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Direct Inhibition of Inositol-1,4,5-trisphosphate-Induced Ca^{2+} Release from Brain Microsomes by K^+ Channel Blockers

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SUMMARY

Tetraethylammonium and 9-tetraethylammonium have previously been reported to inhibit inositol-1,4,5-trisphosphate (IP_3)-induced Ca^{2+} release from brain microsomes, purportedly by blocking potassium channels [*Biochem. J.* **258**:617–620 (1988)]. The effects of these and other K^+ channel blockers have been studied here in greater detail using a spectrophotometric assay for Ca^{2+} movements into and out of canine brain microsomes. IP_3-induced Ca^{2+} release was inhibited by substitution of K^+ in the medium with nominally impermeant cations or by addition of most of the K^+ channel blockers tested. Nevertheless, addition of valinomycin to the medium (to provide an alternative pathway for counter-ion K^+ movements) failed to alleviate the inhibition of IP_3-induced Ca^{2+} release caused by K^+ channel blockers. To determine whether these substances act by inhibition of IP_3 binding or by direct interaction with the Ca^{2+} channel of the internal store that promotes IP_3-induced Ca^{2+} release, their effect on [^{3}H]IP_3 binding was investigated. None of the K^+ channel blockers tested inhibited [^{3}H]IP_3 binding. Nearly all the K^+ channel blockers appear to interact directly with a Ca^{2+} channel of the intracellular stores or perhaps interfere with its coupling to the IP_3 receptor. Because of their multiplicity of actions, these substances cannot be presumed to be either selective K^+ channel blockers or selective inhibitors of IP_3-induced Ca^{2+} release from internal stores. Three of them were even found to partially inhibit valinomycin-stimulated ^{86}Rb uptake into liposomes.

IP_3-induced Ca^{2+} release from internal stores of many cells has been implicated in the effects of many hormones and neurotransmitters (1). Proof of IP_3 involvement in a cellular response would necessitate the demonstration that its levels are elevated in response to the appropriate stimuli within a requisite time span and that IP_3 is capable of releasing Ca^{2+} from internal stores in that tissue. A pharmacologic blocker of IP_3-induced Ca^{2+} release could have utility in preliminary experiments investigating the role of IP_3 in systems where such measurements are not feasible or are too cumbersome. Given the high density of IP_3 binding sites in brain (2), we have chosen brain microsomes as a model system to assess inhibition of IP_3-induced Ca^{2+} release by pharmacologic agents.

Recently, IP_3-induced Ca^{2+} release from brain microsomes has been reported (3) and shown to be inhibited by two K^+ channel blockers (4). It was suggested that these inhibitors acted on a K^+ channel to produce their effects rather than directly on the Ca^{2+} channel (4). As proposed in an earlier report (5), this K^+ channel would play a supportive role by allowing counter-ion movement when Ca^{2+} is released from internal stores.

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Our aim here was to determine whether other K^+ channel blockers also inhibited IP_3-induced Ca^{2+} release and, if so, how. The K^+ channel blockers we have chosen to investigate include quinine (6, 7), 4-aminopyridine (8), TEA and some of its derivatives (9–11), Ba^{2+} (11–13), and 9-aminoacridine (12, 14). Ba^{2+} and 9-aminoacridine have previously been reported to inhibit caffeine-induced Ca^{2+} release from isolated skeletal muscle SR (15).

Materials and Methods

Dog brain microsomes were isolated by minor modifications of the procedures developed by Edelman *et al.* (16) for subfractionation of rat brain. Dogs were either anesthetized with intravenous α-chloralose/urethane and euthanized with KCl or euthanized directly with a pentobarbital/dibucaine mixture administered intravenously. No systematic differences in brain microsome properties were noted between the two methods of euthanasia employed. All steps were performed at 0–4°. Briefly, 5-g portions of whole fresh dog brain were homogenized in 50-ml portions (10 volumes) of 0.32 M sucrose, 5 mM HEPES, pH 7.4, with a wide clearance Potter-Elvehjem Teflon-glass homogenizer. The homogenate was centrifuged 10 min at 3000 rpm (1380 × g_{max}) in a Beckman JA-14 rotor. In some cases, the pellets were resuspended by shaking with an additional 5 volumes of 0.32 M sucrose, 5 mM HEPES, and were recentrifuged at 3000 rpm for 10 min. The pellets were discarded and the supernatant was recentrifuged at 13,500 rpm (28,000

ABBREVIATIONS: IP_3, myo-inositol-1,4,5-trisphosphate; bis G-10, 1,10-bis-guanidino-*n*-decane; MOPS, 3-(*N*-morpholino)propanesulfonic acid; SR, sarcoplasmic reticulum; TEA, tetraethylammonium; TMA, tetramethylammonium; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

× g_{max}) for 45 min. These pellets were also discarded and the supernatants were centrifuged in a Beckman 45 Ti or Type 35 rotor at 35,000 rpm (143,000 × g_{max}) for 60 min. The microsomal pellets were resuspended in 0.32 M sucrose, 5 mM HEPES, pH 7.4, and stored in liquid nitrogen. Ca^{2+} release characteristics appeared to be stable for over a year when preparations were so stored.

Ca^{2+} uptake and release measurements were carried out in a Hewlett Packard 8451A diode array spectrophotometer at 28–30°. 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). In certain experiments, K^+ was replaced by $Tris^+$, $choline^+$, or TMA^+ in the incubation medium. In the $Tris^+$ medium, Na_2phophocreatine was replaced by $Tris_2$ phosphocreatine. Sucrose medium consisted of isoosmotic sucrose with 0.25 mM antipyrylazo III and 2 mM Tris·MOPS, pH 7.0. For most experiments, microsomes were loaded with Ca^{2+} in 5–10-ml batches at room temperature, for ~30 min before spectrophotometric measurements of Ca^{2+} release. With certain batch-loaded samples, control rates of IP_3-induced Ca^{2+} release progressively declined as successive aliquots were assayed. In these cases, controls were run at the beginning and end of each batch, and control rates of release for intermediate aliquots were inferred by linear interpolation.

Calcium calibrations were performed at the end of each experiment, in the presence of all drugs that were present at the time of IP_3-induced Ca^{2+} release. Because certain drugs caused upward deflections in the optical trace, which could be indicative of Ca^{2+} release, such experiments were repeated in the absence of microsomal sample or in the presence of sample that was not preloaded with Ca^{2+}. Upward deflections seen in these circumstances were considered to be artifactual; if no or only small upward deflections were noted, then the upward deflections noted with Ca^{2+}-preloaded microsomes were considered to be indicative of true Ca^{2+} release.

