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Effect of Calmidazolium Analogs on Calcium Influx in HL-60 Cells

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ABSTRACT. The structure–activity relationships of calmidazolium analogs with respect to intracellular calcium levels were investigated in HL-60 cells. Quaternized derivatives of miconazole and clotrimazole, known inhibitors of store-operated calcium (SOC) channels, were synthesized. The quaternary *N*-methyl derivatives of miconazole (**3**) and clotrimazole (**6**) had no effect on intracellular calcium levels, alone or after elevation of calcium induced by ATP. Calmidazolium alone induced a large increase in intracellular calcium levels in HL-60 cells (EC_{50} 3 μM). Similar effects were observed for miconazole derivatives **1** (EC_{50} 15 μM) and **2** (EC_{50} 10 μM), wherein the diphenylmethyl group in calmidazolium was replaced by a 3,5-difluorobenzyl or cyclohexylmethyl group, respectively. The analogous clotrimazole derivatives **4** and **5** had no effect on intracellular calcium levels. The elevation of calcium levels by calmidazolium, **1**, and **2** appears to be comprised of a calcium release component from inositol trisphosphate (IP_3)-sensitive stores followed by a large calcium influx component. Calcium influx was greater than that normally observed due to depletion of IP_3-sensitive calcium stores and activation of SOC channels. In addition, only a small component of the calmidazolium-elicited influx was inhibited by the SOC channel blocker miconazole. Thus, certain quaternized imidazoles substituted with large residues at both nitrogens of the imidazole ring caused both release and influx of calcium, the latter in part through SOC channels but mainly through an undefined cationic channel. Quaternized imidazoles, unlike the parent nonquaternary imidazole miconazole, did not block SOC channels. Inhibitory effects on calmodulin-activated phosphodiesterase did not correlate with effects on calcium release and influx. BIOCHEM PHARMACOL **60**;3:317–324, 2000. © 2000 Elsevier Science Inc.

KEY WORDS. calcium influx and release; calmidazolium; miconazole; phosphodiesterase; capacitative calcium influx; calmodulin

Calmidazolium, a quaternized imidazole, is well known as a potent antagonist of the calcium-binding protein calmodulin [1, 2]. As a result, calmidazolium affects a variety of calcium-dependent cell pathways [3–5], including inhibition of calmodulin-dependent phosphodiesterase [6, 7]. In recent years, calmidazolium has been reported to cause marked increases in intracellular calcium in eukaryotic cells, namely human leukemic HL-60 cells [8] and *Dictyostelium discoideum* amoebae [9]. The mechanism for the elevation of intracellular calcium levels elicited by calmidazolium is not known, but appears in amoebae to involve release of calcium from intracellular calcium stores in conjunction with a large influx of calcium [9]. The concentrations of calmidazolium required to elicit marked increases in intracellular calcium are far in excess of those necessary for complete inhibition of calmodulin-dependent processes.

Calmidazolium is a quaternized derivative of miconazole, itself a recognized inhibitor of SOC† channels [10, 11]. Clotrimazole is another imidazole inhibitor of SOC channels [10, 11]. Such non-quaternized imidazoles have also been reported to cause increases in intracellular calcium in eukaryotic cells [11–13]. A series of *N*-substituted quaternized derivatives of miconazole and clotrimazole were synthesized and tested in HL-60 cells using the fluorescent probe fura-2 to determine the effects of these quaternary compounds on intracellular calcium levels compared with calmidazolium, miconazole, and clotrimazole. In addition, effects on the generation of inositol phosphates and inhibition of calmodulin-dependent phosphodiesterase were determined.

MATERIALS AND METHODS

Materials

Miconazole, clotrimazole, and dibutyryl cyclic AMP were obtained from the Sigma Chemical Co.; SKF 96365 (1-{β-[3-(4-methoxyphenyl)propoxy]-4-methoxyphenethyl}-1*H*-imidazole hydrochloride) was obtained from Biomol® Research Labs Inc. ATP was purchased from Fluka. Roswell Park Memorial Institute (RPMI) 1640 medium, fetal bovine serum, L-glutamine (200 mM), trypsin–EDTA, and penicillin/streptomycin (10,000 U/mL of penicillin G so-

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† *Abbreviations*: IP_3, inositol trisphosphate; and SOC, store-operated calcium.

