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Pharmacologic Differentiation between Inositol-1,4,5-trisphosphate-Induced Ca^{2+} Release and Ca^{2+}- or Caffeine-Induced Ca^{2+} Release from Intracellular Membrane Systems

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SUMMARY
Various known Ca^{2+} channel blockers and intracellular Ca^{2+} antagonists have been tested for effects of inositol-1,4,5-trisphosphate (IP_3)-induced Ca^{2+} release from isolated canine brain microsomes. In agreement with previous reports, heparin, *p*-chloromercuribenzoic acid, W-7, cinnarizine, flunarizine, certain local anesthetics, La^{3+}, and Ca^{2+} inhibit the release of Ca^{2+} induced by addition of IP_3. In addition, we report here pronounced inhibition of IP_3-induced Ca^{2+} release by low levels of Cd^{2+}, by relatively high concentrations of TMB-8, and by phytic acid. In contrast, a number of blockers of other Ca^{2+} channels (nifedipine, verapamil, dantrolene, dithiothreitol, and ruthenium red) have relatively little or no effect on IP_3-induced Ca^{2+} release from brain microsomes. The relative ineffectiveness of substances that inhibit Ca^{2+}- or caffeine-induced Ca^{2+} release from skeletal muscle sarcoplasmic reticulum suggests that release of Ca^{2+} from caffeine- and IP_3-sensitive neuronal Ca^{2+} stores is likely to be mediated by different channels. Further evidence that different channels are involved is presented by way of demonstration of the lack of Ca^{2+}-induced Ca^{2+} release from these brain microsomes and the lack of effect on sarcoplasmic reticulum caffeine-induced Ca^{2+} release of certain inhibitors of IP_3-induced Ca^{2+} release used here. Among IP_3-induced Ca^{2+} release blockers, La^{3+} appeared to be exceptional in its ability to stimulate microsomal Ca^{2+} uptake sufficiently to attenuate release of Ca^{2+} induced by IP_3. Most blockers of IP_3-induced Ca^{2+} release appear not to function by way of inhibiting K^+ counter-ion movements (valinomycin does not reverse the inhibition) but rather by way of direct interaction with the IP_3 receptor or the Ca^{2+} channel that mediates the IP_3-induced Ca^{2+} release. Inhibition of [^{3}H]IP_3 binding to the microsomes by phytic acid, heparin, pyrophosphate, *p*-chloromercuribenzoic acid, and Ca^{2+} could be demonstrated but not by the other substances tested.

IP_3-induced Ca^{2+} release from internal stores of many tissues has been implicated in many cellular processes (1). In certain specialized tissues such as skeletal muscle, larger caffeine-sensitive Ca^{2+} stores mediate excitation-contraction coupling (2). One recent proposal suggests structural similarity between such sets of internal Ca^{2+} stores in different tissues (3). Specific pharmacologic blockers of IP_3-induced Ca^{2+} release could have utility to preliminarily assess IP_3 involvement in a given cellular process. They could also aid in the determination of whether IP_3-sensitive Ca^{2+} stores and caffeine-sensitive Ca^{2+} stores overlap or utilize the same set of Ca^{2+} channels.

Recently, IP_3-induced Ca^{2+} release has been reported from brain microsomes (4) and a few nonspecific inhibitors have been identified (5). Given the high density of IP_3 binding sites in brain (6), we have chosen to assess IP_3-induced Ca^{2+} release and its inhibition in brain microsomes. In addition to putative K^+ channel blockers (7), we have examined the effects of heparin, reportedly a specific antagonist of IP_3 binding and IP_3-induced Ca^{2+} release (6, 8, 9). We have also tested pyrophosphate and phosphorylated sugars such as phytic acid and glucose-6-phosphate, which are expected to function in the same fashion (6, 10). Other substances tested include calmodulin antagonists such as W-7 (11), the Ca^{2+} antagonists flunarizine and cinnarizine, certain local anesthetics, and *p*CMB, which are reported inhibitors of IP_3-induced Ca^{2+} release from platelet membrane fractions (12, 13), as well as La^{3+}, another reported inhibitor of IP_3-induced Ca^{2+} release (14). We have additionally assessed the effects of other known "intracellular" Ca^{2+} antagonists on the system, including dantrolene (15), TMB-8 (16, 17), ryanodine (18), and ruthenium red (19), as well as the surface membrane Ca^{2+} channel blockers nifedipine and verapamil (20, 21). Finally, certain controlled substances, some of whose mechanisms of action on the central nervous

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ABBREVIATIONS: IP_3, myo-inositol-1,4,5-trisphosphate; bis G-10, 1,10-bis-guanidino-n-decane; MOPS, 3-(*N*-morpholino)propanesulfonic acid; *p*CMB, *para*-chloromercuribenzoic acid; SR, sarcoplasmic reticulum; TMB-8, 3,4,5-trimethoxybenzoic acid-8-(diethylamino) octyl ester; W-7, *N*-(6-aminohexyl)-5-chloro-1 naphthalenesulfonamide.

system remain poorly understood (bufotenine, *N,N'*-dimethyltryptamine, Δ^1-tetrahydrocannabinol, methamphetamine, methaqualone, and pentobarbital), were also tested to assess whether their mechanism of action involved effects on IP_3-induced Ca^{2+} release from intracellular stores of central nervous system neurons.

Materials and Methods

All procedures involving brain microsomes were performed as described previously (7), with the exception of ^{45}Ca uptake determinations described below.

