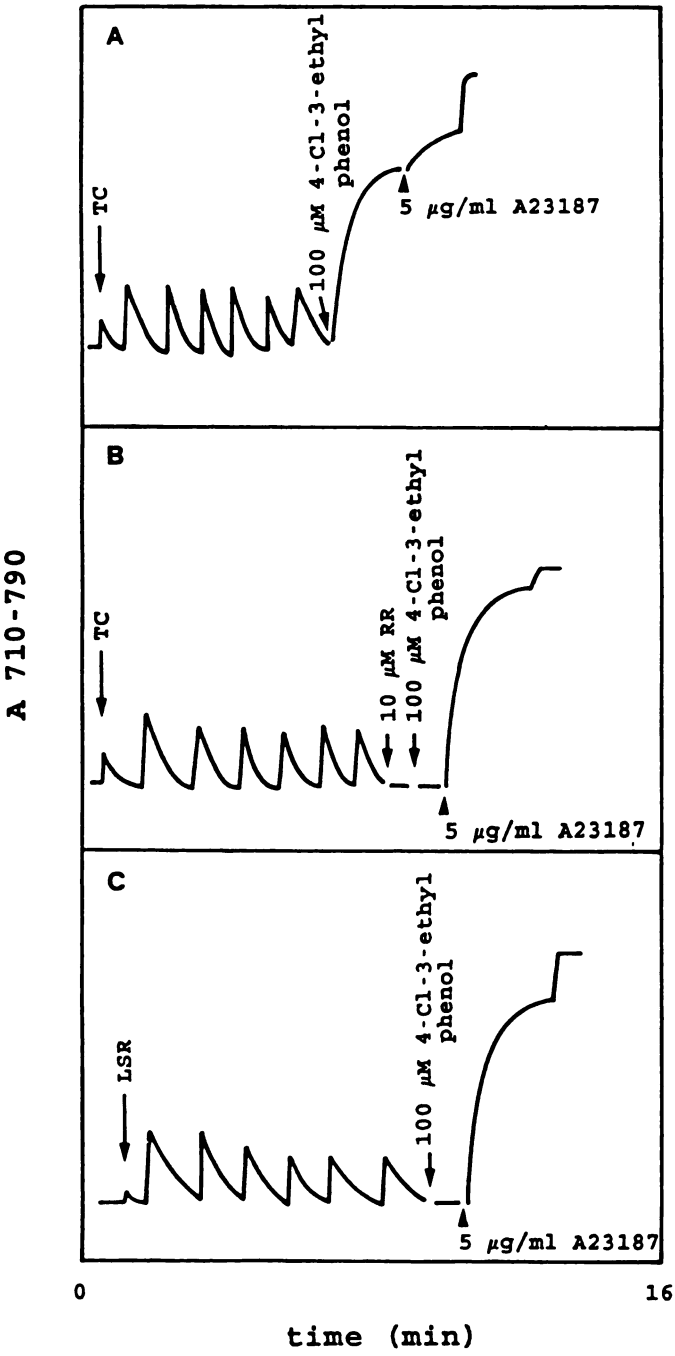

Fig. 1. 4-Chloro-3-ethylphenol induction of Ca^{2+} release from skeletal muscle TC but not LSR. Measurements were performed using the Ca^{2+} indicator antipyrylazo III; where indicated, 50 μg of TC (A and C) or LSR (B) were added to the cuvette, followed by five consecutive additions of 25 nmol of $CaCl_2$. After completion of Ca^{2+} loading, 100 μM 4-chloro-3-etylphenol (A and C) or 10 μM ruthenium red followed by 4-chloro-3-ethylphenol (B) was added. At the end of the experiments, total accumulated Ca^{2+} was released with the ionophore A23187 and the dye response was calibrated by addition of 25 nmol of $CaCl_2$. Upward deflections indicate Ca^{2+} release and downward deflections indicate Ca^{2+} accumulation. Results are representative of experiments carried out at least four times.