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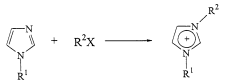
TABLE 1. Synthesis and characterization of quaternary imidazoles (for structures see Fig. 1)



Compound	X^-	Yield (%)	Mol. ion (M^+)	Melting Point (°)
1	Br^-	86	543	150–153
2	Br^-	45	513	119–122
3	I^-	69	431	166–169
4	Br^-	90	359	170–173
5	Br^-	79	471	175–178
6	I^-	49	442	125–130
7	Br	89	285	75–80

dium and 10,000 μg/mL of streptomycin sulfate) were obtained from Gibco BRL, Life Technologies. Bio-Rad AG-1-X8 Resin (100–200 mesh, formate form) and Affigel 601 were purchased from Bio-Rad Laboratories. [^{3}H]Inositol and [^{3}H]cyclic AMP were obtained from NEN Life Science Products. Rat brains were purchased from Pel-Freez Biologicals. 2′-Deoxyadenosine-3′,5′-cyclic monophosphate (2′-deoxy-cyclic AMP) was obtained from Biolog Life Science Institute. All other chemical reagents were purchased from the Aldrich Chemical Co.

Preparation of Quaternary Imidazoles

To a solution of miconazole, clotrimazole, or *N*-benzylimidazole in ethyl acetate was added 5 equivalents of the appropriate organic halide (RX). The reaction mixture was heated under reflux up to 48 hr, with the exception of the MeI reaction, which was stirred at room temperature. The progress of the reaction was followed by TLC (silica gel/ethyl acetate). Upon cooling, a white crystalline solid formed, which was filtered and washed with ice-cold ethyl acetate. Where necessary, recrystallization was achieved using ethyl acetate. In instances where crystals did not form upon cooling of the reaction mixture, the reaction mixture was concentrated *in vacuo*, and the resultant oil was crystallized from ethyl acetate. The results are presented in Table 1. Structures are shown in Fig. 1. The ^{1}H NMR spectrum, ^{13}C NMR spectrum, microanalysis (C, H, N, X), and the EI-mass spectrum were consistent for each compound. NMR spectra were obtained using a Varian Gemini 300 MHz NMR Spectrometer referenced to the solvent signal d_6-DMSO (2.45 ppm). Mass spectroscopy (EI direct probe) was carried out by Noel Whittaker at the National Institutes of Health. Elemental analysis was carried out by Galbraith Laboratories Inc.

Cell Preparation

The human leukemic HL-60 cells were from the American Type Culture Collection and were grown in suspension in RPMI 1640 medium supplemented with 10% fetal bovine serum, 100 U/mL of penicillin, 100 μM streptomycin, and 2 mM L-glutamine. The cells were differentiated in supplemented RPMI 1640 medium containing 500 μM dibutyryl cyclic AMP for 48 hr prior to each experiment. For inositol phosphate assays, [^{3}H]inositol (0.2 μCi/mL) was added with the 500 μM dibutyryl cyclic AMP and incubated for 48 hr prior to each experiment.

Intracellular Calcium Measurements

Aliquots (10–20 mL) of differentiated HL-60 cells were centrifuged at low speed for 10 min at 25°, and the supernatant was removed. The packed cells were resuspended in 10–20 mL of buffered medium containing 1 part supplemented RPMI 1640 medium and 1 part Krebs–Ringer–HEPES buffer (KRH: 125 mM NaCl, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4$, 2 mM $CaCl_2$, 6 mM glucose, 25 mM HEPES, pH 7.4). To the cell suspension was added 10 μL fura-2AM (10 μg in 10 μL dimethyl sulfoxide). After 40 min in the dark at 25°, the cell suspension was centrifuged as described above, and the supernatant was