Ca^{2+} uptake and release measurements were carried out in a Hewlett Packard 8451A diode array spectrophotometer (22) at 28–30°. Briefly, brain microsomes (1 mg) were suspended in 1 ml of the following medium in a spectrophotometer cuvette: 40 mM KCl, 8 mM MOPS, 62.5 mM potassium phosphate, 5 mM Na_2phosphocreatine, 1 mM MgATP, 40 μg/ml creatine phosphokinase, 0.25 mM antipyrylazo III, pH 7.0. Ca^{2+} movements were monitored by subtracting the absorbance at 790 nm (where only contributions from vesicle light scattering occur) from the absorbance at 710 nm (where contributions exist both from vesicle light scattering and antipyrylazo III-Ca^{2+} interaction).

Experiments involving uptake of ^{45}Ca were performed in an identical fashion, except that $^{45}CaCl_2$ was included as part of the loading procedure and the amounts and volumes of all materials were doubled. Aliquots (200 μl) were then passed through 0.45-μm pore size Millipore filters (HAWP), which were immediately washed twice with 3 ml of chilled 40 mM KCl, 8 mM MOPS, 62.5 mM potassium phosphate, pH 7.0, and assayed by liquid scintillation counting. Results were normalized relative to the cpm of an equivalent volume of unfiltered medium.

Experiments involving isolated SR utilized terminal cisternae isolated from rabbit skeletal muscle, as outlined by Saito *et al.* (23). All SR experiments were performed under identical conditions as the brain microsome experiments but using less SR protein (20–50 μg) and a correspondingly greater amount of Ca^{2+} loading per mg of protein. In the case of Ca^{2+}-induced Ca^{2+} release determinations, only 20 μg of SR protein was utilized, in order to slow down the rate of Ca^{2+} release sufficiently to differentiate it from the Ca^{2+} addition that triggered the release. Experiments demonstrating a lack of IP_3-induced Ca^{2+} release from SR terminal cisternae were performed both with 26 μg of SR protein as well as with 1 mg of SR protein, in case the IP_3-induced Ca^{2+} release was manifest only at a low level of SR loading with Ca^{2+}.

All agents were obtained from Fisher Scientific or Sigma Chemical Co., with the following exceptions: IP_3 was obtained from Calbiochem as well as Sigma. Ryanodine and TMB-8 were obtained from Calbiochem. The heparin used was obtained from Sigma, from porcine intestinal mucosa (catalog No. H5640; for the dose-response curves, an average molecular weight of 5000 was assumed). Bis G-10 was a generous gift from Drs. Michael Fill (Baylor College of Medicine) and Philip Best (University of Illinois, Urbana).

Results

Dog brain microsomes were loaded with Ca^{2+} in the presence of ATP and phosphate, with the reaction monitored spectrophotometrically as outlined in Materials and Methods and the accompanying communication (7). Following uptake of 50 nmol of $CaCl_2$/mg of microsomal protein, 10 μM IP_3 was added to the cuvette to elicit Ca^{2+} release.

We have explored the effects of a large number of pharmacologic agents known to inhibit a variety of different Ca^{2+} channels or forms of Ca^{2+} release from other microsomal systems. As seen in Table 1, there is only a small inhibitory effect of ruthenium red, nifedipine, or dithiothreitol on IP_3-induced Ca^{2+} release at the concentrations employed. The agents tested were each applied to the sample after loading with Ca^{2+} and

TABLE 1

Effects of inhibitors of other Ca^{2+} channels and certain controlled substances on IP_3-induced Ca^{2+} release from brain microsomes

The amount of Ca^{2+} present in the brain microsome samples was estimated to be 25 nmol of $CaCl_2$/mg added plus 10–15 nmol of contaminating $CaCl_2$/mg present (as determined by application of 2 μM A23187 to a sample in the absence of any added $CaCl_2$). All data were normalized to a control rate of Ca^{2+} release determined for that particular sample (7). Control rates of Ca^{2+} release varied from 30.5 to 166 nmol/mg·min with the different samples employed here. Extents of Ca^{2+} release in controls varied from 8.5 to 14.7 nmol/mg.

	Normalized rate of Ca^{2+} release[a]
10 μM Ruthenium red	0.73
50 μM Nifedipine	0.73
500 μM Dithiothreitol	0.69
10 μM Verapamil	0.92
100 μM Verapamil	0.50
34 μM Bufotenine monooxalate (10 μg/ml)	1.02
53 μM *N,N'*-Dimethyltryptamine (10 μg/ml)	1.07
32 μM Δ^1-Tetrahydrocannabinol (10 μg/ml)	0.92
100 μM Methamphetamine	1.11
100 μM Methylphenidate	0.97
35 μM Methaqualone HCl (10 μg/ml)	1.18
1 mM Pentobarbital	0.70
3 mM Pentobarbital	0.37

	Normalized rate of Ca^{2+} release after 0.5–1-min exposure[a]	Normalized rate of Ca^{2+} release after 30–40-min exposure[b]
100 nM Ryanodine	0.95	0.96
1 μM Ryanodine	0.92	0.93
10 μM Ryanodine	0.96	0.95
100 μM Ryanodine	1.15	1.08

[a] All experiments were carried out as described for Fig. 1 but with the test drug administered 10–20 sec before addition of 10 μM IP_3.

[b] All experiments were carried out with ryanodine added immediately before the Ca^{2+}-loading procedure.

shortly before IP_3 addition. We also found no effect of ryanodine over a wide range of concentrations, even when the exposure time was increased considerably. Also shown in Table 1 are negative results obtained with a number of controlled substances. The effects of pentobarbital were obtained only at concentrations far higher than in clinical use.

