0026-895X/93/061192-10$03.00/0

MOLECULAR PHARMACOLOGY, 44:1192-1201



# Chlorocresol: An Activator of Ryanodine Receptor-Mediated $Ca^{2+}$ Release

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Received June 16, 1993; Accepted September 9, 1993

**SUMMARY**

In the present study we investigated the effect of the compound chlorocresol on intracellular $Ca^{2+}$ homeostasis. Three different systems that have been shown to express the ryanodine receptor $Ca^{2+}$ channel were chosen, i.e., skeletal muscle sarcoplasmic reticulum, cerebellar microsomes, and PC12 cells. In skeletal muscle sarcoplasmic reticulum, 4-chloro-*m*-cresol was found to be a potent activator of $Ca^{2+}$ release mediated by a ruthenium red/caffeine-sensitive $Ca^{2+}$ release channel. In cerebellar microsomes, this compound released $Ca^{2+}$ from an inositol-1,4,5-trisphosphate-insensitive store, suggesting that there too it was acting at the ryanodine receptor level. When tested on PC12 cells, chlorocresol released $Ca^{2+}$ from a caffeine- and thapsigargin-sensitive intracellular store. In addition, the compound was capable of releasing $Ca^{2+}$ after pretreatment of PC12 cells with bradykinin, suggesting that it acts on a channel contained within an intracellular $Ca^{2+}$ store that is distinct from that sensitive to inositol-1,4,5-trisphosphate. Structure-activity relationship analyses suggest that the chloro and methyl groups in chlorocresols are important for the activation of the ryanodine receptor $Ca^{2+}$ release channel.

The RYR is a homotetramer of four 565-kDa subunits that mediates $Ca^{2+}$ release from intracellular stores (1–3). The primary sequence of this protein has been determined from a number of species and tissues (1, 2, 4–7); Northern blot analyses demonstrated that at least three isoforms exist, one specific for skeletal muscle, one present in cardiac muscle, and a third present in brain. Analysis of the predicted primary structure of the RYR protomer indicates that the amino-terminal portion constitutes a large hydrophilic domain, whereas the last 1000 amino acids form the channel itself (1, 2).

A large number of chemically diverse substances have been reported to release calcium from isolated TC via the RYR $Ca^{2+}$ channel. *In vitro*, caffeine, $Ca^{2+}$ itself, $Ag^{2+}$, thymol, halothane, doxorubicin, and ryanodine (8, 9) all release $Ca^{2+}$ by stimulating the SR RYR. To explain the molecular mechanism by which such diverse substances can act on the same molecule, one can postulate the presence of multiple activator binding sites present on the large hydrophilic portion of the receptor. This region must control a membrane-bound structure encompassing the ion-permeable channel (10). Thus, *in vivo* the RYR could be the target of chemical substances present as additives or preservatives in a variety of drug formulations.

MH, an inherited pharmacogenetic disorder, has been linked to a mutation in the RYR molecule (11). Halothane and other halogenated agents, as well as succinylcholine, have been reported to trigger MH (12, 13). As part of a comprehensive study on MH-inducing compounds, we tested the capacity of pharmacological additives to stimulate contraction of isolated muscle bundles. We found that chlorocresol, a preservative routinely added to commercial preparations of succinylcholine, is in fact a strong activator of muscle contraction.[1]

Here we demonstrate that chlorocresol is capable of releasing $Ca^{2+}$ from a ruthenium red/caffeine-sensitive $Ca^{2+}$ release channel selectively localized in TC. Furthermore, chlorocresol is capable of releasing $Ca^{2+}$ from heparin-treated cerebellar microsomes, indicating that it is not acting via the $Insp_3R$. Finally, we show that in PC12 cells this agonist releases $Ca^{2+}$ from a caffeine/thapsigargin- sensitive intracellular store.

This work was supported by Grants 30 and 185 from Telethon-Italy and Grant CNR 92.02256 and Ministero della Universitè e della Ricerca Scientifica e Technologica, fondi 40% e 60% to Prof. G. Benassi and P. Melandri.

[1] V. Tegazzin, E. Scutari, S. Treves, and F. Zorzato. Chlorocresol induces contracture of malignant hypothermic susceptible muscles. Manuscript in preparation.

**ABBREVIATIONS:** RYR, ryanodine receptor; TC, terminal cisternae; SR, sarcoplasmic reticulum; MH, malignant hyperthermia; $InsP_3R$, inositol-1,4,5-trisphosphate receptor; PMSF, phenylmethanesulfonyl fluoride; $[Ca^{2+}]_i$, intracellular $Ca^{2+}$ concentration; $[Ca^{2+}]_o$, extracellular $Ca^{2+}$ concentration; $InsP_3$, inositol-1,4,5-trisphosphate; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; LSR, longitudinal sarcoplasmic reticulum; MOPS, 3-(*N*-morpholino)propanesulfonic acid.

Thus, chlorocresol could become a new pharmacological tool with which to study intracellular $Ca^{2+}$ homeostasis.

## Experimental Procedures

**Materials.** The experiments presented here were carried out on the parental PC12 line subcultured in our laboratory as described previously (14). Ruthenium red, 4-chloro-*m*-cresol, 4-chloro-*o*-cresol, *o*-cresol, *p*- chlorophenol, and antipyrylazo III were from Fluka (Buchs, Switzerland); bradykinin, heparin, and caffeine were from Sigma Chemical Co. (St. Louis, MO); $Ca^{2+}$ ionophores A23187 and ionomycin were from Calbiochem (San Diego, CA); fura-2 and fluo-3 were from Molecular Probes (Eugene, OR); and $InsP_3$ was from ICN (Costa Mesa, CA). All other materials were analytical grade or of the highest available grade. Thapsigargin was a kind gift of Prof. Tullio Pozzan, Institute of General Pathology, University of Padova (Padova, Italy).

**Subcellular fractionation.** SR was isolated from white muscles of New Zealand White rabbits and was fractionated into longitudinal tubules and TC in the presence of antiproteolytic agents as described by Saito *et al.* (15). The SR fractions were resuspended in 0.3 M sucrose, 5 mM imidazole, pH 7.4, 100 $\mu$M PMSF, 1 $\mu$g/ml leupeptin (buffer A), and were stored in liquid nitrogen until used.

Bovine cerebella were obtained from the local slaughterhouse, from freshly slaughtered animals. The microsomal fraction was obtained by homogenizing the tissue in 0.3 M sucrose, 5 mM imidazole, pH 7.4, 100 $\mu$M PMSF, 1 $\mu$g/ml leupeptin, as described previously (16). The 100,000 $\times$ *g* pellet was resuspended in 0.3 M sucrose, 5 mM imidazole, pH 7.4, 100 $\mu$M PMSF, 1 $\mu$g/ml leupeptin, 1 mM benzamidine, and was stored in liquid nitrogen until used. Protein concentration was determined according to the method of Bradford (17), using bovine serum albumin as the standard.