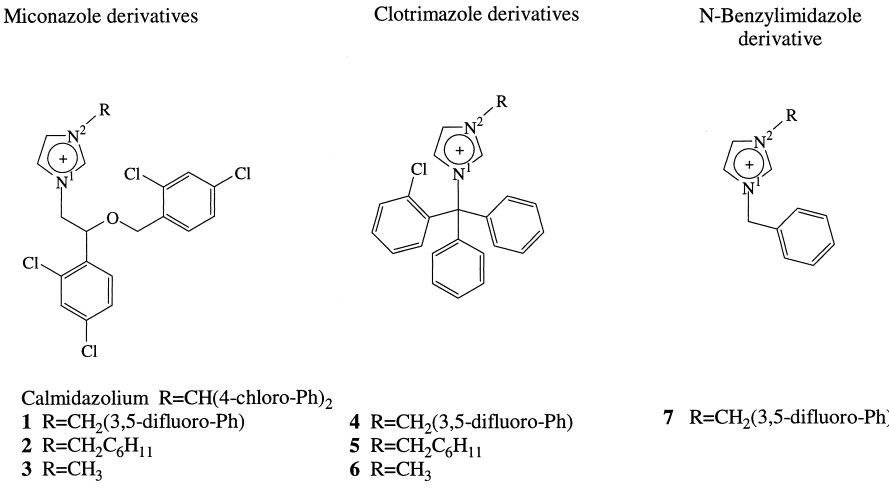


FIG. 1. Structures of imidazole derivatives.

removed. The packed cells were resuspended in KRH buffer.

A 2-mL aliquot of the fura-2-loaded cells was transferred into a cuvette and stirred with a magnetic stirrer bar throughout the entire experiment. Agents were added to the cell suspensions in volumes of 1–20 μL. Intracellular calcium levels were monitored by fluorescent spectroscopy using a PTI Fluorescence Spectrometer (Photon Technology International) set at 380 and 340 nm for excitation and at 500 nm for emission.

Inositol Phosphate Assay

To 1-mL aliquots of differentiated [^{3}H]inositol-labeled HL-60 cells (10^6 cells/mL) was added LiCl (final concentration 10 mM). The cells were incubated at room temperature for 20 min, and then the agents were added. After an incubation of 20 min, the cells were centrifuged and the supernatant was removed. The cells were lysed with 750 μL of 20 mM ice-cold formic acid. The suspensions were neutralized with 60 mM NH_4OH and centrifuged, and the supernatants were transferred onto anion exchange columns (Bio-Rad AG-1-X8 Resin). The columns were washed with 10 mL water, followed by 15 mL formate/borate solution (5 mM sodium borate, 60 mM sodium formate). The [^{3}H]inositol phosphates were eluted with 5 mL ammonium formate buffer (0.1 M formic acid, 0.2 M ammonium formate). Hydrofluor (2:1) was added to the eluent, and the radioactive counts were measured using a Beckman LS 6500 Multipurpose Scintillation Counter.

Phosphodiesterase Assay

Inhibition of soluble Ca^{2+}-dependent phosphodiesterase from rat cerebral cortex [14, 15] was determined as follows: Rat cerebral cortices were washed and homogenized in 2 vol. of ice-cold Tris–HCl buffer A (20 mM Tris–HCl, 1 mM $MgCl_2$, 3 mM 2-mercaptoethanol, 0.1 mM EGTA, pH 7.5). An aliquot (1 mL) of the homogenate was centrifuged, and the supernatant was separated. The supernatant was centrifuged, separated from the pellet, and diluted 10-fold with Tris–HCl buffer B (50 mM Tris–HCl, 5 mM $MgCl_2$, 50 μM $CaCl_2$, pH 7.5). To eliminate any contribution from Ca^{2+}-independent phosphodiesterases, 150 μM 2′-deoxycyclic AMP, a specific inhibitor of such phosphodiesterases, was present in the reaction mixture. Appropriate concentrations of the compounds were added to 0.1 mL of the standard reaction mixture 1 min prior to the addition of 1 μM [^{3}H]cyclic AMP (0.03 μCi). After incubation at 37° for 5 min, the assay was terminated by heating the reaction mixture at 95° for 2 min. After addition of 0.4 mL HEPES–NaCl buffer (0.1 M HEPES, 0.1 M NaCl, pH 8.5), the contents of the tube were loaded onto polyacrylamide–boronate gel columns (Affigel 601, 0.7 × 1.5 mL), which had been washed with 10 mL HEPES–NaCl. The [^{3}H]cyclic AMP was washed from the column with 5 mL HEPES–NaCl buffer. The [^{3}H]5′-AMP was eluted with 7 mL sodium acetate buffer (0.05 M NaOAc, pH 4.8). Hydrofluor (10 mL) was added to the eluent, and the radioactive counts were measured using a Beckman LS 6500 Multipurpose Scintillation Counter. The columns were regenerated with 10 mL of 0.1 N acetic acid and then washed with HEPES–NaCl buffer (2 × 5 mL) prior to each assay.