Several substances inhibited IP_3-induced Ca^{2+} release from brain microsomes. Examples are shown in Fig. 1 for Cd^{2+}, TMB-8, and cinnarizine. With certain substances, immediate upward or downward deflection in the trace accompanied drug additions, as in the trace labeled 100 μM cinnarizine. When these effects were noted, we performed additional experiments adding the drugs to the assay medium in the absence of vesicles or with microsomes that were not loaded with Ca^{2+}. When deflections were also seen under these conditions, they were deemed artifactual (unrelated to microsomal Ca^{2+} release). Cinnarizine and flunarizine added from concentrated ethanolic stock solutions often formed a transient precipitate that redissolved in a few seconds, resulting in the downward movement of the cinnarizine trace before IP_3 addition. With Cd^{2+} at concentrations of >20 μM, we determined that similar deflections (not shown) were also artifactual.

Dose-response curves for these and a number of other related compounds are shown in Fig. 2. The three most potent agents, Cd^{2+}, heparin, and *p*CMB, all exhibited apparent K_i values of less than 20 μM. In some cases, such as with *p*CMB, the dose-response relationship appeared to be steeper than that shown for the one-ligand/one-site model used to generate the curves in Fig. 2. Additionally, the presence of phosphate and ATP in our assay medium might well have reduced the free Cd^{2+} or

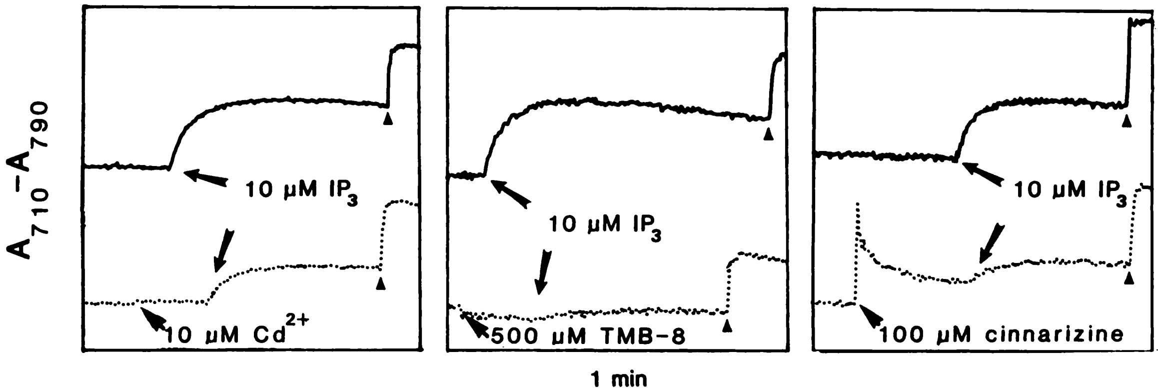

Fig. 1. Inhibition of IP_3-induced Ca^{2+} release by certain drugs. IP_3-induced Ca^{2+} release was measured as described in Materials and Methods. Ca^{2+} release was elicited in response to addition of 10 μM IP_3 in the absence of added drugs (*upper control traces*) or with 10 μM Cd^{2+}, 500 μM TMB-8, or 100 μM cinnarizine added 10–20 sec before addition of IP_3. In this and subsequent figures, the final upward deflections (*arrows*) near the end of each *trace* represent 12.5 nmol of $CaCl_2$ additions for recalibration purposes in the presence of the different agents used.

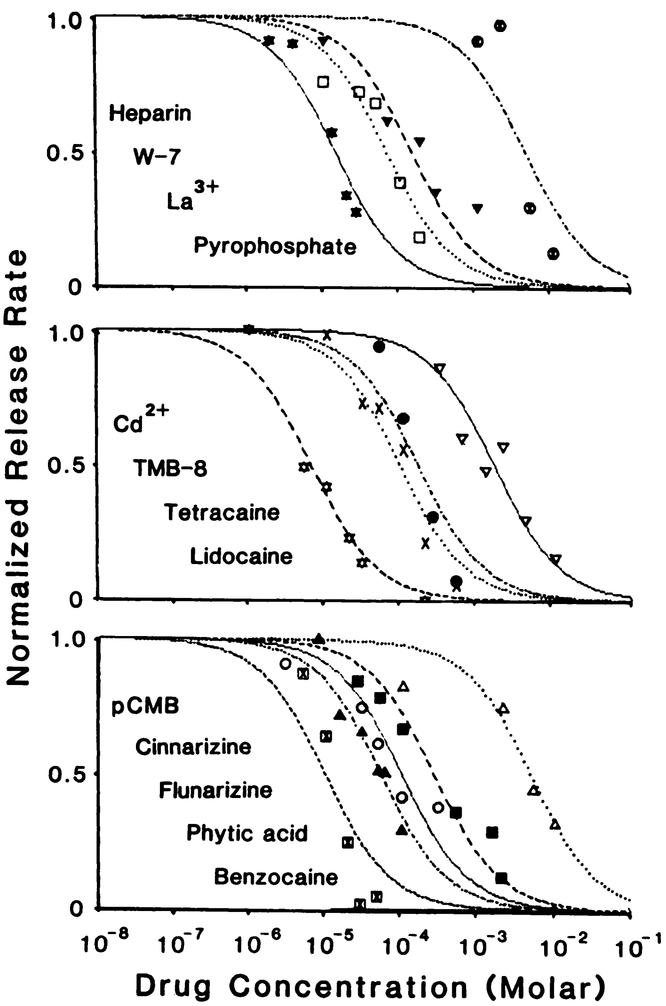

Fig. 2. Dose-response curves for various agents inhibiting IP_3-induced Ca^{2+} release from brain microsomes. All experiments were performed as described in Fig. 1. Release rates were normalized to control Ca^{2+} release rates induced by 10 μM IP_3 in the absence of drug inhibitors. The *curves* were generated by computer fit of the data to a dose-response relationship, using the software program ENZFITTER. All K_i values given below represent apparent K_i determinations. *Upper*, release inhibited by heparin (✱) (K_i = 14 μM), W-7 (□) (K_i = 67 μM), La^{3+} (▼) (K_i = 140 μM), and pyrophosphate (⊗) (K_i = 4.3 mM). *Middle*, release inhibited by Cd^{2+} (☆) (K_i = 6.3 μM), TMB-8 (×) (K_i = 91 μM), tetracaine (●) (K_i = 160 μM), and lidocaine (▽) (K_i = 1.5 mM). *Lower*, release inhibited by *p*CMB (⊠) (K_i = 9.9 μM), cinnarizine (▲) (K_i = 54 μM), flunarizine (○) (K_i = 100 μM), phytic acid (■) (260 μM), and benzocaine (△) (K_i = 4.8 mM).