**$Ca^{2+}$ measurements.** $Ca^{2+}$ release from isolated SR fractions was measured in a Beckmann DU7400 diode array spectrophotometer by monitoring the $A_{710} - A_{790}$ value of the $Ca^{2+}$ indicator antipyrylazo III, as described by Palade (9) and detailed by Treves *et al.* (18). Pulses of 25 nmol of $Ca^{2+}$ were administered to load the SR fractions with approximately 2.3 $\mu$mol 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 TC and cerebellar microsomes was measured in an LS50 thermostatted Perkin Elmer spectrofluorimeter equipped with magnetic stirring, as described by Sayers *et al.* (19). Briefly, TC (final concentration, 100 $\mu$g/ml) or microsomes (final concentration, 200 $\mu$g/ml) were added to a fluorimeter cuvette containing 7.5 mM potassium pyrophosphate, 18.5 mM MOPS, pH 7.0, 100 mM KCl, 5 mM creatine phosphate, 20 $\mu$g/ml creatine phosphate kinase, and 200 nM fluo-3 free acid. $Ca^{2+}$ accumulation was stimulated by the addition of 1 mM MgATP. Pulses of $Ca^{2+}$ were administered and, after steady state was reached, $Ca^{2+}$ release was stimulated by the addition of different agents. At the end of each experiment $F_{min}$ and $F_{max}$ were obtained by the addition of EGTA and $Ca^{2+}$, respectively, and $[Ca^{2+}]$ was calculated according to the formula $[Ca^{2+}] = K_d(F - F_{min}/F_{max} - F)$ ($K_d$ = 900 nM) (19, 20). Loading with fura-2 and $[Ca^{2+}]_i$ measurements were carried out in 140 mM NaCl, 5 mM KCl, 1 mM $MgSO_4$, 20 mM HEPES, 1 mM $NaHPO_4$, 5 mM glucose, pH 7.4, as described (21, 22). In each experiment, $5 \times 10^5$ cells/ml, loaded with 5 $\mu$M fura-2/acetoxymethyl ester, were used.

## Results

**Characterization of chlorocresol-induced $Ca^{2+}$ fluxes in isolated SR fractions.** Fig. 1 shows the chemical strutures of the compounds used to study $Ca^{2+}$ fluxes. In Fig. 2 the effect of 4-chloro-*m*-cresol on the initial rate of $Ca^{2+}$ release from actively loaded longitudinal tubules (LSR) and TC vesicles is shown. $Ca^{2+}$ fluxes were followed spectrophotometrically with the indicator antipyrylazo III, as described previously (18).

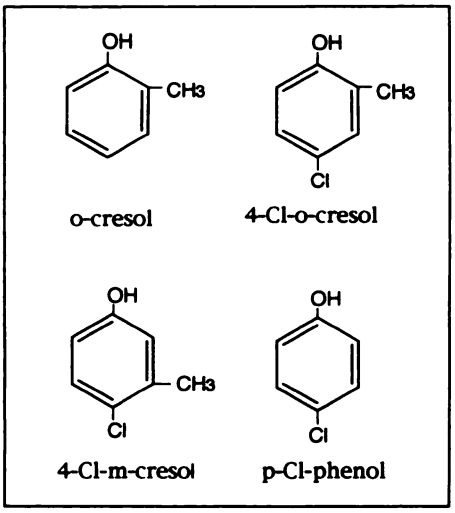


**Fig. 1.** Chemical structures of *o*-cresol, 4-chloro-*o*-cresol, 4-chloro-*m*-cresol, and *p*-chlorophenol.

Addition of 1 mM chlorocresol caused a rapid release of $Ca^{2+}$ from the TC fraction (Fig. 2A), whereas it had no effect on LSR (Fig. 2B), indicating that it was not acting by inhibiting the $Ca^{2+}$-ATPase or damaging the vesicles. To establish whether this agent acts via the RYR $Ca^{2+}$ release channel, we examined the effect of ruthenium red, a known inhibitor of the RYR. Fig. 2C shows that pretreatment of TC with 10 $\mu$M ruthenium red completely abolished 4-chloro-*m*-cresol-induced $Ca^{2+}$ release. Fig. 3 shows the dose-dependent effect of 4-chloro-*m*-cresol on the kinetics of $Ca^{2+}$ release from TC. The half-maximally stimulatory dose of $Ca^{2+}$ release was 300 $\mu$M, whereas at 900 $\mu$M this agonist released $Ca^{2+}$ at an initial rate of approximately 4.8 $\mu$mol of $Ca^{2+}$/min/mg of protein. Such a rate is comparable to $Ca^{2+}$ release activated by caffeine (9).

We next studied whether compounds chemically related to chlorocresol were capable of releasing $Ca^{2+}$. *p*-Chlorophenol at 100 $\mu$M had no effect on $[Ca^{2+}]_0$ (Fig. 4A), whereas at higher concentrations the compound was able to elicit release of $Ca^{2+}$ (Fig. 4B); however, the release rate was approximately 10-fold lower than that observed with similar concentrations of 4-chloro-*m*-cresol. Comparison of Fig. 4, A and B, also shows that the position of the methyl group influences the $Ca^{2+}$-releasing capacity of the compounds; 4-chloro-*m*-cresol was more potent than its *ortho*-analogue.

To further confirm the effects of chlorocresols on $Ca^{2+}$ fluxes, we carried out similar experiments using a different $Ca^{2+}$ indicator, namely fluo-3. In Fig. 5 we show that, at a concentration of 800 $\mu$M, *o*-cresol had no effect on $[Ca^{2+}]_0$, whereas 800 $\mu$M 4-chloro-*m*-cresol caused a rapid release of $Ca^{2+}$ from TC. The subsequent addition of the $Ca^{2+}$ ionophore A23187 caused, as expected, a larger release of $Ca^{2+}$ from *o*-cresol-treated TC than from vesicles that had received 4-chloro-*m*-cresol. We were unable to test the effect of caffeine, because its addition strongly quenched the fluorescent signal (Fig. 5).

**Characterization of chlorocresol-induced $Ca^{2+}$ fluxes in bovine cerebellar microsomes.** Our experiments on isolated SR TC cannot exclude the possibility that chlorocresol is also activating the $InsP_3R$ $Ca^{2+}$ release channel. To address this problem we prepared microsomes from bovine cerebellum, a tissue that is rich in $InsP_3R$ but that also contains a caffeine-sensitive $Ca^{2+}$ release channel (23, 24). Microsomes were pre-