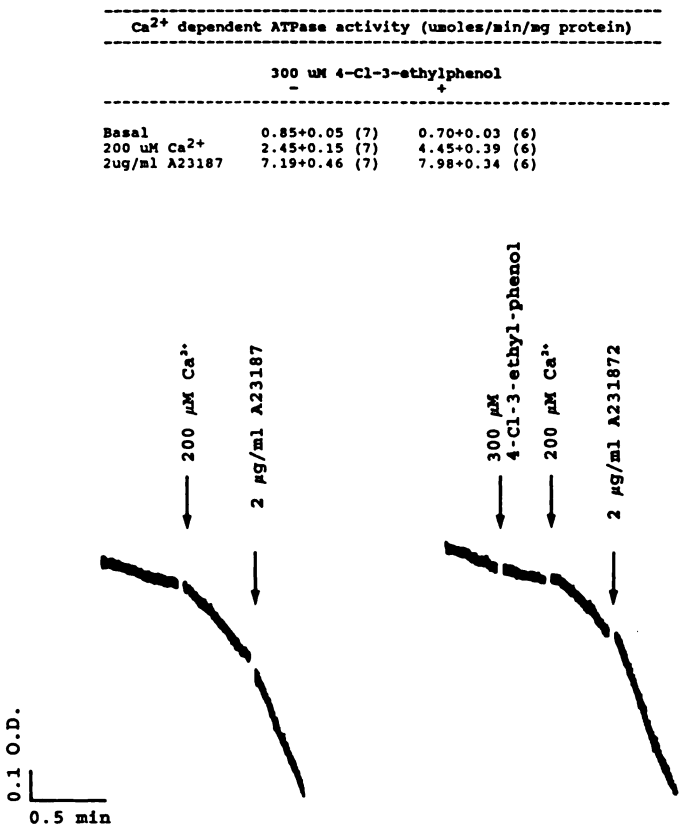

Fig. 2. Effect of 4-chloro-3-ethylphenol on SERCA activity of skeletal muscle LSR. ATPase rates were measured with a spectrophotometric enzyme-coupled assay, by monitoring the absorbance change at 340 nm, as described in Experimental Procedures. Rabbit LSR vesicles (15 μg/ml) were added to a 37° thermostatted cuvette and basal ATPase activity was recorded in the presence or absence of 300 μM 4-chloro-3-ethylphenol. Where indicated, 200 μM $CaCl_2$ and 2 μg/ml A23187 were added to obtain Ca^{2+}-activated and maximal Ca^{2+}-dependent ATPase, respectively. *Inset*, mean ± standard error of Ca^{2+}-dependent ATPase activity of the experiments given in parentheses.

after the addition of 10 μM IP_3 (Fig. 3A). Ca^{2+} release produced by 4-chloro-3-ethylphenol was unaffected by the addition of 200 μg/ml heparin (Fig. 3B), a known inhibitor of the IP_3R (30), whereas the net increase in the $[Ca^{2+}]_{med}$ induced by IP_3, after the addition of 300 μM 4-chloro-3-ethylphenol, was reduced (Fig. 3C). Table 1 summarizes the effects of 4-chloro-3-ethylphenol and IP_3 on the Ca^{2+} pools contained in bovine cerebellar microsomes; when saturating concentrations of 4-chloro-3-ethylphenol were added first, IP_3 released 77.8 ± 10.6 nM Ca^{2+} or approximately 25% of the agonist-sensitive pool, whereas, when microsomes were pretreated with 10 μM IP_3 (i.e., a maximal concentration), 4-chloro-3-ethylphenol released 171.8 ± 13.8 nM Ca^{2+} or approximately 50% of the agonist-sensitive pool. Similar results were obtained when 500 μM 4-chloro-3-ethylphenol was used (data not shown). These results demonstrate the specificity of 4-chloro-3-ethylphenol for an intracellular Ca^{2+} channel that is pharmacologically distinct from the IP_3R. We next investigated whether the Ca^{2+}-releasing activity of 4-chloro-3-ethylphenol could be blocked by ruthenium red. Fig. 4 shows that, in bovine cerebellar microsomes, increasing concentrations of ruthenium red specifically decreased the net Ca^{2+} released by 300 μM 4-chloro-3-ethylphenol, although total inhibition of Ca^{2+} release was never achieved. Greater concentrations of ruthenium red could not be tested because they greatly affected fluo-3 fluorescence.

Effect of 4-chloro-3-ethylphenol on the $[Ca^{2+}]_i$ of intact cells. A number of studies have documented that in the rat pheochromocytoma cell line PC-12 two distinct intracellular