La^{3+} concentrations to significantly lower levels than those indicated in Fig. 2. These dose-response curves were determined within 30 sec of drug addition, but we saw little additional inhibition of release with 30–40 min exposures (not shown). Thus, we presume that the site of action of all membrane-impermeant agents is on the external surface of IP_3-sensitive microsomes. Only lidocaine and tetracaine appeared to be capable of any time-dependent inhibition, and their effects in that regard were far less pronounced than a time-dependent local anesthetic inhibition of a form of spontaneous Ca^{2+} release from isolated skeletal muscle SR (24).

Inhibitors of IP_3-induced Ca^{2+} release were tested for their ability to inhibit microsomal Ca^{2+} loading. Only 30 μM *p*CMB, 50 μM Cd^{2+}, and 10 mM pyrophosphate appeared to appreciably inhibit Ca^{2+} loading at concentrations that greatly inhibited Ca^{2+} release, and only *p*CMB caused a >50% inhibition of uptake (not shown). W-7 (200 μM), which is reported to inhibit liver microsomal Ca^{2+} uptake (25) and to open SR Ca^{2+} release channels (26), had only a minor effect (~15% inhibition) on brain microsomal Ca^{2+} uptake (not shown). Strikingly, La^{3+} caused a large (5–6-fold) stimulation of Ca^{2+} uptake that could contribute to its attenuation of IP_3-induced Ca^{2+} release. The La^{3+} effect was reinvestigated using ^{45}Ca uptake, and its stimulatory effect was confirmed (Fig. 3). All other substances tested had little effect on Ca^{2+} uptake, regardless of whether exposure to the agents was of short duration or ≥20 min (not shown).

Valinomycin was unable to restore release inhibited by any of these agents (not shown). This suggests that interference with K^+ ion movements contributes little to the effects of any of these agents.

We have attempted to further distinguish among these agents to determine whether their site of action was at the level of interference with IP_3 binding to its receptor or via blockade of the Ca^{2+} channel it opens. We first tested for competitive antagonism between the drugs and IP_3 by testing the drugs at a second, higher, IP_3 concentration. As seen in Fig. 4, addition of 50 μM IP_3 in the presence of blocking concentrations of *p*CMB did little to restore Ca^{2+} release. However, 50 μM IP_3

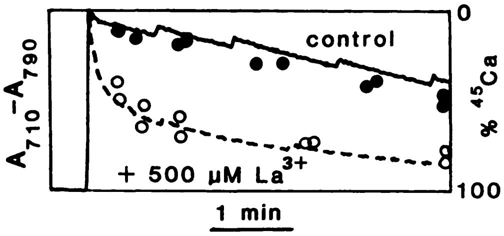

Fig. 3. La^{3+} stimulation of Ca^{2+} uptake by brain microsomes. Two milligrams of microsomal protein were suspended in 2.0 ml of medium to which 50 nmol of ^{45}Ca were added. Absorbance ($A_{710} - A_{790}$) measurements provided the *traces*, while 200-μl aliquots were removed at the jumps in the traces and filtered with a vacuum manifold, as described in Materials and Methods, yielding the data points referable to the *right ordinate*. The experiments were performed in duplicate in the absence (●) and presence (○) of 500 μM La^{3+}.

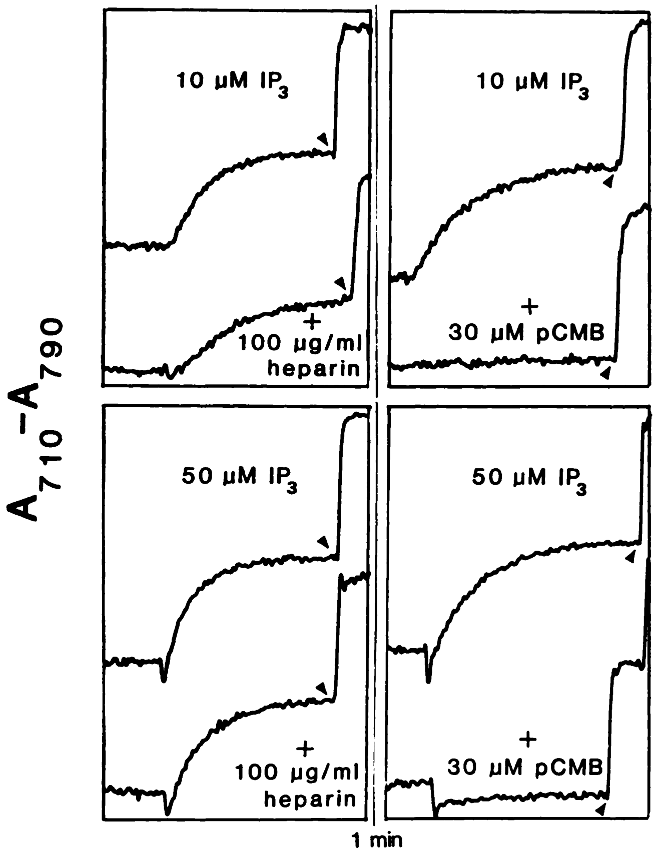

Fig. 4. Test for competitive antagonism of IP_3-induced Ca^{2+} release by drugs. Experiments were performed as described in Fig. 1. Each *pair of traces* represents a control Ca^{2+} release elicited by addition of 10 or 50 μM IP_3, as indicated, together with an experiment performed in the presence of the indicated concentration of release blocker. *Arrowheads* near the end of each *trace* indicate 12.5-nmol $CaCl_2$ additions for calibration purposes.

was able to greatly enhance release that had been blocked by heparin when challenged with only 10 μM IP_3. This suggests that heparin competitively antagonized IP_3 binding to its receptor and that *p*CMB might not.