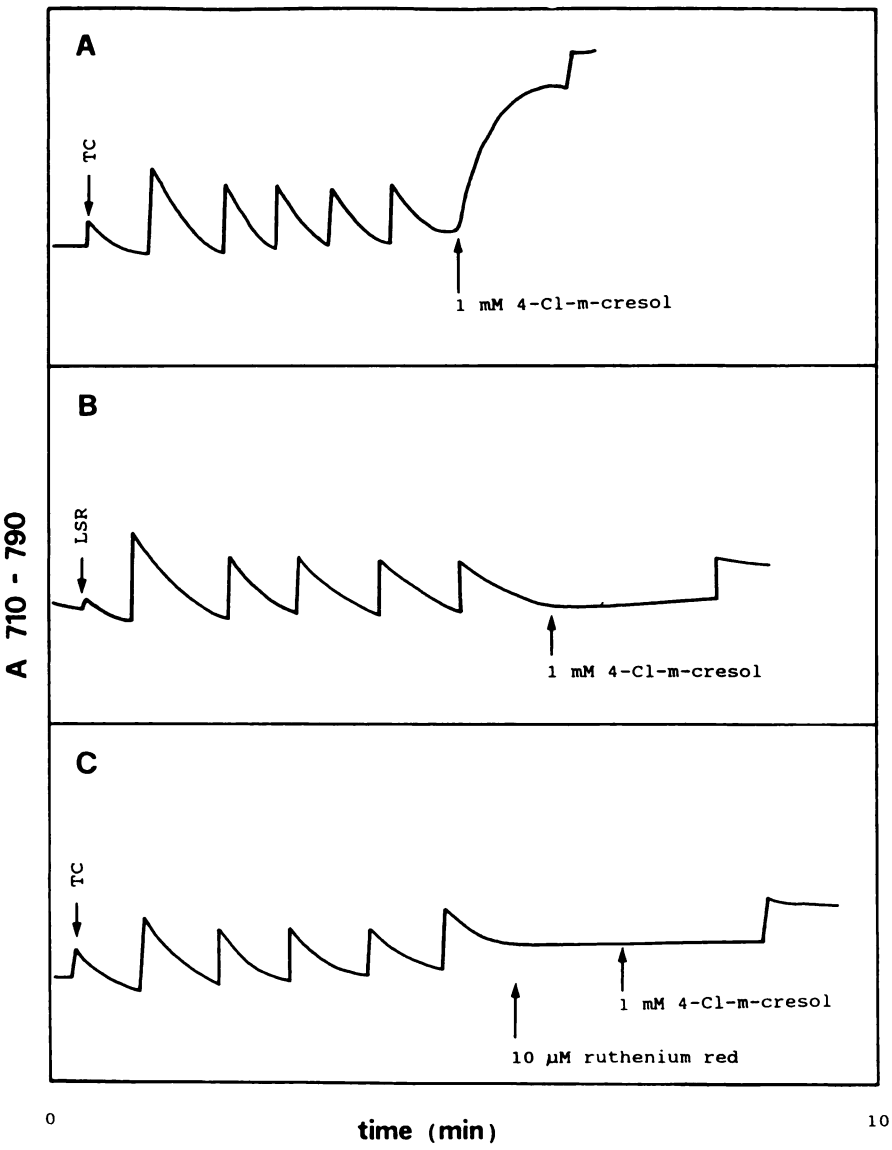


**Fig. 2.** 4-Chloro-*m*-cresol induces $Ca^{2+}$ release from skeletal muscle TC but not LSR. The experiments were carried out as described in Experimental Procedures, using the $Ca^{2+}$ indicator antipyrylazo III. Where indicated, 45 μg of TC (A and C) or LSR (B) were added to the cuvette. Five consecutive additions of 25 nmol of $CaCl_2$ were administered; after completion of $Ca^{2+}$ loading, 1 mM 4-chloro-*m*-cresol (A and B) or 10 μM ruthenium red followed by 1 mM 4-chloro-*m*-cresol (C) was added. At the end of the experiments, 25 nmol of $CaCl_2$ were added to calibrate the dye response. Upward deflections indicate $Ca^{2+}$ release, and downward deflections indicate $Ca^{2+}$ accumulation.

pared as described in Experimental Procedures, and $Ca^{2+}$ accumulation was stimulated by the addition of MgATP. Changes in $[Ca^{2+}]_o$ were followed with the fluorescent indicator fluo-3. As expected, 5 μM $InsP_3$ caused release of $Ca^{2+}$ from microsomes (Fig. 6A); the subsequent addition of $InsP_3$ caused no additional release of $Ca^{2+}$, whereas addition of 800 μM 4-chloro-*o*-cresol caused a small release of $Ca^{2+}$. Fig. 6B shows that 800 μM chlorocresol was capable of releasing $Ca^{2+}$ from microsomes. Furthermore, when microsomes were pretreated with heparin, a known inhibitor of the $InsP_3R$, $InsP_3$ failed to elicit $Ca^{2+}$ release; on the other hand, chlorocresol was still capable of releasing $Ca^{2+}$ (Fig. 6C). These results exclude a direct action of chlorocresol on the $InsP_3R$ and also indicate that microsomes may contain at least three intracellular $Ca^{2+}$-containing vesicles, i.e., a small population containing only a RYR-like $Ca^{2+}$ release channel, another containing both channels, and a third that can be depleted only by ionophore treatment. The latter store is thapsigargin insensitive (data not shown) and may represent either resealed membrane vesicles or contaminating mitochondria. Unfortunately, we were unable to activate the RYR $Ca^{2+}$ release channel with caffeine or doxorubicin, because addition of both compounds strongly quenched the fluorescent signal.

**Effect of chlorocresol on $[Ca^{2+}]_i$ of PC12 cells.** A number of studies have documented that in the rat pheocromocytoma cell line PC12 two distinct intracellular $Ca^{2+}$ release channels exist, one being $InsP_3$ sensitive and the other caffeine sensitive (14). Caffeine has thus been used to check for the presence of RYR-like $Ca^{2+}$ channels in these as well as other cells. Unfortunately the use of this agonist has several drawbacks; (i) very high concentrations (10–40 mM) must be used to obtain an effect and (ii) at such high doses caffeine quenches fluo-3 fluorescent signals, whereas it causes fluorescence artifacts when cells are loaded with low concentrations of fura-2.[2] Because of the problems linked to the use of caffeine, we tested whether chlorocresol could be used to study intracellular $Ca^{2+}$ pools. All the experiments carried out in intact cells were performed in $Ca^{2+}$-free medium. Fig. 7 shows the effect of cresol

[2] S. Treves, unpublished observations.

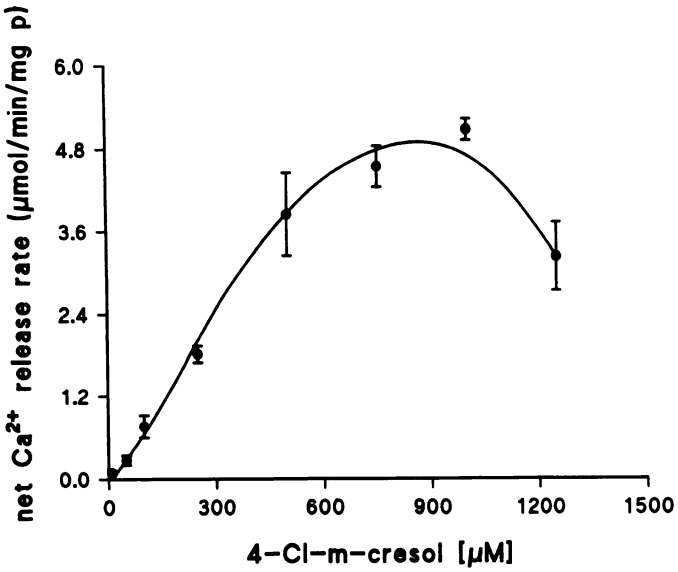


**Fig. 3.** Dose dependence of 4-chloro-*m*-cresol-induced net $Ca^{2+}$ release from skeletal muscle TC. Experiments were carried out as described for Fig. 2A, and $Ca^{2+}$ release was stimulated by the addition of different concentrations of 4-chloro-*m*-cresol. $Ca^{2+}$ release is expressed as the mean ± standard error of four experiments.

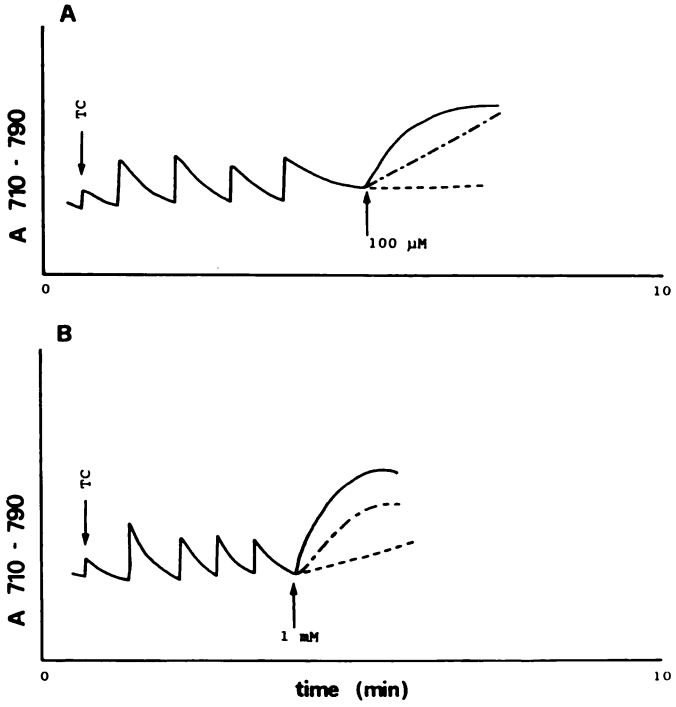


**Fig. 4.** $Ca^{2+}$-releasing properties of chlorocresols and *p*-chlorophenol on isolated TC. Conditions were as in Fig. 2. $Ca^{2+}$ release was stimulated by the addition of 4-chloro-*m*-cresol (———), 4-chloro-*o*-cresol(– — – —), or *p*-chlorophenol (– – – –). *Arrows*, addition of 100 μM (A) or 1 mM (B) of each compound.