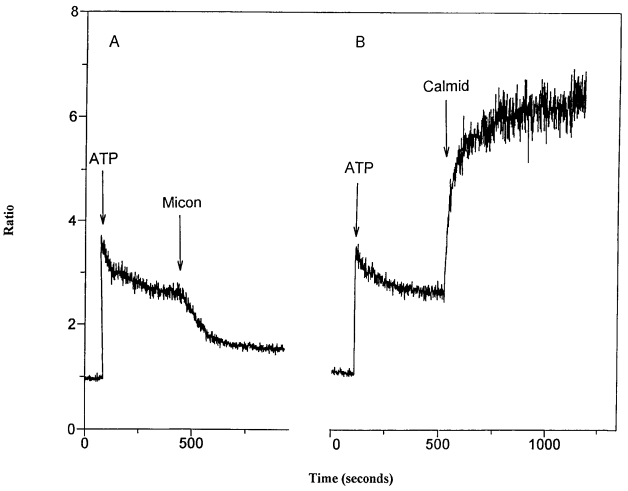


FIG. 2. Effect of miconazole or calmidazolium on intracellular calcium levels after ATP. Intracellular calcium levels were elevated with 10 μM ATP prior to the addition of (A) 10 μM miconazole or (B) 10 μM calmidazolium as indicated by the arrows. A typical experiment is shown. Cells had been loaded with fura-2 and were assayed as described in Materials and Methods. Basal values for intracellular levels of calcium were typically about 100 nM.

RESULTS

Intracellular Calcium

Miconazole (10 μM) caused a decrease in calcium levels after elevation of calcium levels by ATP (Fig. 2A). In contrast, calmidazolium (10 μM) caused a large additional increase in intracellular calcium levels after elevation of intracellular calcium levels by ATP (Fig. 2B). Calmidazolium (10 μM) also caused a large additional increase in intracellular levels of calcium after elevation of intracellular calcium by thapsigargin (data not shown). A similar large additional increase in calcium levels was observed for 30 μM *N*-3,5-difluorobenzylmiconazole (**1**) and 30 μM *N*-cyclohexylmethylmiconazole (**2**) after elevation of intracellular calcium levels by ATP (data not shown). The *N*-methylmiconazole **3**, the clotrimazole derivatives **4–6**, *N*-benzylimidazole, and the *N*-benzylimidazole derivative **7** had no effect on intracellular calcium levels after ATP (data not shown). Clotrimazole at 20 μM caused a decrease in calcium levels after elevation of intracellular levels by ATP ([13] and data not shown).

In the presence of external calcium, calmidazolium alone caused a concentration-dependent elevation in intracellular calcium levels (Fig. 3). The EC_{50} was calculated to be 3 μM, and a maximal response was observed at 10 μM (Table 2). The miconazole derivatives **1** and **2** caused a concen-