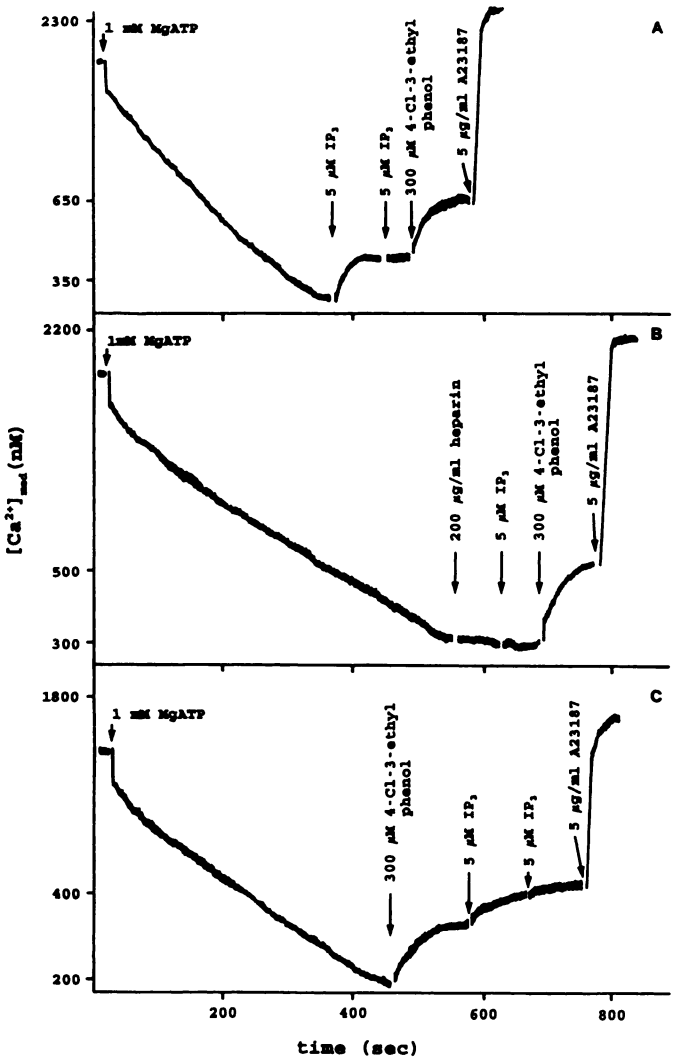

Fig. 3. Effect of 4-chloro-3-ethylphenol on bovine cerebellar microsomes. Changes in $[Ca^{2+}]_{med}$ were monitored with the fluorescent indicator fluo-3 (final concentration, 200 nM). Ca^{2+} accumulation was stimulated by the addition of 1 mM MgATP to the microsomes (final concentration, 200 μg of protein/ml), in the presence of 5 mM NaN_3. At the end of the experiment, all of the accumulated Ca^{2+} was released by the addition of 5 μg/ml A23187.

release channels exist, one sensitive to IP_3 and the other sensitive to caffeine (31). We thus tested the ability of 4-chloro-3-ethylphenol to release Ca^{2+} from the intracellular stores of PC-12 cells. Fig. 5 shows that the maximal Ca^{2+}-releasing activity was obtained at a concentration of 300 μM, a result similar to that obtained in cerebellar microsomes and skeletal muscle TC. This concentration of 4-chloro-3-ethylphenol was utilized to study the intracellular Ca^{2+} pools of a number of cell types. In the human hepatoma cell line PLC/PRF/5, 4-chloro-3-ethylphenol induced a rapid rise in the $[Ca^{2+}]_i$, followed by a slower decay phase (Fig. 6A). The experiments depicted in Fig. 6 also demonstrate that the intracelluar Ca^{2+} pool that is sensitive to bradykinin, i.e., an IP_3-mobilizing agonist, is contained within the 4-chloro-3-ethylphenol-sensitive Ca^{2+} pool. In fact, when PLC/PRF/5 cells were pretreated with 4-chloro-3-ethylphenol, bradykinin no longer caused an increase in the $[Ca^{2+}]_i$ (Fig. 6A). When 4-chloro-3-ethylphenol was added after a maximal concentration of bradykinin (200 nM), however, its

TABLE 1

Ca^{2+} release induced by 4-chloro-3-ethylphenol and IP_3 in bovine cerebellar microsomes

Changes in the $[Ca^{2+}]_{med}$ were monitored with the fluorescent indicator fluo-3, and the increase in $[Ca^{2+}]_{med}$ (which reflects Ca^{2+} release from microsomes) due to the addition of 300 μM 4-chloro-3-ethylphenol and 10 μM IP_3 was calculated. Results are the mean ± standard error of the number of experiments given in parentheses.

Increase in $[Ca^{2+}]_{med}$	
nM	
4-Chloro-3-ethylphenol first	IP_3 second
222.3 ± 18.5 (6)	77.8 ± 10.6 (4)
IP_3 first	4-Chloro-3-ethylphenol second
185.2 ± 15.4 (5)	171.8 ± 13.8 (5)

Ca^{2+}-mobilizing activity was marginally reduced (Fig. 6, compare A and B; also see Table 2). Fig. 6C shows that the addition of thapsigargin, an inhibitor of intracellular Ca^{2+}-ATPase that depletes intracellular pools endowed with SERCA (32), almost completely abolished 4-chloro-3-ethylphenol-induced $[Ca^{2+}]_i$ rises. These results demonstrate the specificity of 4-chloro-3-ethylphenol for SERCA-containing intracellular stores. It should be noted that pretreatment of PLC/PRF/5 cells with bradykinin and 4-chloro-3-ethylphenol did not totally abolish thapsigargin-mediated $[Ca^{2+}]_i$ rises (Fig. 6, A and B), indicating the possible existence of a fourth Ca^{2+} pool in this cell type.