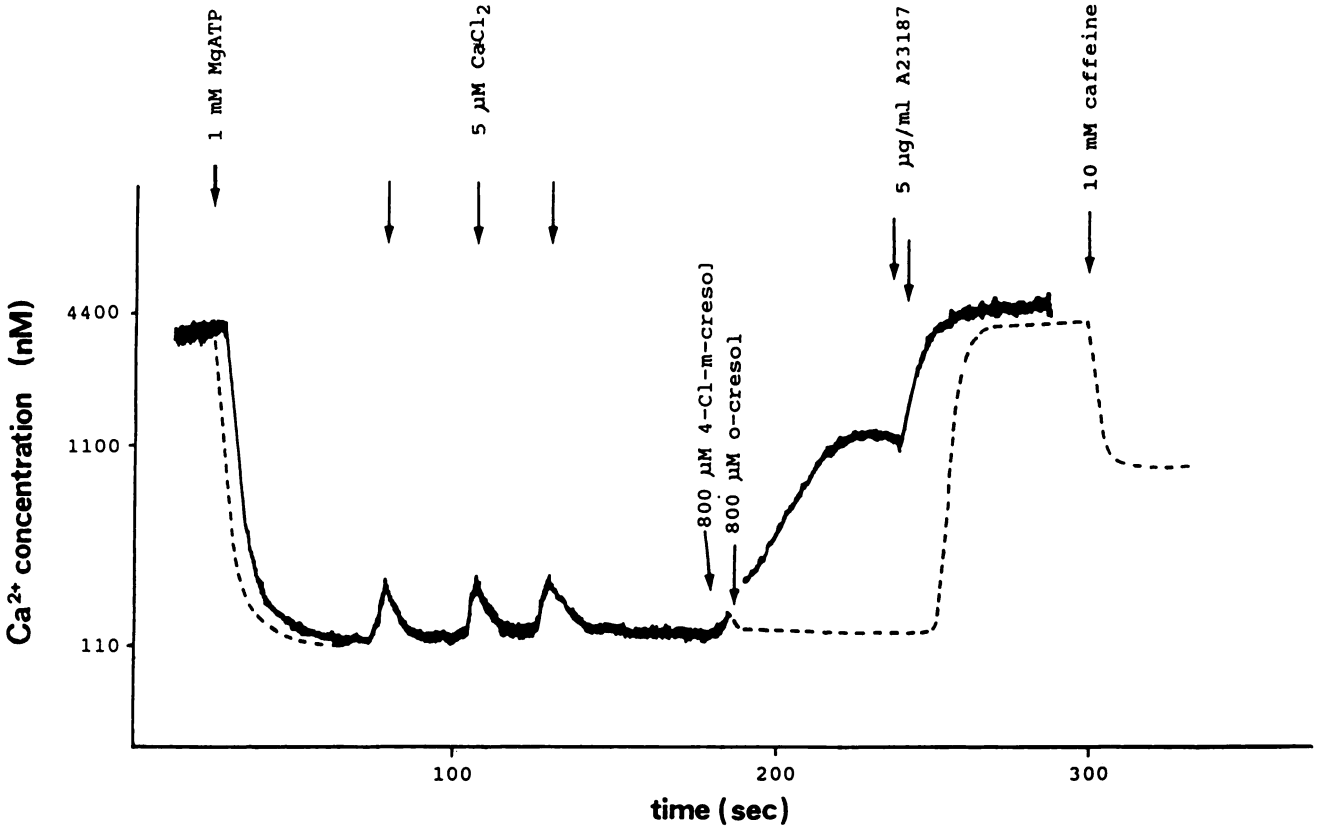


**Fig. 5.** Effects of cresols on $[Ca^{2+}]_o$ of TC. Experiments were carried out as described in Experimental Procedures, using the indicator fluo-3. $Ca^{2+}$ accumulation was stimulated by the addition of 1 mM MgATP to isolated TC (final concentration, 100 μg/ml); three consecutive pulses of 5 μM $CaCl_2$ were administered to the cuvette and after steady state was reached 800 μM 4-chloro-*m*-cresol (———) or 800 μM *o*-cresol (– – –) was added. At the end of the experiment 5 μg/ml A23187 was added to release the accumulated $Ca^{2+}$. Caffeine (10 mM) was also added (– – –), to show fluorescence quenching.

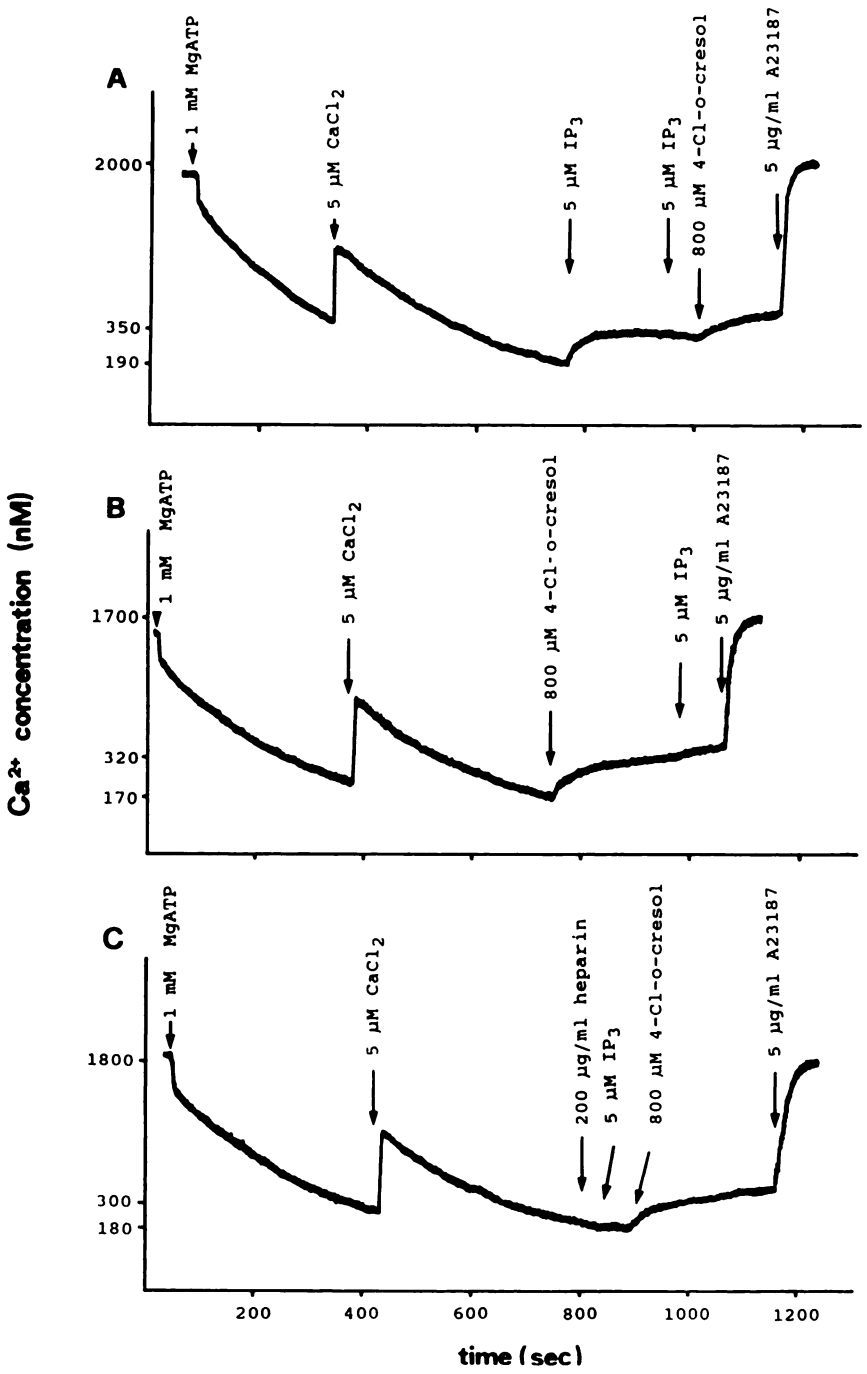


**Fig. 6.** Effects of 4-chloro-*o*-cresol on $[Ca^{2+}]_o$ of bovine cerebellar microsomes. Conditions were as described for Fig. 5; 200 μg/ml bovine microsomes were used in each experiment. In A–C, different compounds were added (*arrows*) as indicated.

and its methylated derivatives on $[Ca^{2+}]_i$ homeostasis. Under these conditions *o*-cresol (Fig. 7A) had no effect on $[Ca^{2+}]_i$. On the other hand, 4-chloro-*m*-cresol (Fig. 7B) and 4-chloro-*o*-cresol (Fig. 7C) were capable of releasing $Ca^{2+}$ from intracellular stores. The effect on $[Ca^{2+}]_i$ was completely abolished by pretreatment with 100 nM thapsigargin (Fig. 7D) or 1 μM ionomycin (data not shown).