In Table 2 we have assessed the ability of several agents to inhibit [^{3}H]IP_3 binding to brain microsomes. As seen, phytic acid, heparin, and pyrophosphate, three agents whose effects were partially overcome by 50 μM IP_3 (not shown), were able to appreciably inhibit IP_3 binding. Flunarizine also was slightly less effective at inhibiting release in the presence of 50 μM IP_3 (not shown) but it did not significantly inhibit IP_3 binding (Table 2). Significant inhibition of IP_3 binding was noted with *p*CMB and with Ca^{2+}. This effect of *p*CMB undoubtedly contributes more to the inhibition of IP_3-induced Ca^{2+} release than does its inhibition of Ca^{2+} uptake. It is difficult to infer any causal relationship between the increased IP_3 binding observed in the presence of W-7 and the inhibitory effects of that compound on IP_3-induced Ca^{2+} release.

We additionally wished to determine whether certain inhibitors of IP_3-induced Ca^{2+} release also inhibited caffeine-induced Ca^{2+} release from SR isolated from skeletal muscle. For this we also tested effects of some of the K^+ channel blockers utilized in the companion communication (7). Previously, it has been reported that similar concentrations of tetracaine, Ba^{2+}, and 9-aminoacridine (26) inhibit SR caffeine- and Ca^{2+}-induced Ca^{2+} release, as do even lower concentrations of neomycin (22). We tested certain substances on caffeine-induced Ca^{2+} release under the same experimental conditions as for our brain microsome experiments. We determined that many blockers of IP_3-induced Ca^{2+} release also inhibited caffeine-induced Ca^{2+} release at similar concentrations and, thus, were quite nonspecific in their effects. However, as shown in Fig. 5 or listed in Table 3, tetrapentylammonium, heparin, and quinine did not inhibit caffeine-induced Ca^{2+} release, suggesting that these agents were more specific in their actions on these two different forms of Ca^{2+} release from intracellular stores. Other agents that inhibited IP_3-induced Ca^{2+} release from brain microsomes actually induced Ca^{2+} release from isolated SR but not from the brain microsomes.

Another determinant of IP_3-induced Ca^{2+} release is the extravesicular free [Ca^{2+}]. The experiments shown in Fig. 6 contrast the Ca^{2+} dependence of IP_3-induced Ca^{2+} release from brain microsomes with that of Ca^{2+}-induced Ca^{2+} release from rabbit skeletal muscle terminal cisternae under similar conditions. First, there was no Ca^{2+}-induced Ca^{2+} release from the brain microsomes, and extravesicular Ca^{2+} instead produced an inhibition of subsequent IP_3-induced Ca^{2+} release. In contrast, the terminal cisternae were unresponsive to IP_3 under these conditions (not shown), and the Ca^{2+}-induced Ca^{2+} release observed exhibited a markedly different dependence on extravesicular free Ca^{2+} than did the IP_3-induced release from brain microsomes.

Discussion

We have assessed the ability of a large number of substances to inhibit IP_3-induced Ca^{2+} release from brain microsomes. The substances reported here are more heterogeneous in their actions than those reported in the companion communication (7). We have corroborated earlier reports of the inhibitory effects of many of these compounds, further elucidated the mechanism of action of several, and attempted to determine some additional effects so as to guide other investigators who might wish to utilize "specific" inhibitors of IP_3-induced Ca^{2+} release for *in situ* studies. Finally, we have provided an example of the use of such pharmacologic agents for the purposes of further distinguishing two different forms of Ca^{2+} release from intracellular stores.

As in the accompanying communication (7), the results reported here all involved determinations of drug effects on the rate of IP_3-induced Ca^{2+} release. Most previous reports lacked the time resolution to assess more than drug effects on the

TABLE 2

Effects of IP_3-induced Ca^{2+} release inhibitors on specific $[^3H]IP_3$ binding by brain microsomes

Experiments were performed by incubating 0.5 mg of brain microsomes in a medium consisting of 40 mM KCl, 62.5 mM potassium phosphate, 2.5 mM EDTA, and 8 mM MOPS, pH 7.0, for 30 min at 4°. Subsequently, the samples were sedimented in a Beckman airfuge, the supernatants were removed, and the pellets were rinsed once with binding medium lacking $[^3H]IP_3$ (7) and then transferred to liquid scintillation vials. Nonspecific binding was assessed in the presence of 5 μM unlabeled IP_3. Specific binding was calculated as the difference between total and nonspecific binding.