Contaminating Ca^{2+} present in the sample and assay medium was assessed by first allowing it to be taken up by the sample. This was measured either by monitoring the amplitude of the downward movement in the trace following addition of microsomes to the cuvette (see Fig. 1) or by permitting Ca^{2+} uptake to reach completion (flat absorbance baseline) and then applying 2 μM A23187 to release all the internalized Ca^{2+}. Both procedures gave similar estimates.

The effects of selected substances on the ability of valinomycin to transport K^+ were explored by ^{86}Rb uptake experiments, using a modified version (17) of the procedures described by Garty *et al.* (18). Asolectin (40 mg) was sonicated in the presence of 40 mM KCl, 62.5 mM potassium phosphate, and 8 mM MOPS, pH 7.0 (1 ml), until clear. Fifty microliters of this liposome suspension were then passed rapidly through a cation exchange column (Dowex 50-X8, Tris form, in a glass Pasteur pipette), and eluted with 0.95 ml of an isoosmotic solution of sucrose buffered to pH 7.0 with 10 mM Tris·HEPES. This procedure exchanges all cations outside the vesicles for $Tris^+$. Valinomycin (10^{-10} M) and K^+ channel blockers were added to the collection tube. After a 30-sec incubation at room temperature, ^{86}Rb was added (1 μl of 1 mCi/ml) to initiate the measurement. Initial rates of ^{86}Rb uptake were determined by terminating the reaction at 0.5, 1, and 2 min by passing 100 μl of the reaction mixture through another Dowex column with a 0.9-ml wash of isoosmotic sucrose. This procedure removes all ^{86}Rb outside the vesicles and permits subsequent assessment of ^{86}Rb associated with the vesicles by liquid scintillation counting. Results were normalized relative to 5-μl aliquots of reaction mixture.

^{86}Rb uptake into brain microsomes was determined in a similar fashion. Microsomes (2 mg/ml) were equilibrated overnight on ice in 40 mM KCl, 62.5 mM potassium-phosphate, 8 mM MOPS, pH 7.0. Subsequently, 100 μl of microsomal suspension were passed through a Dowex column, with the addition of 0.9 ml of an isoosmotic solution of sucrose buffered with 10 mM Tris·HEPES into a glass tube containing 10^{-8} M valinomycin, 10^{-6} M valinomycin, 10 μM IP_3 (final concentrations), K^+ channel blockers, or none of the above. Aliquots (150–200 μl) were passed through additional Dowex columns at various time points to determine intravesicular ^{86}Rb content.

$[^3H]IP_3$ binding was performed at 4° in 0.5 ml of EDTA-supplemented release medium containing 40 mM KCl, 62.5 mM potassium phosphate, 2.5 mM EDTA, and 8 mM MOPS, pH 7.0. Total $[^3H]IP_3$ binding was measured in the presence of 50 nM $[^3H]IP_3$ only. Nonspecific binding was determined in the presence of both 50 nM $[^3H]IP_3$ and 5 μM unlabeled IP_3. Specific binding was determined as the difference between total and nonspecific binding. Brain microsomes (1 mg/ml) were incubated on ice for 30 min with occasional vortexing. A 20-μl aliquot was assayed for total bath radioactivity. After the incubation period, another 430-μl aliquot was centrifuged in a Beckman Airfuge (A-95 rotor) for 10 min at room temperature. The supernatant was carefully removed and discarded. The pellet was rinsed once gently with chilled binding medium containing no IP_3, without further centrifugation. The pellet was then solubilized by addition of 430 μl of 10% (w/v) glycerol, 5% (v/v) 2-mercaptoethanol, 2.3% (w/v) sodium dodecyl sulfate, and 62.5 mM Tris·HCl, pH 6.8, for ≥3 hr; the entire tube was then assayed for radioactivity by liquid scintillation counting.

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, tetraalkylammonium compounds were obtained as bromide salts from Aldrich or Sigma, and tetramethylammonium hydroxide was from Matheson, Coleman & Bell. Bis G-10 was a gracious gift of Drs. Michael Fill (Baylor College of Medicine) and Philip Best (University of Ilinois, Urbana). $[^3H]IP_3$ was obtained from Dupont/NEN, and ^{86}Rb from Amersham.

Stock solutions were prepared in water or in ethanol. The final ethanol concentration in any experiment never exceeded 1% (218 mM), except when valinomycin or gramicidin was also present (maximum 2% ethanol). We tested the effects of ethanol on one of our samples. Rates of Ca^{2+} release in the presence of 0.1–1.0% ethanol were not significantly different from control rates. These results do not confirm the recently reported effects of low concentrations of ethanol in inducing Ca^{2+} release from brain microsomes (19). However, our experiments were all performed at 30°; Shah and Pant (19) reported ethanol-induced Ca^{2+} release at 37° but not at 0°.

All K_i values represent apparent K_i determinations obtained by computer fit of the data to a single-drug molecule/single-binding site model, using commercially available software (ENZFITTER; Elsevier). In some cases, the dose-response relationships appeared to be steeper

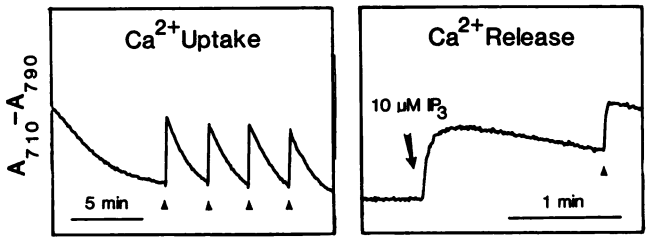

Fig. 1. IP_3-induced Ca^{2+} release from brain microsomes. *Left*, the experimental *trace* demonstrates uptake of four 12.5-nmol $CaCl_2$ additions (*arrowheads*) by 1 mg of brain microsomes in 62.5 mM potassium phosphate, 40 mM KCl, 8 mM MOPS, 5 mM Na_2phosphocreatine, 1 mM MgATP, 40 μg/ml creatine phosphokinase, 0.25 mM antipyrylazo III, pH 7.0. *Right*, following completion of this uptake, 10 μM IP_3 was added where indicated, eliciting Ca^{2+} release. The final upward deflection in this and subsequent figures represents another 12.5-nmol $CaCl_2$ addition (*arrowhead*) designed to recalibrate the system following any drug additions. Note the difference in time scales between the two parts of the figure. The *ordinate* in this and subsequent figures represents the absorbance difference $A_{710} - A_{790}$ (see Materials and Methods). When Ca^{2+} is added or released, the trace goes up; when Ca^{2+} is taken up by the vesicles, the trace goes downward.