In Fig. 8 the dose dependence curves for 4-chloro-*m*-cresol and 4-chloro-*o*-cresol effects on $[Ca^{2+}]_i$ are given; the order of potency of the two compounds in PC12 cells is 4-chloro-*o*-cresol > 4-chloro-*m*-cresol. To rule out the possibility that these agents were indirectly affecting $[Ca^{2+}]_i$ by damaging the cell membrane, we tested their effects on membrane potential using the fluorescent dye bis-oxonol. Addition of chlorocresol had no apparent damaging effect, because it caused plasma membrane hyperpolarization, as would be expected for an agent causing release of $Ca^{2+}$ from intracellular stores (data not shown).

The next set of experiments were aimed at studying the effect of chlorocresol on the $InsP_3$-sensitive and/or caffeine-sensitive intracellular $Ca^{2+}$ pools. We observed that, in $Ca^{2+}$-free medium, the $Ca^{2+}$ transients elicited by saturating concentrations of chlorocresol were drastically reduced if the cells were first exposed to caffeine (Fig. 9A). Similarly, if PC12 cells were pretreated with chlorocresol, caffeine failed to elicit an increase in $[Ca^{2+}]_i$ (Fig. 9B). On the other hand, pretreatment of PC12 cells with 200 nM bradykinin, an agonist that causes activation of $InsP_3$-sensitive $Ca^{2+}$ channels, did not abolish the response to 4-chloro-*o*-cresol (Fig. 9C).

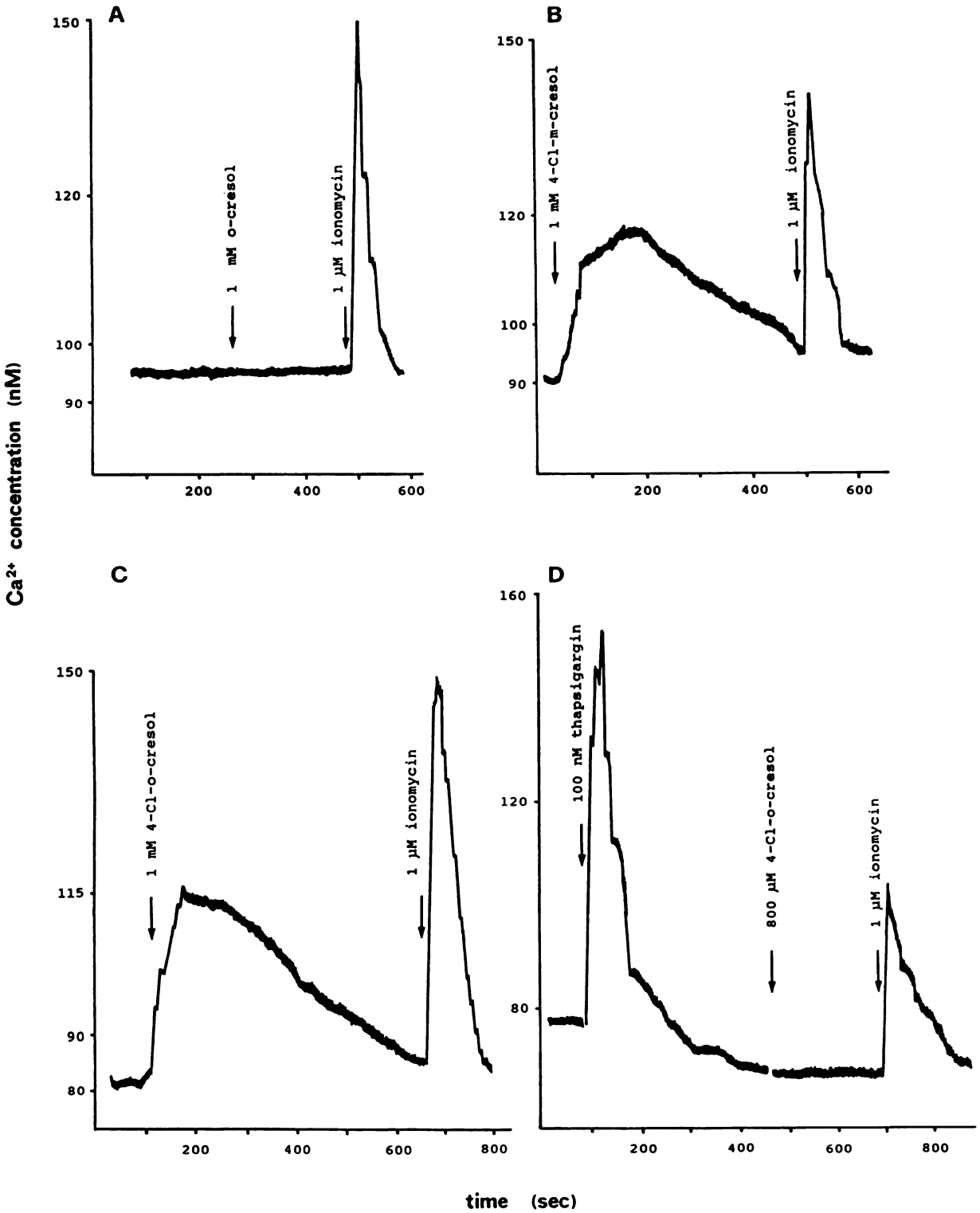


**Fig. 7.** Effects of various cresol derivatives on $[Ca^{2+}]_i$ of PC12 cells. Suspensions of $0.5 \times 10^6$ PC12 cells/ml, loaded with 5 µM fura-2, were used. Experiments were performed in $Ca^{2+}$-free medium plus 2 mM EGTA. The results shown are representative of at least four experiments.

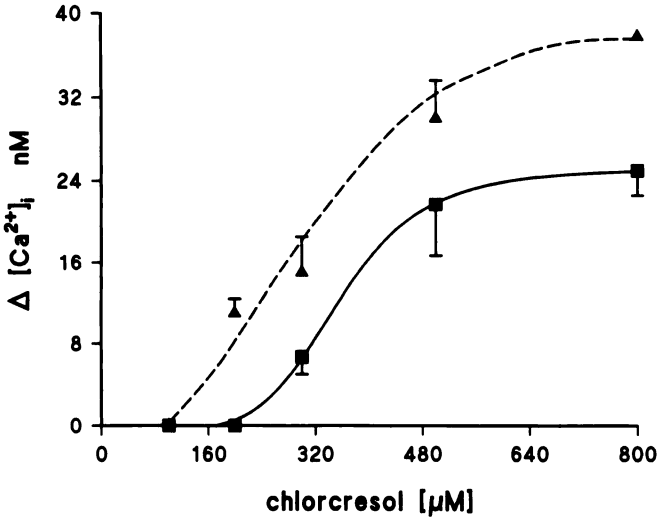


**Fig. 8.** Dose-response curves for chlorocresols in PC12 cells and effects on $[Ca^{2+}]_i$. $[Ca^{2+}]_i$ was measured in PC12 cells that had been loaded with 5 μM fura-2 and treated with different concentrations of 4-chloro-*o*-cresol (▲) or 4-chloro-*m*-cresol (■). $[Ca^{2+}]_i$ was obtained by subtracting the maximal $[Ca^{2+}]_i$ increase from the basal $[Ca^{2+}]_i$. Values are the mean ± standard error of four experiments.