	Total binding	Nonspecific binding	Specific binding
		pmol/mg	
Control	0.609 ± 0.083	0.284 ± 0.049	0.325 ± 0.053
100 μM Cinnarizine	0.582 ± 0.052	0.276 ± 0.012	0.306 ± 0.065
100 μM Flunarizine	0.604 ± 0.134	0.333 ± 0.052	0.272 ± 0.083
500 μM TMB-8	0.666 ± 0.102	0.291 ± 0.048	0.375 ± 0.112
200 μM W-7	1.289 ± 0.093	0.371 ± 0.013	0.918 ± 0.106
10 mM Benzocaine[a]	0.596	0.282	0.314
5 mM Lidocaine	0.580 ± 0.089	0.269 ± 0.031	0.298 ± 0.101
500 μM Tetracaine	0.643	0.329	0.314
2 mM Phytic acid	0.229 ± 0.003	0.231 ± 0.008	−0.002 ± 0.007
100 μg/ml Heparin	0.265 ± 0.029	0.237 ± 0.062	0.028 ± 0.033
10 mM Pyrophosphate	0.381	0.251	0.130
30 μM *p*CMB	0.367	0.318	0.049
13 μM Ca^{2+} free[b]	0.272	0.257	0.015

[a] Some precipitation was noted in these determinations with benzocaine this might have masked an inhibitory effect.
[b] The total Ca^{2+} present was 34 μM, which was calculated to yield the same free $[Ca^{2+}]$ = 13 μM as the highest concentration used in Fig. 6. To achieve this concentration, the EDTA was omitted from the assay.

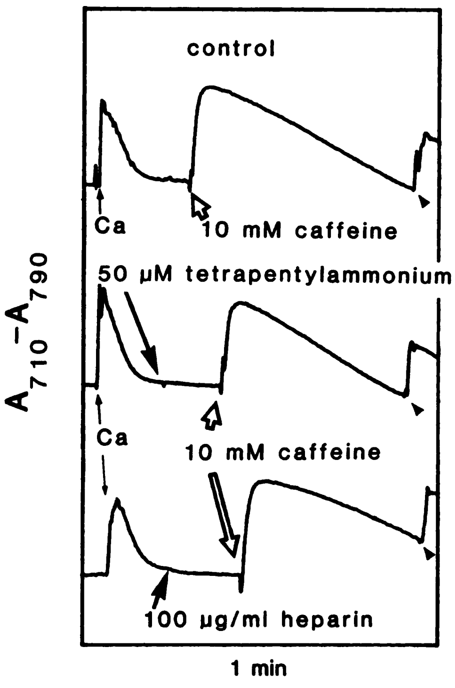

Fig. 5. Lack of effect of heparin or tetrapentylammonium on caffeine-induced Ca^{2+} release from isolated skeletal muscle terminal cisternae. Experiments were performed as described for Fig. 1, except with 50 μg of terminal cisternae substituted for brain microsomes, five additions of $CaCl_2$ (12.5 nmol each), and addition of 10 mM caffeine instead of IP_3. The *traces* shown include the administration and uptake of the fifth aliquot of $CaCl_2$, the transient releases elicited by caffeine, and, finally, additions of $CaCl_2$ for recalibrating purposes (*arrowheads*).

amount of Ca^{2+} released, which would be less likely to be affected except at higher drug concentrations. None of the drug effects presented here involved indirect effects via inhibition of counter-ion K^+ movements or possible elevations of free $[Ca^{2+}]$ to a level (Fig. 6) that by itself would have been inhibitory to a subsequent IP_3-induced Ca^{2+} release (6). We cannot at present rule out an increased sensitivity to released Ca^{2+} as a means of attenuating the responses.

TABLE 3

Effects of selected inhibitors of IP_3-induced Ca^{2+} release on caffeine-induced Ca^{2+} release from skeletal SR terminal cisternae

SR terminal cisternae (50 μg) were loaded with Ca^{2+} as described in Fig. 4 and then exposed to the substances listed to the left, followed by 10 mM caffeine.

	Rate of caffeine-induced Ca^{2+} release
	μmol/mg·min
Control	18.6 ± 3.2
75 μM Cinnarizine	R[a]
100 μM TMB-8	18.1
500 μM TMB-8	R
200 μM W-7	R
500 μM Tetracaine	0
5 μM Cd^{2+}	22.3
30 μM Cd^{2+}	R
1 mM La^{3+}	11.3
100 μg/ml Heparin	17.1 ± 3.2
100 μM Quinine	22.9
50 μM Tetrapentylammonium	14.8
500 μM Tetrapentylammonium	22.2
200 μM Ba^{2+}	5.8
30 μM 9-Aminoacridine	9.1
200 μM Neomycin	0
500 μM Zn^{2+}	10.2
3 μM Bis G-10	9.4
30 μM Bis G-10	5.2 ± 0.8

[a] R, the IP_3-induced Ca^{2+} release inhibitor actually caused a release of Ca^{2+} from the SR vesicles under these conditions, before caffeine addition. Following this release, caffeine failed to induce further release, possibly due to emptying of the caffeine-sensitive Ca^{2+} stores.

Among the substances found to inhibit IP_3-induced Ca^{2+} release in this communication, we found several that appeared likely to competitively inhibit IP_3 binding to its receptor (heparin, pyrophosphate, and phytic acid), as well as one that appeared more likely to inhibit IP_3 binding noncompetitively (*p*CMB). One agent (La^{3+}) appeared to be capable of inhibiting IP_3-induced Ca^{2+} release by stimulating the net rate of Ca^{2+} uptake by the brain microsomes utilized here. The remaining agents (cinnarizine, flunarizine, TMB-8, W-7, benzocaine, lidocaine, tetracaine, and Cd^{2+}) appeared to be similar in their actions to the K^+ channel blockers studied previously (7), in