TABLE 1

Effects of increased and decreased K^+ permeability on IP_3-induced Ca^{2+} release from brain microsomes

All experiments were carried out as described for Fig. 1. K^+ and $Tris^+$ media contained 102.5 mM K^+ and 102.5 mM $Tris^+$, respectively. A, $Tris^+$ medium was substituted for K^+ medium before Ca^{2+} loading in the second, third, and fourth lines, with 0.25 or 1.0 volumes of K^+ medium added immediately before IP_3 addition. Ca^{2+} loading required considerably longer time when K^+ was not present. However, all release determinations were performed following loading of 50 nmol of $CaCl_2$/mg of microsomal protein. B, Utilizing a different sample, valinomycin was added 10–20 sec before IP_3 in the experiments indicated. C, These experiments were performed to determine whether cation substitutes could affect release in the presence of K^+. Microsomes were loaded in 1 ml of K^+ medium, following which 1 ml of K^+, $Tris^+$, TMA^+, $choline^+$, or isosmotic sucrose medium (without ATP or regenerating system) was added. After allowing time for uptake of any contaminating Ca^{2+} in the added medium, 10 μM IP_3 was added. Chloride and phosphate were present at the same concentrations in all media except the sucrose-substituted one. Values in parentheses represent determinations performed with a different sample. The data presented represent individual determinations, but we have obtained similar results with other samples that cannot readily be pooled together into this table.

		Rate of Ca^{2+} release	Amount of Ca^{2+} released
		nmol/mg · min	*nmol/mg*
A.	K^+ medium (control)	278	16.7
	$Tris^+$ medium	25	10.0
	82 mM $Tris^+$ + 20.5 mM K^+ medium	56	14.6
	51.25 mM $Tris^+$ + 51.25 mM K^+ medium	93	16.3
B.	K^+ medium (control)	185	18.5
	TMA^+ medium	0	0
	$Choline^+$ medium	0	0
	K^+ medium + 1 μM valinomycin	239	18.8
	K^+ medium + 3 μM valinomycin	273	22.4
	K^+ medium + 10 μM valinomycin	266	24.3
C.	K^+ medium + K^+ medium	74.1 (49.5)	14.3 (8.1)
	K^+ medium + $Tris^+$ medium	15.5	9.0
	K^+ medium + TMA^+ medium	25.5	10.8
	K^+ medium + $choline^+$ medium	20.1	9.6
	K^+ medium + sucrose medium	(27.6)	(6.9)

than a simple 1:1 relationship would indicate, but these differences were not explored further.

Results

Dog brain microsomes were loaded with Ca^{2+} in the presence of ATP and phosphate, with the reaction being monitored spectrophotometrically as outlined in Materials and Methods. Following uptake of 50 nmol of $CaCl_2$/mg of microsomal protein, IP_3 was added to the cuvette at different concentrations to elicit Ca^{2+} release. An example of such Ca^{2+} uptake and subsequent IP_3-induced Ca^{2+} release is given in Fig. 1. When the contaminating Ca^{2+} content of this sample was taken into account, we determined that 29% of the Ca^{2+} that was loaded into the vesicles was released by IP_3. The proportion of Ca^{2+} released from the brain microsomes under these conditions varied from 15 to 30%, depending upon the sample. From 16 separate determinations of initial release rates on three different microsomal samples, we estimated an EC_{50} of 1.4 μM for IP_3 (not shown).

In order to determine whether counter-ion K^+ entry was an obligate requirement for IP_3-induced Ca^{2+} release, we performed two types of experiments, 1) substituting ostensibly impermeant cations for K^+ and 2) artificially increasing the K^+ permeability by the addition of the K^+ ionophore valinomycin. First, as seen in Table 1, total substitution of K^+ by $Tris^+$, $choline^+$, or TMA^+ in the loading medium (leaving the chloride and phosphate concentrations unaltered) resulted in a greatly diminished rate of microsomal Ca^{2+} release in response to IP_3.[1] These results suggest that IP_3-induced Ca^{2+} release is electrogenic and does require counter-ion movements or that a coupled Ca^{2+}/K^+ counter-transport system is involved. Second, as seen in Table 1B, addition of valinomycin to the normal K^+-containing solution caused only small stimulations of the rate and extent of IP_3-induced Ca^{2+} release. Other samples showed even less effect of valinomycin. Taken together, these results suggest that the native K^+ permeability of these vesicles is high enough to support near-maximal IP_3-induced Ca^{2+} release.[2]

Several substances used as K^+ channel blockers in other systems inhibited IP_3-induced Ca^{2+} release from these brain microsomes. Examples are shown in Fig. 2 for tetraethylammonium and Ba^{2+}. In the case of Ba^{2+} addition, note the increase in absorbance accompanying the addition. Control experiments performed without microsomes (or with microsomes that were not loaded with Ca^{2+}) displayed the same absorbance changes. This result indicated that the absorbance increase was not a manifestation of a Ca^{2+} release that might have depleted the stores before IP_3 application but instead represented an artifact due to Ba^{2+} interaction with antipyrylazo III. Similar artifacts were observed with quinine and high concentrations of certain tetraalkylammonium compounds. Such artifacts did not obscure subsequent IP_3-induced Ca^{2+} release or its inhibition.

Dose-response curves for these and a number of other related compounds are shown in Fig. 3 and Table 2. In most cases, there was no indication of a release of Ca^{2+} induced by these compounds at concentrations sufficient to cause marked inhibition of IP_3-induced Ca^{2+} release. In the ≤30-sec exposure to these drugs before IP_3 application, it is highly unlikely that a release slow enough to be masked by Ca^{2+} uptake by the

[1] To test for possible pharmacologic effects of the cation substitutes ($Tris^+$, $choline^+$, or TMA^+) on the release, we performed control experiments in which microsomes that were loaded with calcium in the normal K^+-containing medium were then diluted with K^+, $Tris^+$, $choline^+$, TMA^+, or sucrose medium shortly before IP_3 addition. The results, shown in Table 1C, suggest that there are direct effects due to the presence of the cation replacements. Reducing the K^+ concentration by one half caused a >50% inhibition of the IP_3-induced Ca^{2+} release rate, and the decrease in rate of release was larger when other cations were partially substituted for K^+ than when the presumably inert sucrose was substituted. Nevertheless, it is clear from the sucrose substitution experiment that a reduction in K^+ concentration by itself inhibits the rate of IP_3-induced Ca^{2+} release. The effect is more severe when all the K^+ is replaced (Table 1, A and B). The K^+ effect is unlikely to be due to interference with IP_3 binding to its receptor, binding which has been shown to occur with $choline^+$ and in the absence of K^+ (20).