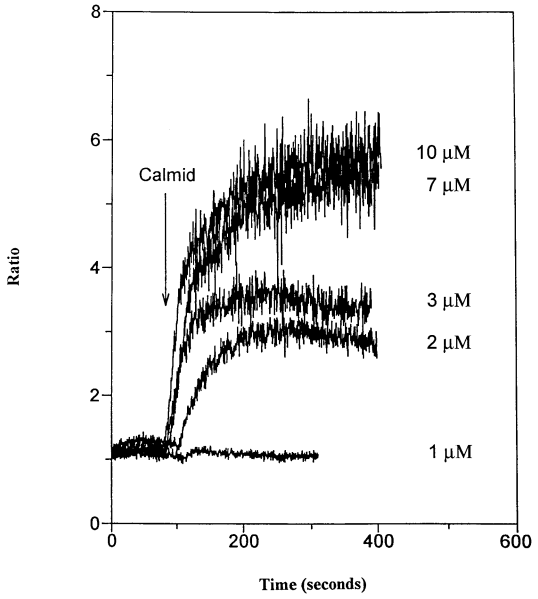


FIG. 3. Effect of calmidazolium on intracellular calcium levels. Calmidazolium was added as indicated by the arrow to cells at 1, 2, 3, 7, and 10 μM. Similar effects were observed for miconazole derivatives 1 and 2 except that both 1 and 2 were about 3- to 5-fold less potent (see Table 2). Cells had been loaded with fura-2 and were assayed as described in Materials and Methods.

tration-dependent elevation of intracellular calcium with EC_{50} values of 15 and 10 μM, respectively (Table 2). Miconazole derivatives **1** and **2** showed a maximal elevation of intracellular calcium at 30 μM. Miconazole alone caused a concentration-dependent transient elevation of intracellular calcium levels (Fig. 4). The EC_{50} was calculated to be 3 μM, and a maximal response was observed at 10 μM. When added alone, the *N*-methylmiconazole **3**, the clotrimazole derivatives **4–6**, *N*-benzylimidazole, and the 1-benzyl-3-(3,5-difluorobenzyl)imidazole **7** had no effect on intracellular calcium levels.

In the absence of external calcium, calmidazolium alone

TABLE 2. Elevation of intracellular calcium levels in HL-60 cells by calmidazolium analogs*

Compound	Ca^{2+} elevation	
	EC_{50} (μM)	EC_{max} (μM)
Calmidazolium	3	10
Miconazole	3	10
1	15	30
2	10	30
3	No effect	
4	No effect	
5	No effect	
6	No effect	
7	No effect	
Benzylimidazole	No effect	

*Compounds at concentrations up to 100 μM were incubated with HL-60 cells, and calcium levels were monitored with fura-2 as described in Materials and Methods. The EC_{50} and EC_{max} values were from at least 3 experiments. Compounds **3–7** and benzylimidazole had no effect at 100 μM.

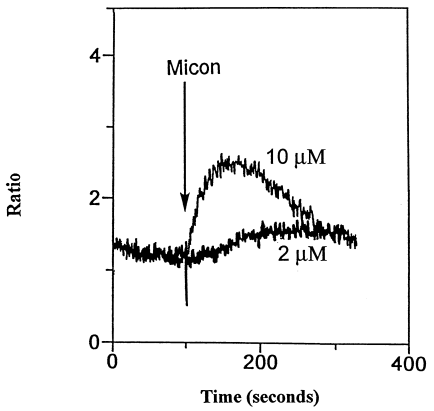


FIG. 4. Effect of miconazole on elevation of intracellular calcium levels. Miconazole was added as indicated by the arrow to cells at 2 and 10 μM (lower and upper trace, respectively). The elevation of intracellular calcium levels was similar when EGTA was present (data not shown). Cells had been loaded with fura-2 and were assayed as described in Materials and Methods.

caused a concentration-dependent elevation of intracellular calcium (Fig. 5A). Subsequent reintroduction of external calcium resulted in a sustained elevation of intracellular calcium levels. Similar effects were observed for miconazole derivatives **1** and **2** (Fig. 5, B and C). Both clotrimazole and miconazole alone caused an elevation of intracellular calcium levels ([8, 14] and data not shown).

A variety of different calcium antagonists were tested with respect to their ability to block the elevation of intracellular calcium levels elicited by 10 μM calmidazolium, 30 μM **1**, or 30 μM **2**. These antagonists were nifedipine, nitrendipine, diltiazem, methoxyverapamil (D600), proadifen, trifluoperazine, chlorpromazine, loperamide, and SKF 96365. None of the compounds had any effect at concentrations up to 100 μM (data not shown). Miconazole inhibited the elevation of intracellular calcium induced by a maximal 10 μM concentration of calmidazolium with an EC_{50} of 25 μM and inhibited the elevation of intracellular calcium induced by a submaximal 10 μM concentration of **2** with an EC_{50} of 7 μM (Fig. 6). Thus, the potency of miconazole as an inhibitor probably was similar for both calmidazolium and **2**. However, miconazole did not cause complete inhibition of elevation of intracellular calcium levels by either calmidazolium or **2**. Remarkably, miconazole at concentrations up to 100 μM did not inhibit the elevation of intracellular calcium by **1** (data not shown). Prior addition of 5 mM $NiCl_2$ had no effect on the calmidazolium-elicited elevation of intracellular calcium, but did block influx through SOC channels that are activated after 10 μM ATP (data not shown).