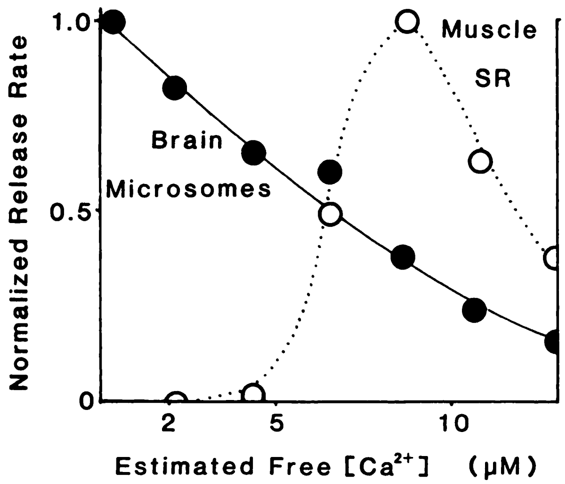

Fig. 6. Differences in Ca^{2+} dependence of IP_3-induced Ca^{2+} release from brain microsomes and Ca^{2+}-induced Ca^{2+} release from isolated SR terminal cisternae. Brain microsome experiments were performed as described in Fig. 1, except that additions of $CaCl_2$ were made ~10 sec before administration of 10 μM IP_3. SR experiments were performed in the same medium in the same fashion but with only 26 μg of SR protein. This enabled discrimination between the $CaCl_2$ addition and the Ca^{2+} release it produced. The SR was preloaded with only two 12.5-nmol $CaCl_2$ additions before the addition that elicited release. Free [Ca^{2+}] was calculated according to a computer program previously described (22, 25), with 72.5 μM total Ca^{2+} resulting in a free [Ca^{2+}] of 13 μM. Release rates were normalized for each form of release to the highest rate obtained at any [Ca^{2+}] tested.

that they did not inhibit IP_3 binding and appeared not to function by way of inhibiting obligate K^+ counter-ion movements. Most likely, they directly interfere with ion flow through the IP_3-activated Ca^{2+} channel or with the coupling between that channel and the IP_3 receptor, if the two are separate entities. An IP_3 receptor has been purified from brain cerebellum (27). An associated membrane protein that confers sensitivity to inhibition by Ca^{2+} (calmedin) has also been purified from cerebellum membranes (28). As yet we do not know whether either of these proteins functions additionally as the Ca^{2+} channel.

The inhibitory effect of extravesicular Ca^{2+} on IP_3-induced Ca^{2+} release is apparent only at higher concentrations than the inhibition of [^{3}H]IP_3 binding demonstrated by Worley *et al.* (6), possibly because of the differences in pH between the two studies (pH 7.0 versus pH 8.3). In their study, Worley *et al.* (6) also demonstrated a profound influence of pH on IP_3 binding. This may indicate that at physiological intracellular pH the required concentration of Ca^{2+} might be intermediate between their value and ours. It also remains possible that our control IP_3-induced Ca^{2+} release is partially inhibited by residual Ca^{2+} not taken up by the microsomes (estimated at 0.3 μM in these experiments from Ca^{2+} electrode measurements) (see also Ref. 29).

Heparin, pyrophosphate, and phytic acid were all previously shown to inhibit IP_3 binding to cerebellar homogenates (6) and heparin to inhibit IP_3-induced Ca^{2+} release in a number of other systems (8, 9). The results obtained with pyrophosphate and phytic acid, consequently, were expected. In our hands, higher heparin concentrations were required, perhaps because of the presence of phosphate, another polyvalent anion, in our assay. We attribute most of the effects of these substances on IP_3-induced Ca^{2+} release to their ability to inhibit IP_3 binding competitively. The lack of additional effect of the Ca^{2+}-precipitating anion pyrophosphate with increasing exposure time suggests that it is not as permeant to the IP_3-sensitive vesicles as to muscle Sr (26) or as permeant to the IP_3 sensitive vesicles as is phosphate.

Although *p*CMB also inhibits IP_3 binding, its effects are not significantly diminished at high IP_3 concentrations; thus its effects on IP_3 binding are likely to be due to noncompetitive inhibition, perhaps by virtue of its effects on SH groups. Previously, *p*CMB had been reported to inhibit IP_3-induced Ca^{2+} release from platelet membranes (13) but its mechanism of action was not explored.

The La^{3+} effect uncovered here was quite unexpected, and La^{3+} was the only agent assayed that appreciably stimulated net Ca^{2+} uptake by the brain microsomes. The enormous stimulation of uptake by La^{3+} indubitably contributed to its attenuation of IP_3-induced Ca^{2+} release. Because we were unable to assess IP_3 binding in the presence of La^{3+}, La^{3+} might have other sites of action inhibitory to IP_3-induced Ca^{2+} release as well. In this regard, La^{3+} has been reported to inhibit IP_3-induced Ca^{2+} release from platelet membranes without appreciable effect on platelet membrane Ca^{2+} uptake (14). The effect of La^{3+} on Ca^{2+} uptake seen here is reminiscent of the ryanodine-induced stimulation of Ca^{2+} uptake by cardiac SR (30), but the effect is unlikely to be due to block of either IP_3- or ryanodine-sensitive Ca^{2+} channels here, because numerous other blockers of each were without similar effect. We note that La^{3+} need not be stimulating a microsomal Ca^{2+} pump, however. An active La^{3+}-inhibitable Na^+/Ca^{2+} exchange system is known to exist in brain microsomal preparations (31). La^{3+} is also known to inhibit neuronal surface membrane Ca^{2+} channels (32), but other inhibitors of such channels (verapamil, nifedipine, and Cd^{2+}) had no such stimulatory effect on Ca^{2+} uptake.

Among the other effective agents presumed to interact more directly with the IP_3-sensitive Ca^{2+} channel, Cd^{2+}, W-7, flunarizine, and cinnarizine are also all known to inhibit surface membrane Ca^{2+} channels (33, 34), although for the same reason cited above this is unlikely to be related to their effects on IP_3-induced Ca^{2+} release. Inhibition of IP_3-induced Ca^{2+} release has been previously reported for W-7 (11), flunarizine, and cinnarizine (12) but not for Cd^{2+}.