[2] A less likely possible explanation for the effect of valinomycin on Ca^{2+} release would involve clamping the membrane potential in all IP_3-sensitive vesicles during a release closer to the potassium equilibrium potential. This could increase the electrochemical gradient for Ca^{2+} efflux or impede a voltage-sensitive inactivation process that might normally close the Ca^{2+} channels.

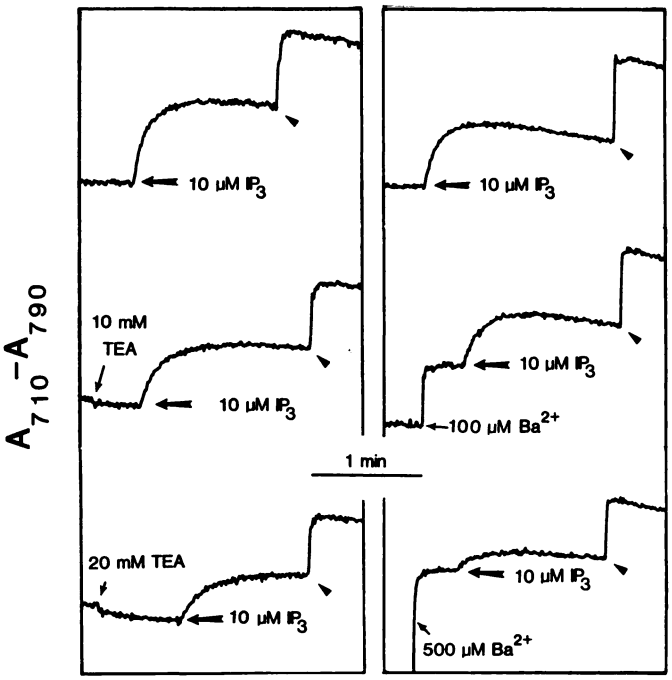

Fig. 2. Inhibition of IP_3-induced Ca^{2+} release by tetraethylammonium and by barium. *Left*, IP_3-induced Ca^{2+} release was measured as shown in Fig. 1 and with 10 or 20 mM tetraethylammonium (*TEA*) added, followed within 1 min by addition of IP_3. *Right*, equivalent experiments were performed with 100 or 500 μM Ba^{2+}, using a different sample. The addition of Ba^{2+} caused an upward deflection in the trace, causing it to rise from its baseline. As discussed in the text, this does not represent a release of Ca^{2+}. *Arrowheads* near the end of each trace indicate 12.5-nmol $CaCl_2$ additions for calibration purposes.

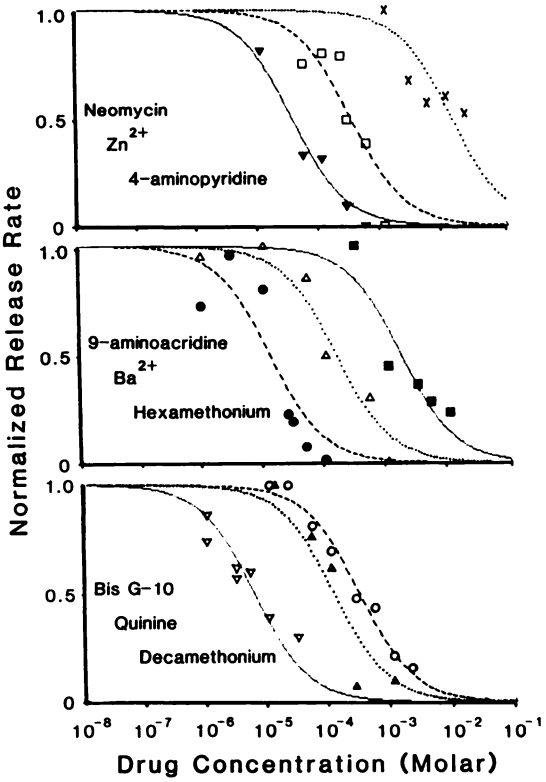

Fig. 3. Dose-response curves for various agents inhibiting IP_3-induced Ca^{2+} release from brain microsomes. All experiments were performed as described for Fig. 2. Release rates were normalized to control Ca^{2+} release rates induced by 10 μM IP_3 in the absence of drug inhibitors. The *curves* were generated by computer fit of the data to a dose-response relationship, using the software program ENZFITTER. From these curves, an apparent K_i was obtained for each inhibitor; these are the K_i values detailed below. *Upper*, release inhibited by neomycin (▼) (K_i = 32 μM), Zn^{2+} (□) (K_i = 290 μM), and 4-aminopyridine (×) (K_i = 11.6 mM). *Middle*, release inhibited by 9-aminoacridine (●) (K_i = 12 μM), Ba^{2+} (△) (K_i = 140 μM), and hexamethonium (■) (K_i = 1.3 mM). *Lower*, release inhibited by bis G-10 (▽) (K_i = 5.6 μM), quinine (▲) (K_i = 110 μM), and decamethonium (○) (K_i = 280 μM).

remaining vesicles could deplete the IP_3-sensitive pool enough to account for the inhibition. With higher concentrations of certain agents (Table 2), there were indications of some Ca^{2+} release. This occurred primarily with longer chain tetraalkylammonium and alkyltrimethylammonium derivatives. Hexadecyltrimethylammonium in particular appeared to be unable to inhibit IP_3-induced Ca^{2+} release at concentrations that did not cause Ca^{2+} release (not shown). In other experiments, only optical artifacts (Table 2) were detected.

These dose-response curves were generally determined within 30 sec of drug addition. Because we were interested in assessing the potential utility of these agents as inhibitors of IP_3-induced Ca^{2+} release *in situ*, for which longer term exposures are generally utilized, we tested the effect of longer term exposures to moderate doses of the agents. We found little additional inhibition of release with 30–40-min drug treatments before IP_3 addition (not shown).

Short and long term exposure to such agents could also affect the refilling of internal Ca^{2+} stores if Ca^{2+} uptake were also inhibited. Therefore, inhibitors of IP_3-induced Ca^{2+} release were also tested for their ability to inhibit ATP-dependent microsomal Ca^{2+} loading. Only 500 μM Zn^{2+} appeared to inhibit Ca^{2+} loading (by 60%) at concentrations that greatly inhibited Ca^{2+} release (not shown).

Because these substances have been reported to block K^+ channels in other systems and were claimed to produce inhibition of IP_3-induced Ca^{2+} release by way of blocking K^+ channels (4), we tested whether valinomycin was able to restore Ca^{2+} release inhibited by any of these agents. We found that release blocked by K^+ channel blockers could not be restored

TABLE 2

Inhibition of IP_3-induced Ca^{2+} release by tetraalkylammonium derivatives

All experiments were performed as in Fig. 2, by adding tetraalkylammonium ions to microsomes previously loaded with 50 nmol of $CaCl_2$/mg of microsomal protein in K^+ medium. Subsequently, 10 μM IP_3 was added within 1 min. Five or six experimental determinations were used to estimate each apparent K_i.