In the experiments shown in Fig. 10, we examined the effect of chlorocresol on PC12 cells in the presence of both bradykinin and caffeine. Bradykinin (200 nM) strongly reduced but did not abolish the increase in $[Ca^{2+}]_i$ produced by 40 mM caffeine. The subsequent addition of 4-chloro-*o*-cresol had no additional effect on $[Ca^{2+}]_i$, although ionomycin was still capable of releasing $Ca^{2+}$ from membrane-bound intracellular stores.

## Discussion

**Effect of chlorocresol on $[Ca^{2+}]_i$ homeostasis.** In the present study we report the effect of chlorocresol on $Ca^{2+}$ homeostasis and demonstrate that this compound is a strong activator of the RYR $Ca^{2+}$ release channel. $Ca^{2+}$ release experiments with isolated SR vesicles indicate that the effect is specific for the RYR, because (i) pretreatment of TC vesicles with ruthenium red, a known inhibitor of this $Ca^{2+}$ release channel, completely blocks the effect of chlorocresol and (ii) chlorocresol has no effect on LSR vesicles and thus its mode of action does not involve inhibition of the $Ca^{2+}$-ATPase, which comprises approximately 80% of the latter fraction (15), or damage to the vesicles. Chlorocresol does not activate the $InsP_3R$, because its $Ca^{2+}$-releasing effects on cerebellar microsomes were maintained even after blockage of the $InsP_3R$ with heparin.

In PC12 cells, a caffeine-sensitive cell line, the effect of chlorocresol could not be attributed to nonspecific damage of the membranes by the compound because, under the experimental conditions used, it did not cause plasma membrane depolarization but rather a slight hyperpolarization, a consequence of its ability to release intracellular $Ca^{2+}$. The kinetics of chlorocresol-induced $Ca^{2+}$ transients are slower than those of the transients induced by bradykinin. In fact, under our experimental conditions, in the presence of the latter agonist the rise in $[Ca^{2+}]_i$ occurred within 5–10 sec and the $[Ca^{2+}]_i$ returned to basal levels within 20–30 sec. Similar transients could be obtained by addition of ionomycin. In the presence of 800 μM 4-chloro-*o*-cresol, however, peak $[Ca^{2+}]_i$ was reached after approximately 160 sec and the $[Ca^{2+}]_i$ did not return to resting levels for at least 250 sec. As far as the rising phase is concerned, the different kinetics may reflect permeability differences. In fact, bradykinin, a phosphatidylinositol-4,5-bisphosphate-mobilizing agonist, acts by stimulating surface receptors, whereas chlorocresol must pass through the plasma membrane to activate its receptor. With regard to the slower decay phase seen with chlorocresol, it is unlikely that it reflects inhibition of the microsomal $Ca^{2+}$-ATPase, because as mentioned earlier this compound has no apparent effect on the SR $Ca^{2+}$ pump. The slower decay phase may be due to the extent of activation of the $Ca^{2+}$-ATPase (25, 26), because the activity of this enzyme is regulated by $Ca^{2+}$ (27). Bradykinin causes a rapid increase in $[Ca^{2+}]_i$; thus, it activates the $Ca^{2+}$-ATPase to a greater extent than does a slower rise in $[Ca^{2+}]_i$. In this context, it should be mentioned that, when PC12 cells are pretreated with bradykinin, the chlorocresol-induced $[Ca^{2+}]_i$ rise is slower than that seen in cells receiving the latter agonist alone. This observation may reflect the fact that the $Ca^{2+}$ content of the stores is diminished; on the other hand, it may be a consequence of preactivation of the $Ca^{2+}$ATPase by bradykinin.

**Use of chlorocresol to study intracellular $Ca^{2+}$ pools.** Thapsigargin, a specific inhibitor of SR/endoplasmic reticulum $Ca^{2+}$-ATPase-like $Ca^{2+}$ pumps (28, 29), inhibited the chlorocresol-induced rise in $[Ca^{2+}]_i$, indicating that chlorocresol releases $Ca^{2+}$ from a membrane-bound intracellular store equipped with a SR/endoplasmic reticulum $Ca^{2+}$-ATPase-type ATPase.

In PC12 cell clones, we found that (i) bradykinin did not completely abolish caffeine-mediated $Ca^{2+}$ release; (ii) chlorocresol was consistently able to release $Ca^{2+}$ from intracellular stores, even after treatment with bradykinin; (iii) intracellular $Ca^{2+}$ transients caused by caffeine and chlorocresol were strongly reduced, if not abolished, upon pretreatment of the cells with either agonist; and (iv) there was an intracellular $Ca^{2+}$ store that could be depleted only with ionomycin. The simplest way to explain these results is to postulate the existence of at least two types of intracellular $Ca^{2+}$ stores, one containing both $InsP_3R$ and thapsigargin-sensitive RYR (14) and another that can be released only with ionomycin. If this were the case, treatment with bradykinin, which causes only a rapid release of $Ca^{2+}$, would partially deplete the $Ca^{2+}$ pool and chlorocresol, which appears to be more potent than caffeine, would still be capable of releasing $Ca^{2+}$. The other possibility is that three types of intracellular $Ca^{2+}$ stores exist, one equipped with both $InsP_3R$ and RYR, another equipped with only RYR, and a third that is insensitive to thapsigargin and releases $Ca^{2+}$ only after ionophore treatment. Thus, stimulation with bradykinin, a phosphatidylinositol-4,5-bisphosphate-mobilizing agonist, would release $Ca^{2+}$ only from vesicles containing both RYR and $InsP_3R$, whereas chlorocresol would release $Ca^{2+}$ from both RYR/$InsP_3R$- and RYR-containing intracellular stores. The results obtained using bovine cerebellar microsomes confirm in part the results obtained in whole cells, because they suggest the existence of at least three populations of vesicles, one that is chlorocresol sensitive, another that is sensitive to both $InsP_3$ and chlorocresol, and a third that releases $Ca^{2+}$ only after treatment with a $Ca^{2+}$ ionophore.

Regarding the lack of effect of chlorocresol in PC12 cells pretreated with both bradykinin and caffeine, we can only