The same protocol was applied to several other cell lines and gave qualitatively similar results, that is 4-chloro-3-ethylphenol is capable of releasing Ca^{2+} from a thapsigargin-sensitive pool in PC-12 cells, Jurkat cells, HL-60 cells, and human fibroblasts. Table 2 shows the net changes in the $[Ca^{2+}]_i$ induced by 4-chloro-3-ethylphenol in different cell lines. Cells were treated either with 4-chloro-3-ethylphenol alone or with maximal stimulatory concentrations of IP_3-mobilizing agonists followed by 4-chloro-3-ethylphenol. Thus, in the five cell lines that we examined, 4-chloro-3-ethylphenol was able to cause an increase in the $[Ca^{2+}]_i$; however, its capacity to do so and the net amount of Ca^{2+} released by 4-chloro-3-ethylphenol after pretreatment with an IP_3-mobilizing agonist varied in the different cell lines.

Effect of 4-chloro-3-ethylphenol in permeabilized cells. The results presented for intact cells and cerebellar microsomes support the hypothesis that most cell types are endowed with two intracellular Ca^{2+} release channels, the IP_3R and a 4-chloro-3-ethylphenol-sensitive Ca^{2+} release mechanism. To gain direct access to the intracellular Ca^{2+} stores, we carried out experiments on permeabilized cells. Ca^{2+} accumulation was stimulated by the addition of 1 mM MgATP and, once steady state was reached, Ca^{2+} release was triggered by different compounds. At the end of each experiment 2 μM ionomycin or 5 μg/ml A23187 was added to release total accumulated Ca^{2+}. All experiments were carried out in the presence of 5 mM NaN_3 or 10 μM carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone (data not shown). Fig. 7A shows that pretreatment of permeabilized PLC/PRF/5 cells with heparin abolished IP_3-mediated Ca^{2+} release but did not affect that mediated by 4-chloro-3-ethylphenol (the increase in the $[Ca^{2+}]_{med}$ obtained after the addition of 300 μM 4-chloro-3-ethylphenol was 250.0 ± 22.3 nM; four experiments). When cells were pretreated with 15 μM IP_3 (the increase in $[Ca^{2+}]_{med}$ obtained was 128.3 ± 11.4 nM; three experiments), 4-chloro-3-ethylphenol was still capable of releasing Ca^{2+} from the intracellular stores (the increase in $[Ca^{2+}]_{med}$ was 149.6 ± 29.1 nM; three experiments) (Fig. 7B). Fig. 7C demonstrates that pretreatment of cells with 4-chloro-

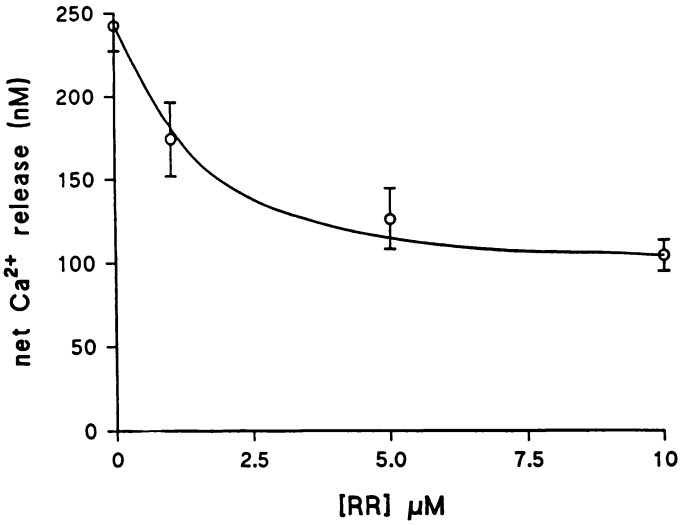

Fig. 4. Effect of increasing concentrations of ruthenium red (*RR*) on the Ca^{2+}-releasing capacity of 300 μM 4-chloro-3-ethylphenol in bovine cerebellar microsomes. Changes in the $[Ca^{2+}]_{med}$ were monitored using the fluorescent indicator fluo-3, as described for Fig. 3. Cerebellar microsomes were treated with 300 μM 4-chloro-3-ethylphenol in the presence or absence of the indicated concentration of ruthenium red and the net changes in the $[Ca^{2+}]_{med}$ were calculated as described in Experimental Procedures. Each *point* represents the mean ± standard error of at least three experiments.