Inositol Phosphates

Calmidazolium, miconazole, **1**, and **2** all caused a concentration-dependent increase in [^{3}H]inositol phosphates in HL-60 cells (Table 3). Calmidazolium, miconazole, and the miconazole derivative **1** had similar effects, with EC_{50} values

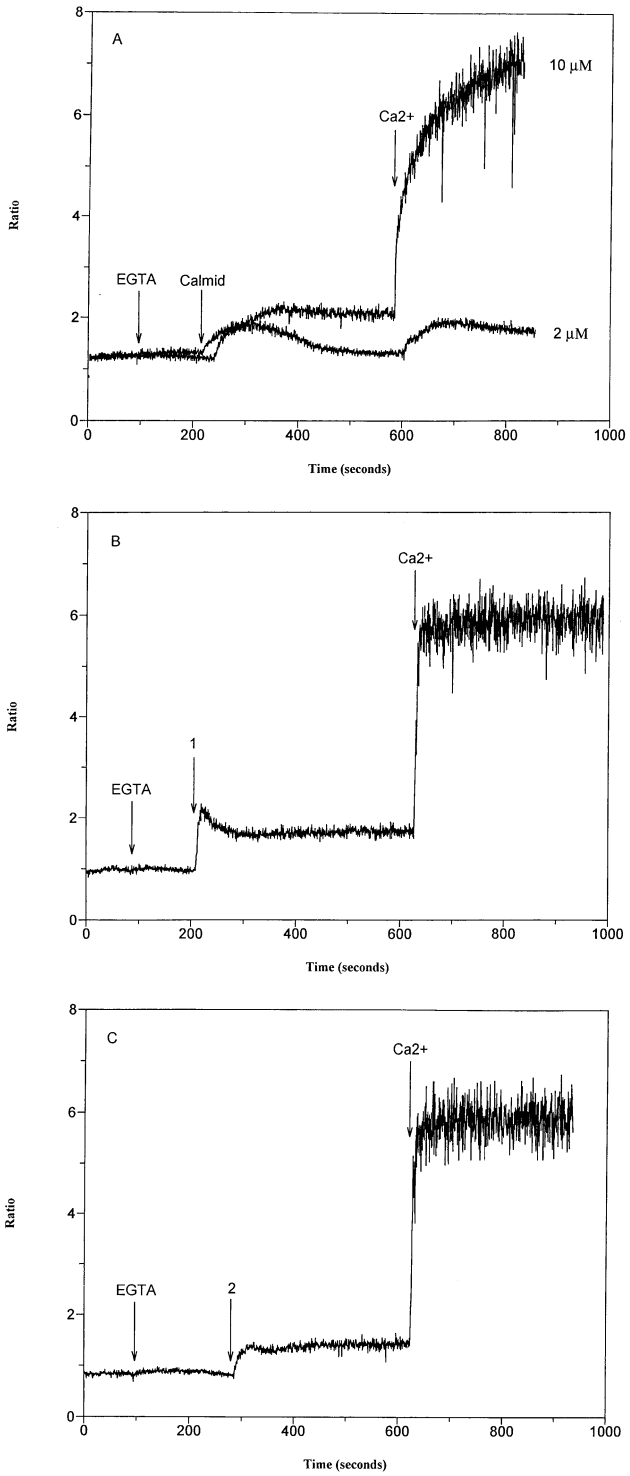


FIG. 5. Effect of calmidazolium and miconazole derivatives 1 and 2 on intracellular calcium release and calcium influx. Cells were treated with 2 mM EGTA for 2 min prior to the addition of (A) 2 or 10 μM calmidazolium followed by reintroduction of 2 mM Ca^{2+}, (B) 30 μM 1 followed by 2 mM Ca^{2+}, or (C) 30 μM 2 followed by 2 mM Ca^{2+} as indicated by the arrows. Cells had been loaded with fura-2 and were assayed as described in Materials and Methods.