	Apparent K_i for release inhibition	Minimum concentration eliciting release[a]	Minimum concentration eliciting ΔA unrelated to release[b]
	μM	μM	μM
Tetraethylammonium	7140	>20,000	>10,000
Tetrabutylammonium	410	>10,000	>10,000
Tetrapentylammonium	12.5	>1,000	>1,000
Tetrahexylammonium	4.0	>50	>50
Tetraheptylammonium	16.3	20	100
Tetraoctylammonium	24.5	10	100
Tetradecylammonium	66.2	50	>1000

[a] Certain of the longer chain tetraalkylammonium compounds elicited Ca^{2+} release by themselves, some even at concentrations that only partially inhibited IP_3-induced Ca^{2+} release. With these compounds, Ca^{2+} release might have contributed to the inhibition of IP_3-induced Ca^{2+} release. The shorter chain derivatives did not produce any Ca^{2+} release up to the highest concentrations tested.

[b] Upward deflections in the absorbance trace were obtained when these drug concentrations were administered to microsomes that were not loaded with Ca^{2+} previously. With certain compounds, the highest value tested gave no response.

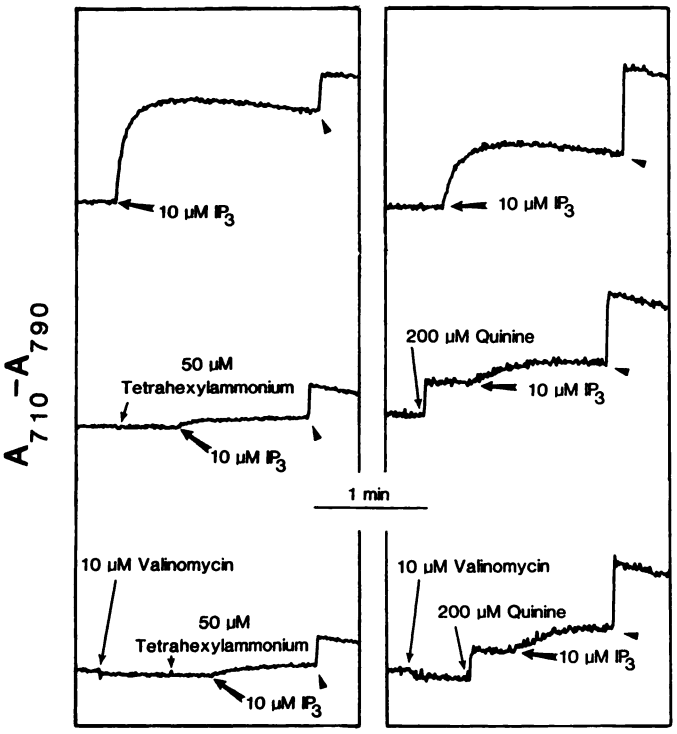

Fig. 4. Increasing the K^+ permeability of the brain microsomes does not reverse the inhibition of IP_3-induced Ca^{2+} release. *Left*, IP_3-induced Ca^{2+} release was elicited as described for Fig. 1 or with 50 μM tetrahexylammonium added ~30 sec before IP_3 or with 10 μM valinomycin added before the tetrahexylammonium and IP_3. *Right*, identical experiments were performed except with a different sample, and 200 μM quinine was substituted for tetrahexylammonium. The upward deflections in the traces accompanying quinine additions represent optical artifacts similar to those induced by Ba^{2+} in Fig. 2. They are also seen with quinine additions in the absence of microsomes and do not represent Ca^{2+} release. *Arrowheads* near the end of each trace indicate 12.5-nmol $CaCl_2$ additions for calibration purposes.

TABLE 3

Effects of valinomycin on the inhibition of IP_3-induced Ca^{2+} release by various drugs

All experiments were carried out as described for Fig. 4 with 10 μM valinomycin, if present, added 10–20 sec before the K^+ channel blockers, with 10 μM IP_3 added last. Numbers in parentheses denote equivalent experiments performed with 10 μg/ml gramicidin D instead of valinomycin.

	Normalized rate of release in absence of valinomycin (gramicidin D)	Normalized rate of release in presence of valinomycin (gramicidin D)
250 μM Quinine	0.13 (0.07)	0.08 (0.07)
10 mM 4-Aminopyridine	0.48	0.59
500 μM Decamethonium	0.44	0.32
20 μM Tetrahexylammonium	0.10 (0.04)	0.06 (0.07)
50 μM Tetrahexylammonium	0.03	0.02
250 μM Ba^{2+}	0.23 (0.24)	0.24 (0.26)
100 μM 9-Aminoacridine	0 (0.02)	0 (0.02)
500 μM Neomycin	0	0
500 μM Zn^{2+}	0.13	0.21
30 μM Bis G-10	0.33	0.30

by either valinomycin or gramicidin D (Fig. 4; Table 3). These two ionophores should have rendered the membranes K^+ permeable, even if native K^+ channels had been blocked by the K^+ channel blockers under examination. These results suggested that the K^+ channel blockers were exerting direct effects on IP_3-induced Ca^{2+} release, rather than by solely inhibiting K^+ counter-ion movement as postulated by Shah and Pant (4).

However, the K^+ channel blocker effects on Ca^{2+} release in the presence of valinomycin might alternatively have been due to simultaneous block of both endogenous microsomal K^+ channels and valinomycin-stimulated K^+ transport. Therefore, we determined the effects of 50 μM tetrahexylammonium, 250 μM quinine, and 250 μM $BaCl_2$ on initial rates of valinomycin-stimulated ^{86}Rb uptake into asolectin liposomes, as described in Materials and Methods. The results shown in Fig. 5 suggest that all three do appreciably affect valinomycin-stimulated K^+ movements but that the inhibition at these concentrations was still far from complete. Valinomycin at even higher concentrations failed to restore the releases inhibited by these agents (Table 3), even though it should have introduced a parallel potassium permeability that was partially insensitive to the blockers. Consequently, at least with quinine, tetrahexylammonium, and Ba^{2+}, effects on endogenous microsomal K^+ channels alone could certainly not have accounted for their inhibition of IP_3-induced Ca^{2+} release.