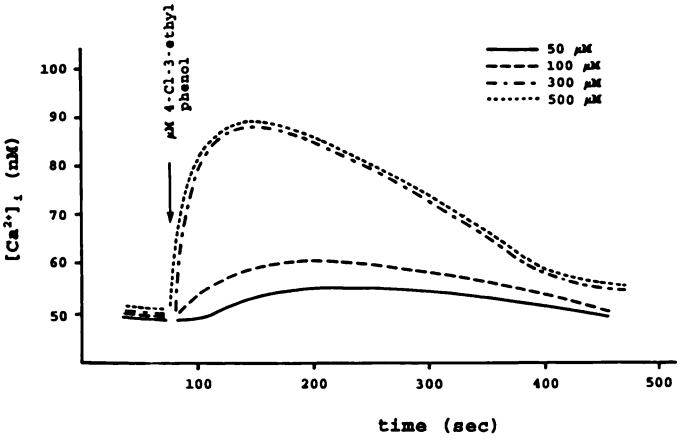

Fig. 5. Dose-dependent increases in the $[Ca^{2+}]_i$ of PC-12 cells produced by 4-chloro-3-ethylphenol. Cells (0.5×10^6 cells/ml) loaded with 5 μM fura-2/AM were used. Experiments were performed in Ca^{2+}-free medium with 1 mM EGTA. The results shown are representative of at least three experiments.

3-ethylphenol prevented IP_3-mediated Ca^{2+} release. Thus, the results for permeabilized cells largely confirm our observations with intact cells, i.e., that 4-chloro-3-ethylphenol releases Ca^{2+} from intracellular stores that only partially overlap with the intracellular Ca^{2+} stores that are sensitive to IP_3. To characterize in more detail the Ca^{2+} release pathway activated by 4-chloro-3-ethylphenol, we studied the effect of ruthenium red. The presence of 10 μM ruthenium red greatly reduced fluo-3 fluorescence intensity. Nevertheless, we were able to calibrate the Ca^{2+} signal and determine the effect of ruthenium red on 4-chloro-3-ethylphenol-induced Ca^{2+} release. As examples, we chose three cell lines, namely PC-12 cells, which have been shown to contain both IP_3-sensitive and caffeine-sensitive intracellular Ca^{2+} stores, Jurkat cells, which grow in suspension, and PLC/PRF/5 cells, which grow attached. Table 3 shows

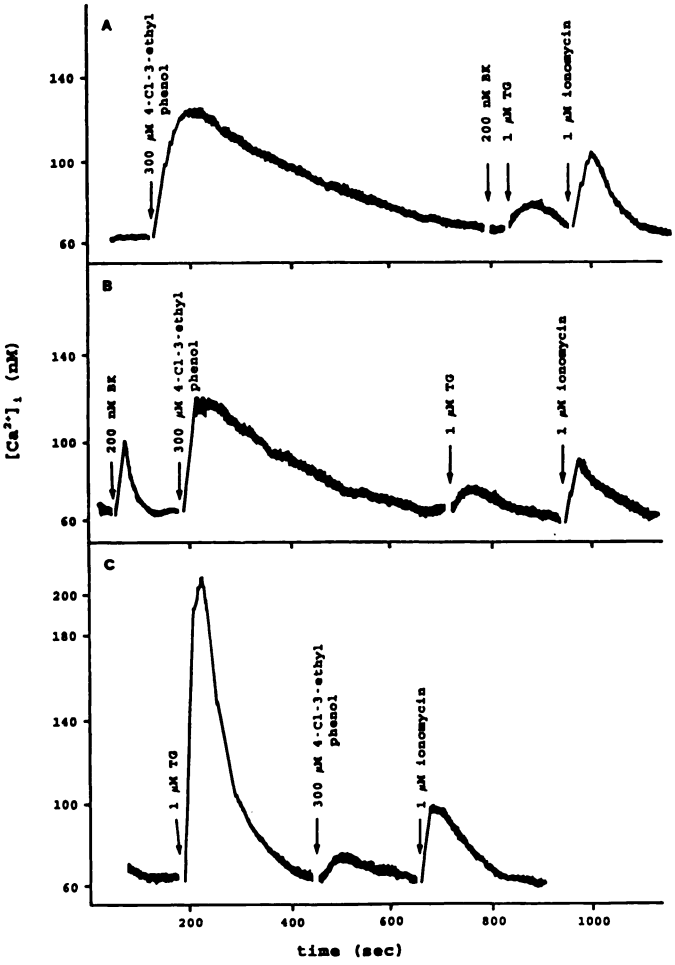

Fig. 6. Effect of 4-chloro-3-ethylphenol on the $[Ca^{2+}]_i$ of PLC/PRF/5 cells. In each experiments, 0.5×10^6 cells/ml were used. Conditions were as in Fig. 5. *TG*, thapsigargin; *BK*, bradykinin.