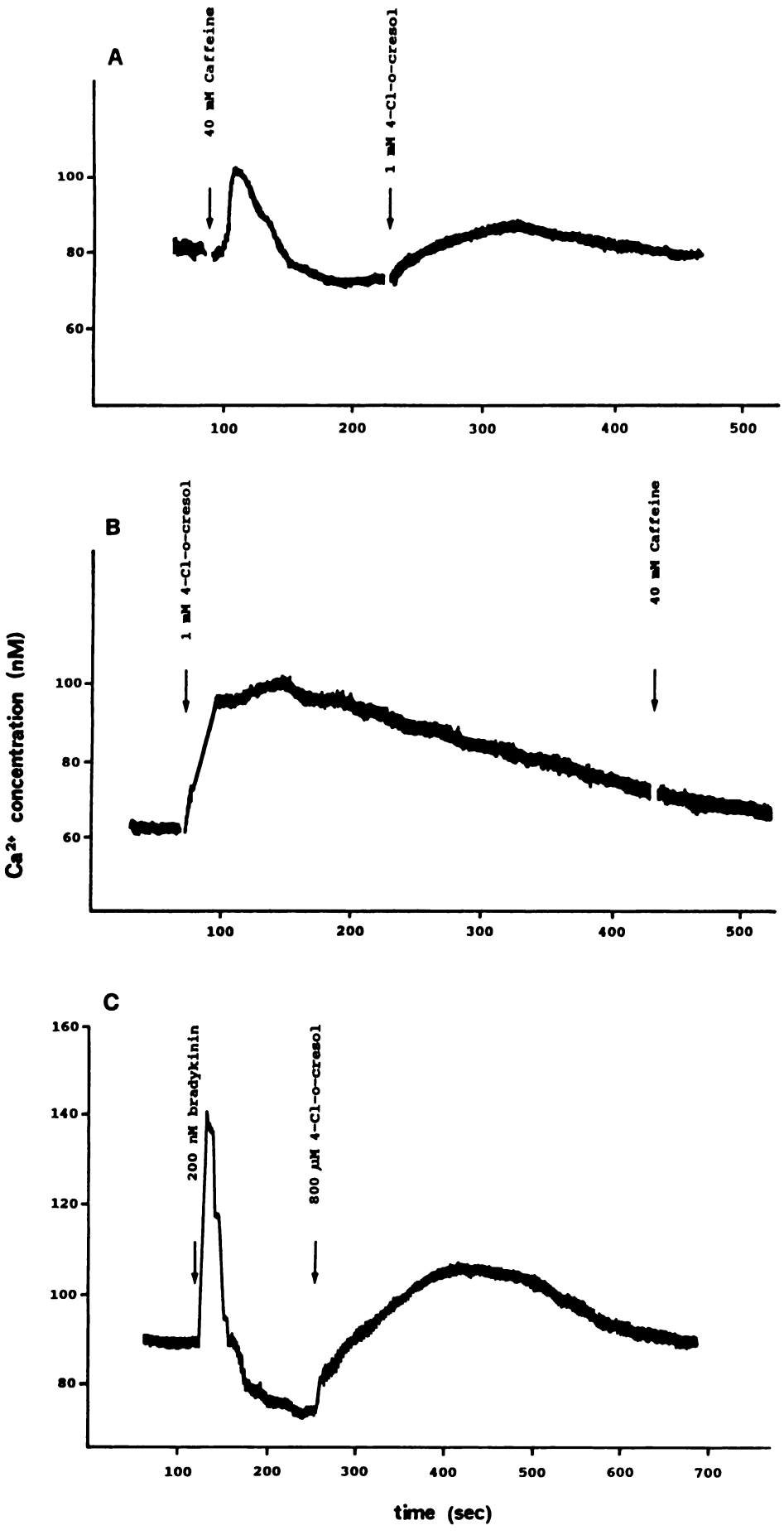


**Fig. 9.** Effects of 4-chloro-o-cresol on the intracellular $Ca^{2+}$ pools of PC12 cells. Conditions were as in Fig. 7.

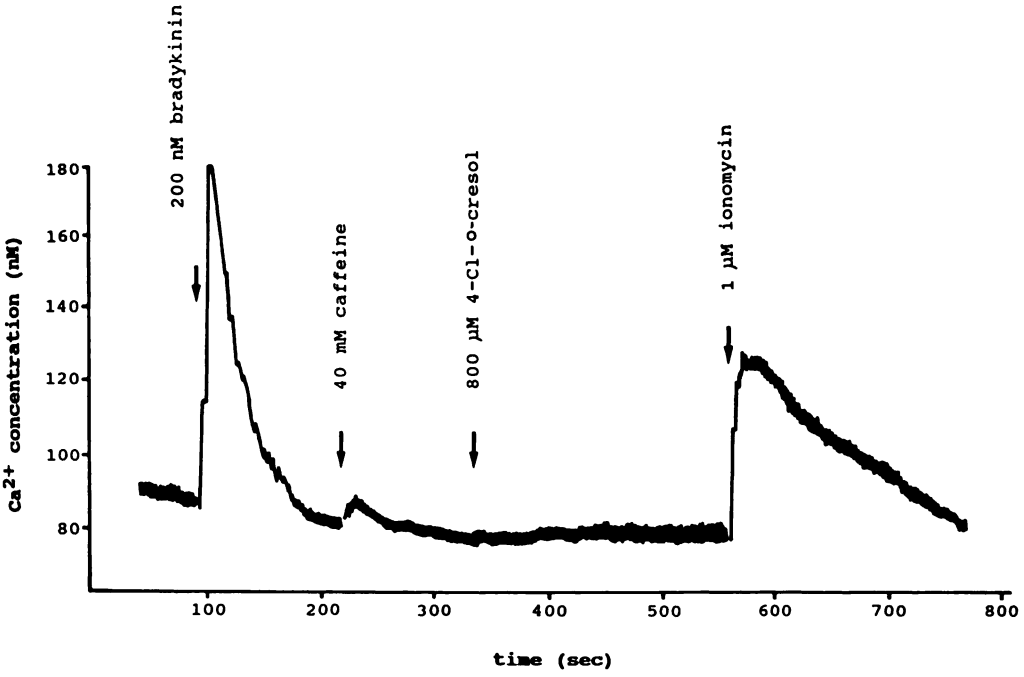


Fig. 10. Effects of 4-chloro-o-cresol on intracellular $Ca^{2+}$ transients of PC12 cells in the presence of bradykinin and caffeine. Conditions were as in Fig. 7.

advance an hypothesis. The RYR is a substrate for a variety of protein kinases including protein kinase C (30), and phosphorylation of the RYR appears to inactivate channel activity (31). Prestimulation of PC12 cells with bradykinin followed by an unusually high concentration of caffeine may thus activate an inhibitory mechanism mediated by protein kinases, which would in turn down-regulate the $Ca^{2+}$ channel response to additional $Ca^{2+}$-mobilizing stimuli.

**Structure-activity relationship of chlorocresols.** A wide variety of unrelated drugs have been shown to induce SR $Ca^{2+}$ release (8, 9). In the present report we used cresol and its methylated derivatives and found that (i) the presence of a chloride atom plus a methyl group is necessary for the activation of $Ca^{2+}$ release, (ii) the position of such a methyl group influences the ability of the compound to release $Ca^{2+}$, and (iii) skeletal RYR was more sensitive to the effect of 4-chloro-*m*-cresol, whereas in PC12 cells 4-chloro-*o*-cresol was more potent in releasing $Ca^{2+}$ from intracellular stores.

Chlorocresol has a relatively simple chemical structure; thus, one can postulate that there are two important structural groups that confer reactivity to the molecule; a methyl group, which partitions in a hydrophobic pocket, is an electron donor and most likely renders more electronegative the chloride at position 4 of the aromatic ring. Substitution of the methyl group with a bulkier hydrophobic side chain may produce a more potent RYR agonist. It is tempting to speculate that the electronegative chloride interacts with positively charged amino acids in the RYR gating domain. This conclusion is substantiated by the finding that *o*-cresol, which lacks the chloride group, is not capable of stimulating $Ca^{2+}$ release from either isolated TC or PC12 cells, whereas *p*-chlorophenol, which lacks the hydrophobic side chain, is approximately 10 times less potent than 4-Cl-cresol in activating $Ca^{2+}$ release from isolated TC. *p*-Chlorophenol had no visible effect on $[Ca^{2+}]_i$ in PC12 cells; however, this compound was quite fluorescent at the fura-2 wavelengths, and thus autofluorescence artifacts may have masked small changes in $[Ca^{2+}]_i$.

**Acknowledgments**

We thank Prof. Francesco DiVirgilio for his suggestions and for critically reading the manuscript and Prof. Tullio Pozzan and Prof. Mario Rippa for helpful suggestions. We would also like to acknowledge Paola Menegazzi for the artwork.