We have attempted to determine whether K^+ channel blockers inhibit IP_3-induced Ca^{2+} release through interference with IP_3 binding to its receptor or via blockade of the IP_3-sensitive Ca^{2+} channel. Addition of 50 μM IP_3 in the presence of blocking concentrations[3] of several of these K^+ channel blockers was no

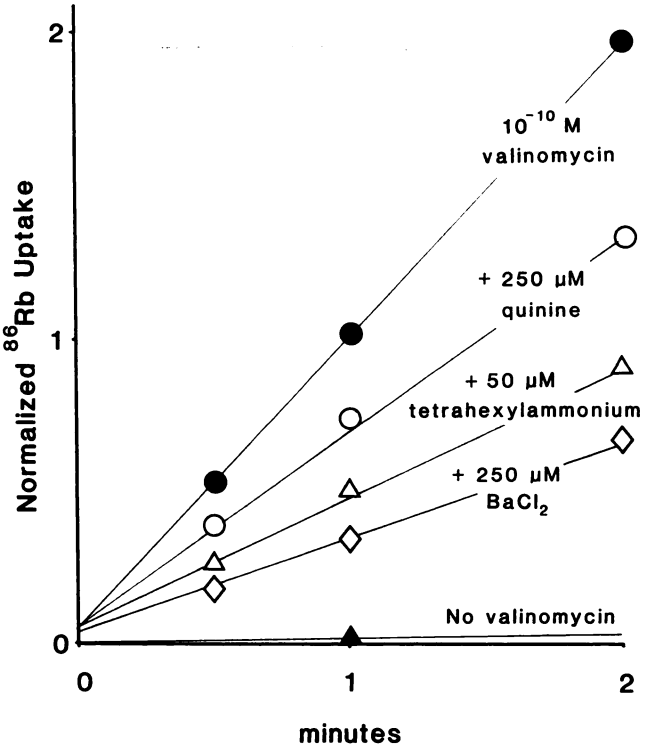

Fig. 5. Effects of selected K^+ channel blockers on valinomycin-stimulated ^{86}Rb transport. Experiments were performed as described in Materials and Methods, utilizing 10^{-10} M valinonycin to stimulate ^{86}Rb uptake into asolectin liposomes against a steep K^+ concentration gradient. Data shown include results obtained in the presence of 10^{-10} M valinomycin alone (●) or with the same concentration of valinomycin but also containing 250 μM quinine (○), 50 μM tetrahexylammonium (△), or 250 μM $BaCl_2$ (◇). Each of these data points represents the average of two determinations. Also shown are results obtained in the absence of valinomycin and any K^+ channel blocker (▲). Data were normalized by dividing the cpm of each time point passed through a secondary Dowex column by the cpm of 5 μl of that sample's reaction mixture (typically 5000–6000 cpm) before passage through a secondary Dowex column.

[3] The concentrations of inhibitors used resulted in 26–75% inhibition of the release rate caused by addition of 10 μM IP_3.

TABLE 4

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

Experiments were performed by incubating 0.5 mg of brain microsomes in 0.5 ml of uptake medium without ATP, dye, or an ATP-regenerating system for 30 min on ice. Subsequently, the samples were sedimented in a Beckman airfuge, the supernatants were removed, and the pellets were solubilized and transferred in the tubes 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. Specific IP_3 binding at pH 8.3 in the absence of phosphate was twice as high as that obtained in the presence of phosphate at pH 7.0 (not shown; see also Ref. 51), with binding in the presence of phosphate at pH 8.3 intermediate in value. Most of the values given represent individual determinations, except for those for which standard deviation values are additionally given.

	Binding		
	Total	Nonspecific	Specific
		pmol/mg	
Control	0.672 ± 0.015	0.349 ± 0.025	0.323 ± 0.020
Control + ethanol (1%)	0.664	0.307	0.357
+100 μM Quinine	0.705	0.320	0.385
+2.5 mM 4-Aminopyridine	0.645	0.306	0.331
+10 mM 4-Aminopyridine	0.616	0.285	0.339
+20 mM 4-Aminopyridine	0.661 ± 0.151	0.341 ± 0.047	0.320 ± 0.197
+500 μM Decamethonium	0.748 ± 0.047	0.331 ± 0.028	0.417 ± 0.075
+100 μM Tetrapentylammonium	0.778	0.423	0.355
+30 μM 9-Aminoacridine	0.686	0.319	0.367
+200 μM Neomycin	1.149 ± 0.367	0.415 ± 0.014	0.734 ± 0.380
+30 μM Bis G-10	0.647	0.299	0.348

more effective at eliciting Ca^{2+} release than was 10 μM IP_3 (not shown). On the other hand, heparin, a competitive inhibitor of IP_3 binding (20), was considerably less effective in inhibiting IP_3-induced Ca^{2+} release in the presence of elevated [IP_3] (21). These results suggest that the K^+ channel blockers do not compete with IP_3 for receptor occupancy.

This hypothesis was tested directly by measuring the effects of these agents on [^{3}H]IP_3 binding. As seen in Table 4, none of the K^+ channel blockers tested here produced a significant inhibition of IP_3 binding. Identical determinations with heparin, pyrophosphate, and phytic acid (21) demonstrate that our binding assay detects interference with IP_3 binding by compounds previously reported to do so (20). Apparent increases in specific binding of IP_3 to the membranes in the presence of decamethonium and neomycin did not attain statistical significance.

We turn finally to the issue of whether the counter-ion K^+ movement that is associated with IP_3-induced Ca^{2+} release involves K^+ permeation through the IP_3-sensitive Ca^{2+} channel or through a separate pathway that is present in IP_3-sensitive vesicles. Because Rb^+ was able to support equally fast IP_3-induced Ca^{2+} release as K^+ (not shown), we examined ^{86}Rb uptake into brain microsomes against an opposing K^+ gradient. We compared stimulation by a low concentration of valinomycin (10^{-8} M) with stimulation by IP_3 (to test for Rb^+ movements through the IP_3-sensitive Ca^{2+} channels). As seen in Fig. 6A, addition of 10 μM IP_3 caused no detectable increase in ^{86}Rb uptake, even at short times when IP_3 hydrolysis in the absence of Mg^{2+} would not be significant. In contrast, even 10^{-8} M valinomycin produced a significant increase in ^{86}Rb uptake. These results suggest little Rb^+ (or K^+) permeability through the IP_3-sensitive channel.

A native microsomal vesicle Rb^+ permeability that is sensitive to tetrahexylammonium and to quinine is shown in Fig. 6B. As indicated by the large stimulation of ^{86}Rb uptake in the steady state by 10^{-6} M valinomycin shown in Fig. 6C, only about 40% of the native vesicles were permeable to Rb^+ under these conditions in the absence of valinomycin.