TABLE 2

Net change in the $[Ca^{2+}]_i$ induced in different cell lines by 4-chloro-3-ethylphenol

Cells were loaded with the fluorescent Ca^{2+} indicator fura-2 FA (final concentration, 5 μM); changes in the $[Ca^{2+}]_i$ induced by 300 μM 4-chloro-3-ethylphenol alone or after the indicated IP_3-mobilizing agonists were recorded in a Perkin Elmer LS50 spectrofluorimeter. The values represent net changes in the peak $[Ca^{2+}]_i$ (mean ± standard error of the number of experiments given in parentheses).

Cell type	Net change in $[Ca^{2+}]_i$ produced by 300 μM 4-chloro-3-ethylphenol	
	No pretreatment	After IP_3-mobilizing agonist
	nM	
PC-12 cells	51.0 ± 6.4 (5)	36.0 ± 5.8 (5)[a]
PLC/PRF/5 cells	66.7 ± 6.3 (8)	56.6 ± 6.7 (3)[a]
HL-60 cells	45.0 ± 6.0 (7)	23.0 ± 2.4 (5)[b]
Fibroblasts	56.3 ± 5.6 (4)	23.3 ± 0.6 (5)[c]
Jurkat cells	48.8 ± 4.3 (8)	27.5 ± 2.5 (4)[d]

[a] Cells pretreated with 1 μM bradykinin.
[b] Cell pretreated with 1 μM formyl-methionine-leucine-phenylalanine.
[c] Cells pretreated with 500 nM bradykinin.
[d] Cells pretreated with 5-μg/ml OKT3 antibody and 10 μg/ml concanavalin A.

that pretreatment of the permeabilized cells with 10 μM ruthenium red reduced the capacity of 4-chloro-3-ethylphenol to release accumulated Ca^{2+}, although it never abolished the Ca^{2+}-releasing activity of 4-chloro-3-ethylphenol. This result argues in favor of the presence of a Ca^{2+} release channel, with phar-

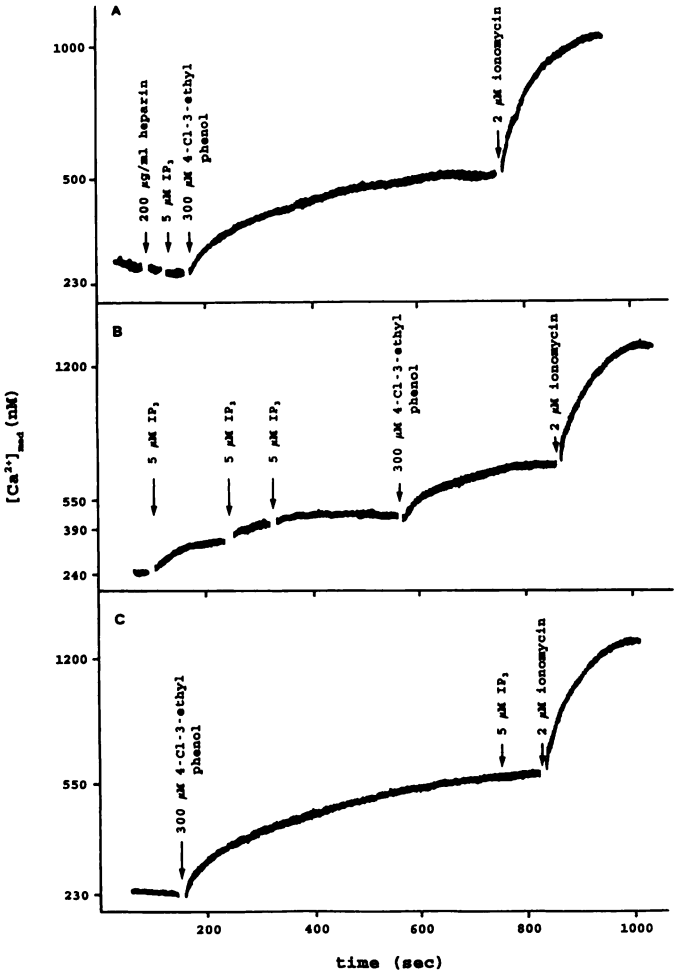

Fig. 7. Effects of heparin, IP_3, and 4-chloro-3-ethylphenol on Ca^{2+} release by permeabilized PLC/PRF/5 cells. Saponin-permeabilized cells were prepared as described in Experimental Procedures. Ca^{2+} accumulation was stimulated by the addition of 1 mM MgATP to 5×10^6 cells/ml in the presence of 5 mM NaN_3. At the end of each experiment, total accumulated Ca^{2+} was released with the Ca^{2+} ionophore ionomycin. Changes in the $[Ca^{2+}]_{med}$ were monitored with 200 nM fluo-3 FA.