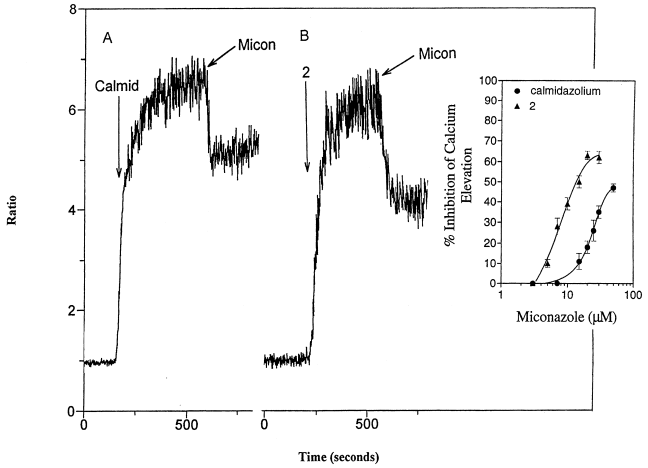


FIG. 6. Effect of miconazole on calmidazolium-induced and miconazole derivative 2-induced elevation of intracellular calcium levels. (A) Addition of 10 μM calmidazolium, followed by 30 μM miconazole. (B) Addition of 30 μM 2, followed by 10 μM miconazole as indicated by the arrow. Inset: Concentration-dependent inhibition curves for miconazole of calmidazolium-induced and miconazole derivative 2-induced elevation of intracellular calcium levels. Calmidazolium and miconazole derivative 2 were present at 10 μM, the EC_{max} for the former and the EC_{50} for the latter, accounting for the apparent difference in potency for miconazole. Elevation of calcium by miconazole derivative 1 was not inhibited by miconazole (data not shown). Cells had been loaded with fura-2 and were assayed as described in Materials and Methods.

of 10, 5, and 7 μM, respectively. The miconazole derivative **2** was 10-fold less potent, with an EC_{50} of 100 μM. The clotrimazole derivatives **4–6**, *N*-benzylimidazole, and the *N*-benzylimidazole derivative **7** had no effect on the production of [^{3}H]inositol phosphates at concentrations up to 100 μM. Clotrimazole has been reported to stimulate production of inositol phosphates in HL-60 cells [8].

TABLE 3. Effect of calmidazolium analogs on the generation of inositol phosphates in HL-60 cells

	[^{3}H]Inositol phosphate generation	
Compound	**EC_{50} (μM)**	**E_{max} (% increase)**
Calmidazolium	10	60
1	7	82
2	100	65
3	No effect	No effect
4	No effect	No effect
5	No effect	No effect
6	No effect	No effect
7	No effect	No effect
Miconazole	5	75

Compounds were incubated with HL-60 cells, and [^{3}H]inositol phosphate generation was determined from the breakdown of [^{3}H]inositol-labeled phosphoinositides as described in Materials and Methods. The EC_{50} values were from at least 3 experiments. The E_{max} is presented as a percentage of inositol phosphate generation by 10 μM ATP. Compounds **3–7** had no effect at 100 μM.