**References**

1. Takeshima, H., S. Nishimura, H. Matsumoto, K. Ishida, N. Kangawa, H. Minamino, M. Matsuo, M. Ueda, M. Hanakoa, T. Horise, and S. Numa. Primary structure and expression from complementary DNA of skeletal muscle ryanodine receptor. *Nature (Lond.)* **339**:439–445 (1989).
2. Zorzato, F., J. Fuji, K. Otsu, M. Phillips, N. M. Green, F. A. Lai, G. Meissner, and D. H. MacLennan. Molecular cloning of cDNA encoding human and rabbit forms of the $Ca^{2+}$ release channel (ryanodine receptor) of skeletal muscle sarcoplasmic reticulum. *J. Biol. Chem.* **265**:2244–2256 (1990).
3. Lai, F. A., H. P. Erickson, E. Rousseau, Q. Y. Liu, and G. Meissner. Purification and reconstitution of the calcium release channel from skeletal muscle. *Nature (Lond.)* **331**:315–319 (1988).
4. Otsu, K., H. F. Willard, V. J. Khanna, F. Zorzato, N. M. Green, and D. H. MacLennan. Molecular cloning of cDNA encoding the $Ca^{2+}$ release channel (ryanodine receptor) of rabbit cardiac muscle sarcoplasmic reticulum. *J. Biol. Chem.* **265**:13472–13483 (1990).
5. Hakamata, Y., J. Nakai, H. Takeshima, and K. Imoto. Primary structure and distribution of a novel ryanodine receptor calcium release channel from rabbit brain. *FEBS Lett.* **312**:229–235 (1992).
6. Nakai, J., T. Imagawa, Y. Hakamata, M. Shigekawa, H. Takeshima, and S. Numa. Primary structure and functional expression from cDNA of the cardiac ryanodine receptor calcium release channel. *FEBS Lett.* **271**:169–177 (1990).
7. Giannini, G., E. Clementi, R. Ceci, G. Marziali, and V. Sorrentino. Identification of a broadly expressed ryanodine receptor $Ca^{2+}$ channel regulated by TGF$\beta$. *Science (Washington D. C.)* **257**:91–94 (1992).
8. Palade, P., C. Dettbarn, D. Brunder, P. Stein, and G. Hals. Pharmacology of calcium release from sarcoplasmic reticulum. *J. Biomembr. Bioenerg.* **21**:295–320 (1989).
9. Palade, P. Drug-induced $Ca^{2+}$ release from isolated sarcoplasmic reticulum. II. Release involving a $Ca^{2+}$-induced $Ca^{2+}$ release channel. *J. Biol. Chem.* **262**:6142–6148 (1987).
10. Kim, D. H., S. T. Ohnishi, and N. Ikemoto. Kinetic studies of calcium release from sarcoplasmic reticulum *in vitro*. *J. Biol. Chem.* **258**:9662–9668 (1983).
11. Fuji, J., K. Otsu, F. Zorzato, S. DeLeon, V. J. Khanna, J. E. Weiler, P. J. O'Brien, and D. H. MacLennan. Identification of a mutation in porcine ryanodine receptor associated with malignant hyperthermia. *Science (Washington D. C.)* **253**:448–451 (1991).
12. Gronert, G. A. Malignant hyperthermia. *Anesthesiology* **53**:395–423 (1980).
13. Sessler, D. I. Malignant hyperthermia. *J. Pediatr.* **109**:9–13 (1986).
14. Zacchetti, D., E. Clementi, C. Fasolato, P. Lorenzoni, M. Zottini, F. Grohovaz, G. Fumagalli, T. Pozzan, and J. Meldolesi. Intracellular $Ca^{2+}$ pools in PC12 cells: a unique rapidly exchanging pool is sensitive to both inositol 1,4,5-trisphosphate and caffeine-ryanodine. *J. Biol. Chem.* **266**:20152–20158 (1991).
15. Saito, A., S. Seiler, A. Chu, and S. Fleischer. Preparation and morphology of

SR terminal cisternae from rabbit skeletal muscle. *J. Cell. Biol.* **99**:875–885 (1984).
16. Treves, S., M. DeMattei, M. Lanfredi, A. Villa, N. M. Green, D. H. MacLennan, J. Meldolesi, and T. Pozzan. Calreticulin is a candidate for a calsequestrin-like function in $Ca^{2+}$ storage compartments (calciosomes) of liver and brain. *Biochem. J.* **271**:473–480 (1990).
17. Bradford, M. M. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **72**:248–254 (1976).
18. Treves, S., P. Chiozzi, and F. Zorzato. Identification of the domain recognized by anti-(ryanodine receptor) antibodies which affect $Ca^{2+}$-induced $Ca^{2+}$ release. *Biochem. J.* **291**:757–763 (1993).
19. Sayers, L. G., G. R. Brown, R. H. Michell, and F. Michelangeli. The effects of thimerosal on calcium uptake and inositol 1,4,5-trisphosphate-induced $Ca^{2+}$ release by cerebellar microsomes. *Biochem. J.* **289**:883–887 (1993).
20. Minta, A., J. P. Y. Kao, and R. Y. Tsien. Fluorescent indicators of cytosolic calcium based on rhodamine and fluorescein chromophores. *J. Biol. Chem.* **264**:8171–8178 (1989).
21. Grynkiewicz, G., M. Poenie, and R. Y. Tsien. A new generation of $Ca^{2+}$ indicators with greatly improved fluorescence properties. *J. Biol. Chem.* **260**:3440–3446 (1985).
22. Treves, S., F. DiVirgilio, V. Cerundolo, P. Zanovello, D. Collavo, and T. Pozzan. Calcium and inositol phosphates in the activation of T cell-mediated cytotoxicity. *J. Exp. Med.* **166**:33–42 (1987).
23. Mignery, G., T. C. Sudhof, K. Takei, and P. DeCamilli. Putative receptor for the inositol 1,4,5-trisphosphate similar to ryanodine receptor. *Nature (Lond.)* **342**:192–195 (1989).
24. Walton, P. D., J. A. Airey, J. L. Sutko, C. F. Beck, G. A. Mignery, T. C. Sudhof, T. J. Deerinck, and M. H. Ellisman. Ryanodine and inositol trisphosphate receptors coexist in avian cerebellar Purkinje neurons. *J. Cell Biol.* **113**:1145–1157 (1991).
25. Fasolato, C., P. Pizzo, and T. Pozzan. Receptor-mediated $Ca^{2+}$- influx in PC12 cells: ATP and bradykinin activate two independent pathways. *J. Biol. Chem.* **265**:20351–20355 (1990).
26. Fasolato, C., M. Zottini, E. Clementi, D. Zacchetti, J. Meldolesi, and T. Pozzan. Intracellular $Ca^{2+}$ pools are distinguished by their turnover and mechanism of $Ca^{2+}$ accumulation, storage and release. *J. Biol. Chem.* **266**:20159–20167 (1991).
27. Scharff, O., B. Foder, and U. Skibsted. Hysteretic activation of the $Ca^{2+}$ pump revealed by calcium transients in human red cells. *Biochim. Biophys. Acta* **730**:295–305 (1983).
28. Thasturp, O., P. J. Cullen, B. K. Drobak, M. R. Hanley, and A. P. Dawson. Thapsigargin, a tumor promoter, discharges intracellular $Ca^{2+}$ stores by specific inhibition of the endoplasmic reticulum $Ca^{2+}$ ATPase. *Proc. Natl. Acad. Sci. USA* **87**:2466–2470 (1990).
29. Lytton, J., M. Westlin, and M. R. Hanley. Thapsigargin inhibits the sarcoplasmic or endoplasmic reticulum Ca-ATPase family of calcium pumps. *J. Biol. Chem.* **266**:17067–17071 (1991).
30. Takasago, T., T. Imagawa, K. Furukawa, T. Ogurusu, and M. Shigekawa. Regulation of the cardiac ryanodine receptor by protein kinase-dependent phosphorylation. *J. Biochem. (Tokyo)* **109**:163–170 (1991).
31. Wang, J., and P. M. Best. Inactivation of the sarcoplasmic reticulum calcium channel by protein kinase. *Nature (Lond.)* **359**:739–741 (1992).

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