TABLE 3

Effects of ruthenium red on the calcium-releasing activity of 4-chloro-3-ethylphenol in permeabilized cells

Changes in the $[Ca^{2+}]_{med}$ were measured with the fluorescent indicator fluo-3 and the increase in $[Ca^{2+}]_{med}$ obtained after the addition of 300 μM 4-chloro-3-ethylphenol in the presence or absence of 10 μM ruthenium red was calculated. Results are the mean ± standard error of four experiments.

Cell type	Increase in $[Ca^{2+}]_{med}$ obtained after addition of 300 μM 4-chloro-3-ethylphenol		Inhibition by ruthenium red
	No pretreatment	Ruthenium red (10 μM) pretreatment	
	nM		%
Jurkat cells	141.3 ± 17.6	43.5 ± 6.8	69.2 ± 4.9
PC-12 cells	112.3 ± 11.5	50.3 ± 6.0	55.3 ± 5.2
PLC/PRF/5 cells	250.0 ± 22.3	54.0 ± 14.1	78.4 ± 5.2

macological similarities to the RYR, in the intracellular Ca^{2+} stores of nonexcitable cells.

Immunological evidence for a RYR-like molecule in human fibroblasts. Having obtained direct functional evidence for the presence of a RYR-like molecule in various cell lines, we investigated its presence immunologically. Monoclonal antibodies were raised against a fusion protein (27) encompassing a region of the skeletal muscle RYR common to the three isoforms. Fig. 8 shows a Western blot of the TC fraction of skeletal muscle (Fig. 8, *lane a*) and total extracts of human fibroblasts (Fig. 8, *lane b*). The other cell lines were also tested but did not yield systematically reproducible results. The only cell type that consistently showed a positive band on Western blots was cultured human fibroblasts (collagen secreting). The Western blot demonstrates that the antibodies recognized the RYR present in skeletal muscle TC, as well as a high molecular mass protein present in cultured human fibroblasts.

Discussion

One of the main issues raised by studies on Ca^{2+} homeostasis is whether only excitable cells posses both the IP_3R and the RYR or whether all cells are endowed with two types of intracellular Ca^{2+} channels (3, 14–20). So far, few reports have provided structural evidence supporting the presence of a RYR in nonexcitable cells; Giannini *et al.* (13) reported that treatment of mink lung epithelial cells with transforming growth factor-B induced the expression of a gene encoding the RYR, whereas Lesh *et al.* (14) used anti-RYR antibodies and cardiac RYR cDNA probes to demonstrate the presence of RYRs in endothelial cells. Those results, together with our Western blots as well as the functional characterization of intracellular Ca^{2+} pools (the present study and Refs. 19 and 20), argue in favor of the presence of a RYR-like molecule in nonexcitable cells.

In the present study we describe the Ca^{2+}-releasing activity of 4-chloro-3-ethylphenol, a new RYR agonist; we report that in skeletal muscle this compound releases Ca^{2+} from a ruthenium red-sensitive Ca^{2+} release channel selectively localized to the TC. Regarding its target in nonmuscle cells, we think that our data strongly support the specificity of 4-chloro-3-ethylphenol for Ca^{2+} release mediated by a RYR-like protein, because (a) heparin, a compound that has been shown to inhibit Ca^{2+} release via the IP_3R (30), did not affect 4-chloro-3-ethyl-

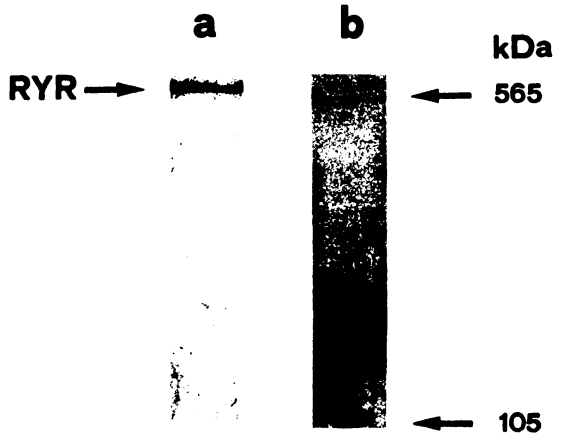

Fig. 8. Indirect immunoenzymatic staining of Western blots using a monoclonal anti-RYR antibody. Proteins present in the TC fraction (30 μg of protein) (*lane a*) and human fibroblasts (300 μg of protein) (*lane b*) were separated on a 5% polyacrylamide gel and transferred to nitrocellulose. Indirect immunoenzymatic staining of Western blots was carried out using monoclonal anti-RYR antibodies (culture supernatant diluted 1/3), followed by peroxidase-conjugated Protein A. The immunological reaction was visualized by enhanced chemiluminescence, using the Boehringer Mannheim kit.