Discussion

The IP_3-induced Ca^{2+} release from brain microsomes studied under our experimental conditions (EC_{50} ~ 1.4 μM) is similar to that reported by Joseph and Rice (22) for forebrain microsomes (EC_{50} ~ 0.65 μM) but quite a bit less sensitive than cerebellum microsomes (EC_{50} ~ 0.18 μM) or the brain micromes of Shah *et al.* (3) (EC_{50} ~ 50 nM). Our determinations relied on measurements of rates of Ca^{2+} release rather than of amounts of Ca^{2+} released (3, 22).[4]

We have chosen to emphasize drug effects on the rate of Ca^{2+} release rather than on the amount of Ca^{2+} released. If there are several IP_3-sensitive channels per releasing vesicle, drugs could well have minimal effects on the amount of Ca^{2+} released even if most channels were blocked. Additionally, any Ca^{2+}-dependent inactivation process (20, 26), if time dependent, could more seriously attenuate the extent of a slower Ca^{2+} release than a more rapid one. Consequently, rate measurements should be more sensitive to drug effects.

Our results confirm reports (3, 5) that IP_3-induced Ca^{2+} release requires the presence of a permeable counter-ion such as K^+. Substitution of K^+ with $Tris^+$, $choline^+$, or TMA^+ could not support rapid IP_3-induced Ca^{2+} release, even though Ca^{2+} could be taken up slowly by the vesicles in the presence of these cations, presumably by co-transport of phosphate. Although some of the effects of these cation substitutes are clearly attributable to pharmacologic effects on the IP_3-sensitive cation channel (or on the postulated counter-ion-transporting K^+ channel), the >90% reduction of release rate caused by complete replacement of K^+ by all three cation substitutes strongly suggests that these ions are much less effective as counter-ions than is K^+ (and that the phosphate and chloride present in our media are less likely to be cotransported together with Ca^{2+}).

Most of the vesicles that are IP_3-sensitive must be quite permeable to K^+. Addition of valinomycin caused only a small stimulation of the rate and extent of IP_3-induced Ca^{2+} release (Table 1), suggesting that most but not all (~70%) IP_3-sensitive

[4] Other factors contributing to these differences might involve variations in the extent of microsomal phosphorylation (23) or in the rate of IP_3 hydrolysis by endogenous phosphatases (cf. Ref. 24) or our use of phosphate, which was shown previously to support IP_3-induced Ca^{2+} release (25) but which might decrease IP_3 binding (cf., legend to Table 4).

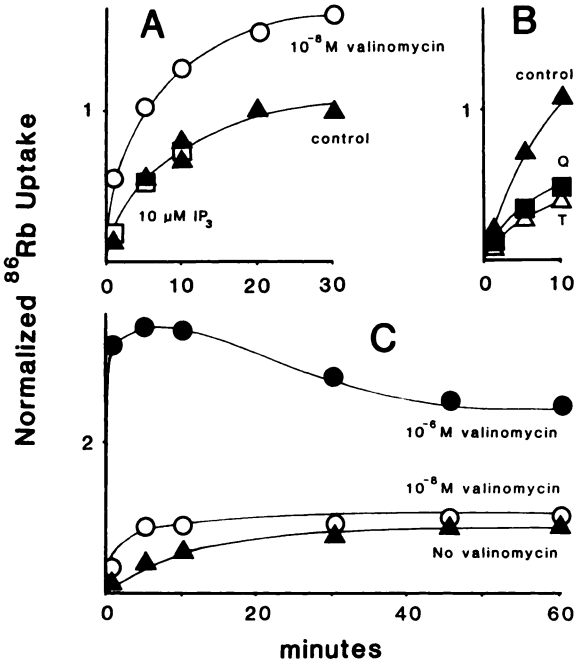

Fig. 6. Effect of valinomycin, IP_3, and selected K channel blockers on ^{86}Rb uptake into brain microsomes. Microsomes were equilibrated overnight in K^+-containing medium, as described in Materials and Methods, and were then assayed for ^{86}Rb uptake as described for liposomes in Fig. 5. A, ▲, Control; ○, 10^{-8} M valinomycin; □, 10 μM IP_3. B, ▲, Control; ■, 250 μM quinine (*Q*); △, 50 μM tetrahexylammonium (*T*). C, ▲, Control (no valinomycin); ○, 10^{-8} M valinomycin; ●, 10^{-6} M valinomycin. Data were normalized as indicated for Fig. 5.

vesicles already contained K^+-permeable channels.[5] Additional evidence for a separate microsomal K^+ permeability pathway distinct from the IP_3-sensitive Ca^{2+} channel is the demonstration that ^{86}Rb uptake is not enhanced by IP_3.[6] Thus, the IP_3-sensitive Ca^{2+} channel may not be as permeable to K^+ as the SR Ca^{2+} release channel from muscle (27, 28). Other reasons for suspecting that the two channels are different are summarized in an accompanying communication (21).

Several blockers of surface membrane K^+ channels were quite inhibitory to IP_3-induced Ca^{2+} release. These included quinine, 4-aminopyridine, Ba^{2+}, and some tetraalkylammonium and alkyltrimethylammonium derivatives, including decamethonium. Additionally, Cs^+, a blocker of several surface membrane K^+ channels (11) and the muscle SR K^+ channel (29), inhibited the rate of IP_3-induced Ca^{2+} release by 50% at 10 mM and by 67% at 50 mM (not shown). Phencyclidine, an inhibitor of certain surface membrane neuronal K^+ channels (30), had less effect, exhibiting an apparent K_i of 250 μM (five experiments, not shown). All inhibitors of SR K^+ channels tested [tetraethylammonium (30, 31), decamethonium (32), Zn^{2+} (33), neomycin (34), bis G-10 (35), and Cs^+ (29)] were effective, many at even lower concentrations than required to inhibit the SR K^+ channel. Neomycin is also known to inhibit phosphatidylinositol-4,5-bisphosphate-induced Ca^{2+} release (36) and Ca^{2+}-induced Ca^{2+} release (37) in other systems and to inhibit formation of IP_3 (38).

Tetraalkylammonium ions have previously been demonstrated to block Ca^{2+} channels in presynaptic nerve terminals (10) and have an inhibitory effect on IP_3-induced Ca^{2+} release, as reported here. Alkyltrimethylammonium ions have similar effects. Ca^{2+} release observed in response to higher concentrations of certain of these agents could be related to their ability to function as cationic detergents. The lack of additional effect of longer drug incubations with vesicles argues against a luminal site of action such as that displayed by many organic cations on the muscle SR K^+ channel (31).