At relatively high concentrations (100–500 μM), the purported "intracellular" Ca^{2+} antagonist TMB-8 (16, 17) also inhibited IP_3-induced Ca^{2+} release. Previously, 50 μM TMB-8 (12) or 1 mM TMB-8 (35) were found not to inhibit IP_3-induced Ca^{2+} release from isolated platelet membranes. This may represent a difference between IP_3-induced Ca^{2+} release in the two systems. In agreement with other reports (12–14), we did find *p*CMB, flunarizine, cinnarizine, and the local anesthetics benzocaine and lidocaine to be inhibitory. To this list we also add the observation that tetracaine is effective. Nevertheless, sufficiently high concentrations of all these agents were required that they would certainly have other actions inside cells when introduced at concentrations inhibitory to IP_3-induced Ca^{2+} release.

If any of these agents were to be used inside cells as potential IP_3-induced Ca^{2+} release blockers, many would certainly have other effects. Local anesthetics, for instance, have many effects on the nervous system, and their misuse as inhibitors of any given process frequently gives rise to unexpected results (36).

Surface membrane Ca^{2+} channel blockers would be impractical to use unless the cells tolerated Ca^{2+}-free media well. Use of *p*CMB, Cd^{2+}, and pyrophosphate would be compromised by their added predisposition to inhibit Ca^{2+} uptake, thereby possibly inhibiting refilling of internal stores.

We have demonstrated a relative lack of effectiveness of several blockers of other ion channels. Ruthenium red, a blocker of the SR Ca^{2+} release channel (19, 37), is only weakly effective, as are nifedipine and verapamil, blockers of L-type surface membrane Ca^{2+} channels (20, 38, 39), dantrolene, a blocker of muscle fiber excitation-contraction coupling (15), and dithiothreitol (40), a blocker of heavy metal-induced Ca^{2+} release from isolated skeletal muscle SR (41). The results with ruthenium red, dantrolene, and nifedipine are in agreement with previous reports (1, 5, 12) and help to demonstrate the uniqueness of IP_3-sensitive Ca^{2+} channels of intracellular stores. At high concentrations (>10 μM) ruthenium red has been reported to have inhibitory effects on IP_3-induced Ca^{2+} release (35), but there are also numerous reports of IP_3-induced Ca^{2+} release in the presence of similarly high ruthenium red concentrations (e.g., Ref. 1). At 50 μM concentrations, verapamil was previously reported not to inhibit IP_3-induced Ca^{2+} release (12). The effects noted at still higher concentrations here are unlikely to be related to its actions on surface membrane Ca^{2+} channels.

Considering that ryanodine and caffeine open ruthenium red-sensitive SR Ca^{2+} channels in muscle (26, 42), it is not surprising that pretreatment of brain microsomes with a wide range of ryanodine concentrations failed to deplete IP_3-sensitive microsomes of Ca^{2+}. These results suggest that ryanodine- and IP_3-sensitive pools are distinct. At least partial separation of IP_3- and caffeine-sensitive pools has been reported recently for different regions of individual neurons (43).

To test this point further, we have investigated the effects of selected inhibitors of IP_3-induced Ca^{2+} release on caffeine-induced Ca^{2+} release from isolated skeletal muscle SR. Agents like tetracaine (26), Ba^{2+} (26), 9-aminoacridine (26), neomycin (22), and bis G-10 (44) appear to inhibit both processes, and other IP_3-induced Ca^{2+} release inhibitors cause caffeine-sensitive stores to release Ca^{2+}. Heparin, quinine,[1] and tetrapentylammonium appear to be quite specific in their actions on IP_3-induced Ca^{2+} release. In this regard, they are more specific than ruthenium red, the well known blocker of Ca^{2+}-induced Ca^{2+} release, which also appears to inhibit IP_3-induced Ca^{2+} release somewhat. These results provide further justification for the use of heparin as a specific IP_3 antagonist by Somlyo and co-workers (9, 46). Their contention (46) that IP_3-induced Ca^{2+} release is unimportant for normal physiologic excitation-contraction coupling in vertebrate skeletal muscle is nevertheless compromised by the lack of demonstration of inhibition by heparin of responses of skeletal fibers to IP_3 (46, 47).

Under similar conditions, brain microsomes displayed no Ca^{2+}-induced Ca^{2+} release, whereas skeletal muscle terminal cisternae displayed no IP_3-induced Ca^{2+} release. There are also clear differences in the dependence of the two kinds of release on extravesicular [Ca^{2+}], even when the comparison was made under nearly identical conditions, as here (Fig. 6). This conclusion confirms a similar one derived from studies with platelet membranes by Adunyah and Dean (48). Because both forms of release do demonstrate an inhibition by Ca^{2+} in excess of 10 μM, they could share some common features of a Ca^{2+}-dependent inactivation process.

In summary, it is likely that the Ca^{2+} channels involved in IP_3-induced Ca^{2+} release and Ca^{2+}-induced Ca^{2+} release are quite different. This conclusion would be in agreement with certain results obtained from single-channel recording from muscle SR (49, 50) but would be inconsistent with others (51–53) that suggest that the high conductance ruthenium red-sensitive channels that mediate Ca^{2+} release may also be opened by IP_3. Final resolution of the uniqueness or identity of the two stores and channels mediating the two forms of Ca^{2+} release will have to await full characterization of Ca^{2+}- (or caffeine-) induced and IP_3-induced Ca^{2+} release from the same tissue.

[1] Quinine, however, is also known to release Ca^{2+} from SR under certain conditions (45).

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