phenol-induced Ca^{2+} release from bovine cerebellar microsomes or permeabilized cells, (b) pretreatment of cerebellar microsomes with 4-chloro-3-ethylphenol did not abrogate IP_3-induced Ca^{2+} release, and (c) ruthenium red affected the ability of 4-chloro-3-ethylphenol to release Ca^{2+}. In fact, in the presence of ruthenium red the net amount of Ca^{2+} released by 4-chloro-3-ethylphenol was always reduced (ranging from an inhibition of 55% in PC-12 cells to 78% in PLC/PRF/5 cells), although total inhibition was never achieved. The different effects of ruthenium red on 4-chloro-3-ethylphenol-mediated Ca^{2+} release from TC and cerebellar microsomes/permeabilized cells may be due to the existence, in the latter case, of two efflux pathways with different ruthenium red sensitivities. This argument is supported by the recent report of Takeshima *et al.* (33), who described the existence in rabbit brain of a truncated RYR transcript encoding a 75-kDa protein. Whether the truncated RYR protein can function as a Ca^{2+} channel and whether its sensitivity to ruthenium red is identical to that of the larger RYR channel remain to be established. The partial inhibitory effect of ruthenium red in permeabilized cells may indicate the (co)existence in nonexcitable cells of a 75-kDa protein (or its homologue) and/or a RYR-like channel that shares only some pharmacological similarities with its muscle/brain counterpart. Such an hypothesis may also explain the results of Cheek *et al.* (34) in bovine adrenal chromaffin cells. Those authors showed that 10 μM ruthenium red inhibited caffeine-activated Ca^{2+} release by 62.8% (34), whereas McNulty and Taylor (35) reported that the same concentration of ruthenium red blocked approximately 20% of the caffeine-mediated Ca^{2+} release from rat hepatocytes.

The present study also indicates that different cell types are endowed with different intracellular Ca^{2+} stores but generally (a) pools gated by the IP_3R and RYR-like channel are endowed with SERCA-type Ca^{2+} pumps, (b) the IP_3R and RYR-like channel coexist in the same Ca^{2+} store, and (c) there is another Ca^{2+} store that contains only the RYR-like Ca^{2+} channel. These results are only partially in agreement with reports by Guse *et al.* (20) and Tanaka and Tashjian (19), who showed that Jurkat cells and GH_4C_1 cells contain three types of intracellular Ca^{2+} pools, with one of these being caffeine sensitive but thapsigargin insensitive. The discrepancy may be due to the different experimental approaches; we probed Ca^{2+} pools both in permeabilized and in intact cells with an agonist that is at least 2 orders of magnitude more potent than caffeine, is freely permeable across the plasma membrane, and does not cause the fluorescence artifacts that have been reported to be associated with high concentrations of caffeine (19).

Regarding the physiological function of the Ca^{2+} pool endowed soley with the RYR-like channel, several hypothesis can be put forward. (a) It may function via a Ca^{2+}-induced Ca^{2+} release mechanism, activated by the influx of Ca^{2+} that occurs after ligand-receptor interactions. Thus, this Ca^{2+} pool may be involved in the phenomenon of Ca^{2+} oscillations, an event that has been observed in a variety of cell types, ranging from fibroblasts and lymphocytes to hepatocytes and cells of neuronal origin (16, 17, 36, 37). (b) As suggested by others (38), it may be the target of cADP-ribose, a newly identified second messenger. (c) This intracellular Ca^{2+} store may not be involved in receptor-mediated events but may partake in Ca^{2+} homeostasis and refilling of IP_3-sensitive intracellular pools. (d) The pool may be nonfunctional, representing nascent intracellular Ca^{2+} pools in which the IP_3R has not yet been inserted into the membrane. (e) This Ca^{2+} store may contain an IP_3R in an inactive state. Such a situation would parallel the behavior of the skeletal muscle dihydropyridine receptor Ca^{2+} channel, which, although present in large amounts in the transverse tubule membrane compartment, appears to be scarce, mainly because only a small percentage of the receptors function as Ca^{2+} channels at one time (39).

Our Western blot analysis of total cell extracts failed to demonstrate the presence of a 560-kDa RYR band in many cell types except for fibroblasts. This may be due in part to the high susceptibility of the protein to degradation and in part to the low density of RYR-like Ca^{2+} channels present on each cell. Further studies on the mode of action of 4-chloro-3-ethylphenol, as well as identification of its intracellular binding protein(s), are clearly needed to resolve the puzzling issue of intracellular Ca^{2+} channels in nonexcitable cells.

Acknowledgments

We would like to thank Drs. E. Clementi and G. Rispoli for constructive discussions and helpful suggestions.

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