The inability of valinomycin or gramicidin to reverse the effects of any inhibitors of IP_3-induced Ca^{2+} release suggests that K^+ channel blockers do not exert their inhibitory effects on IP_3-induced Ca^{2+} release solely or even primarily by inhibiting K^+ counter-ion movements. These ionophores should have restored any Ca^{2+} release indirectly inhibited by blocking of counter-ion movements, with restoration due to a parallel counter-ion pathway inserted into the membrane. We employed two different ionophores in case one of them incidentally also happened to be completely inhibited by the K^+ channel blockers under study here. Additionally, we directly demonstrated a considerable stimulation of ^{86}Rb transport by valinomycin even in the presence of 250 μM $BaCl_2$, 250 μM quinine, or 50 μM tetrahexylammonium.

Care was taken to assess whether K^+ channel blockers caused Ca^{2+} release. This could have led to inhibition of a subsequent IP_3-induced Ca^{2+} release, either by depleting the IP_3-sensitive store or by elevating the extravesicular free Ca^{2+} to levels at which binding to calmedin causes inhibition of IP_3 binding (26). Such Ca^{2+}-dependent inhibition of IP_3 binding has been observed to result in decreased IP_3-induced Ca^{2+} release (39, 40).

We have performed experiments to distinguish whether these K^+ channel blockers exert their effects by directly inhibiting IP_3 binding or, by default, by affecting the IP_3-sensitive Ca^{2+} channels. Previously, Ba^{2+} has been shown not to inhibit [^{3}H]IP_3 binding (20). Although our determinations suggest that none of the K^+ channel blockers directly interfere with IP_3 binding, caution must be exercised in this interpretation, due to the differences in assay conditions between determinations of IP_3 binding (absence of ATP[7] and phosphocreatine, low temperature) and IP_3-induced Ca^{2+} release. We have, however, performed IP_3 binding under similar ionic conditions and pH as encountered in our Ca^{2+} release experiments, even though [^{3}H]IP_3 binding is not optimal under these conditions (20) (see legend to Table 4). Our failure to detect inhibition of [^{3}H]IP_3 binding by K^+ channel blockers was not a result of the assay conditions, because heparin, a reported competitive antagonist at the IP_3 receptor (20), did inhibit binding under the same conditions.

[5] This stands in contrast to the microsomal vesicle population as a whole, for which our ^{86}Rb uptake results suggest that only about 40% of the vesicles are permeable to K^+ in the absence of valinomycin (Fig. 6C). Possible contributory factors for this difference include differences in assay conditions (absence of intravesicular Ca, extravesicular K, and IP_3), as well as potential differences in membrane potential in the ^{86}Rb uptake experiments. Nevertheless, it is also possible that prportionately more IP_3-sensitive vesicles are K^+ permeable than IP_3-insensitive ones are. Rb^+ appears to be as permeable to IP_3-sensitive vesicles as K^+ is.

[6] An IP_3-induced increase in ^{86}Rb uptake would have been expected had the IP_3-sensitive channel been the only K^+ permeability mechanism in IP_3-sensitive stores. Our assay conditions for ^{86}Rb uptake [and those of Muallem *et al.* (5)] would have resulted in a negative inside membrane potential, had the IP_3-sensitive channels been K^+ permeable, and this could conceivably shut down the channel. The increased ^{86}Rb uptake reported by Shah and Pant (4) in response to addition of IP_3 was only observed when Ca^{2+} was available to be released; their failure to stimulate ^{86}Rb uptake with IP_3 alone was determined under conditions more likely to result in zero membrane potential.

[7] We have used the same Sigma ATP in our release studies that was reported to inhibit [^{32}P]IP_3 binding (42). ATP has also been reported to inhibit [^{3}H]IP_3 binding to cerebellar membranes (43).

IP_3 has been reported to activate Ca^{2+}-permeable channels in plasma membrane (43, 44), presumably by binding to surface membrane receptors, but the IP_3-induced Ca^{2+} release studied here is unlikely to involve plasma membrane vesicles, because the release is not diminished by saponin treatment (45). Although IP_3 receptors appear not to reside in brain plasma membrane or mitochondria (46), IP_3 has been shown to bind to discrete inositol 1,3,4,5-tetrakisphosphate receptors in the brain with moderately high affinity (47). Therefore, the possibility exists that not all IP_3 binding will be intimately associated with the IP_3-induced Ca^{2+} release process. However, our inability to reverse blockade with 50 μM IP_3 tentatively suggests a lack of competitive antagonism at the relevant IP_3 receptor.

These K^+ channel blockers are frequently applied to the outside of cells. Determination of whether any of their effects are due to inhibition of IP_3-induced Ca^{2+} release from internal stores remains unclear. In most cases, the permeability of these compounds across cell membranes is only poorly known. An exception is quinine, which is known to elicit Ca^{2+} release from SR even when it is applied outside muscle fibers (48) so it may be presumed to be permeant. Conversely, Ba^{2+} and Zn^{2+} may be presumed to be relatively impermeant in resting cells, although both are known to carry current through voltage-gated Ca^{2+} channels in various cell types (49) and, thus, could build up to low concentrations in cells that became depolarized. Tetraethylammonium is known to be largely impermeant, but less is known about its more lipophilic longer side chain analogs or the alkyltrimethylammonium compounds.[8]

Most of these agents have been used in both electrophysiologic (11) and ion transport (4) experiments as specific blockers of membrane K^+ channels. Undoubtedly, the effectiveness of these agents on surface membrane K^+ channels and IP_3-sensitive Ca^{2+} channels of internal Ca^{2+} stores reflects a considerable degree of homology between the two channel types. The results reported here clearly indicate a lack of specificity in the action of these substances. It remains uncertain whether effects on IP_3-sensitive Ca^{2+} stores ("calciosomes") (50) could have contributed to effects of quinidine that are ascribed to K^+ channel blockade in intact neurons (e.g., Ref. 7).

Finally, our results demonstrating inhibition of valinomycin-stimulated ^{86}Rb uptake by several of these K^+ channel blockers further illustrate their lack of specificity. Therefore, caution must be exercised in the interpretation of experiments involving both valinomycin and K^+ channel blockers.

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[8] Dr. Alan Finkelstein (Albert Einstein College of Medicine; personal communication) suggests that 20 μM tetrahexylammonium exhibits a conductance of 100 pS across a 1-mm^2 diphytanoyl phosphatidylcholine planar lipid bilayer, whereas 20 μM tetraheptylammonium exhibits a conductance of 20 nS. Calculations of the corresponding fluxes into 20-μm diameter cells suggest that tetrahexylammonium would equilibrate within minutes, and tetraheptylammonium even faster. Presumably, tetrapentylammonium and shorter chain analogs would be relatively impermeable.

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