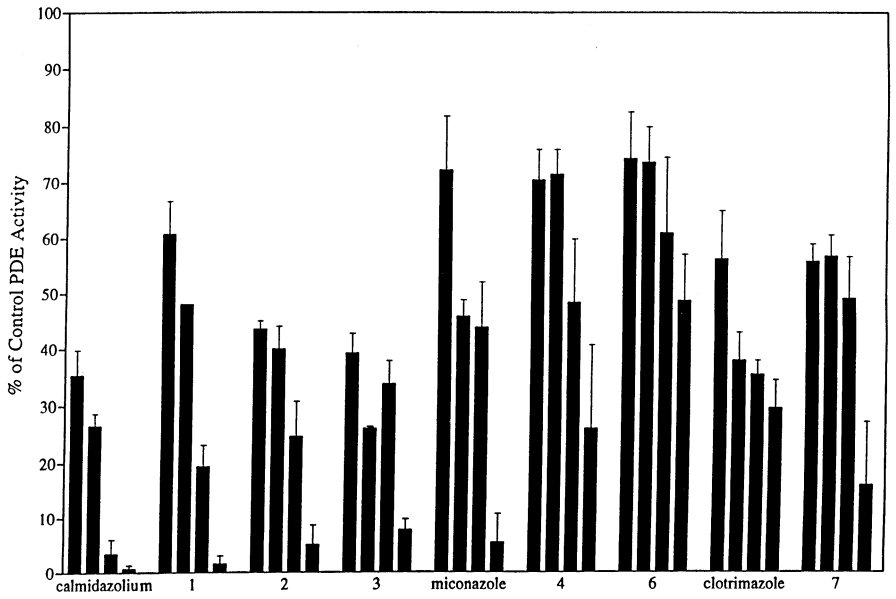


FIG. 7. Inhibition of rat cerebral cortical calmodulin-dependent phosphodiesterase by calmidazolium and calmidazolium analogs. Agents (at 1, 10, 100, or 1000 μM) were added to soluble calmodulin-dependent phosphodiesterase preparations 1 min prior to addition of [^{3}H]cyclic AMP, and activity was assayed, as described in Materials and Methods. Values are means ± SEM (N = 3), relative to a control value of 1500–2000 dpm/assay.

Phosphodiesterase Assay

Calmidazolium, miconazole, the miconazole derivatives **1**, **2**, and **3**, clotrimazole, the clotrimazole derivatives **4–6**, *N*-benzylimidazole, and the *N*-benzylimidazole derivative **7** each caused a concentration-dependent inhibition of calcium-dependent phosphodiesterase (Fig. 7). Miconazole derivatives **2** and **3** were nearly as potent as calmidazolium as inhibitors of the phosphodiesterases, whereas the other compounds were less potent (Fig. 7 and data not shown).

DISCUSSION

Miconazole is considered to be a classical imidazole inhibitor of SOC channels [10, 11], but it also causes a concentration-dependent depletion of IP_3-sensitive calcium stores, resulting in activation of SOC channels and elevation of intracellular calcium [8, 12, 16]. The miconazole derivative calmidazolium is a recognized inhibitor of the calcium-binding protein calmodulin. In addition, this quaternary imidazole elicits an increase in intracellular calcium that is substantially greater than that observed for miconazole [8]. Quaternary derivatives of miconazole, clotrimazole, and *N*-methylimidazole were prepared to probe structure–activity requirements for inhibition of SOC channels and for elevation of intracellular calcium. Clotrimazole is another imidazole-based inhibitor of SOC channels [8, 13].

N-Methylation of miconazole and clotrimazole resulted in a loss of inhibitory effects at SOC channels. Thus, either a charge on the imidazole ring or the presence of a hydrophobic moiety at the *N*-2 position cannot be accommodated at the site of blockade of SOC channels by imidazoles, such as miconazole and clotrimazole. When added alone, these *N*-methyl quaternary imidazoles did not cause an increase in intracellular calcium and, thus, appeared ineffective at the calmidazolium site(s) responsible for release and influx of calcium. The quaternary derivatives of miconazole that have bulkier N-2 substituents (**1**, **2**), analogous to the N-2 substituent present in calmidazolium, did cause elevation of intracellular calcium both alone and after ATP. However, the analogous clotrimazole derivatives **5** and **6** did not cause an elevation of intracellular calcium either alone or after ATP. Thus, both of the *N*-substituents on the imidazole ring seem to be critical to elevations in calcium elicited by calmidazolium. Clotrimazole has more steric bulk at the N-1 position, whereas miconazole has a more extended N-1 substituent with more flexibility. The quaternary derivative **7** has less bulk than the clotrimazole derivative **4** at the N-1 position, but also had no effect on calcium levels.

A concentration-dependent increase in intracellular calcium levels was observed for calmidazolium alone as well as for the miconazole derivatives **1** and **2** (Fig. 3). The elevation of intracellular calcium levels by these three compounds was found to be comprised of two components: first, the release of calcium from intracellular calcium stores, followed by the influx of calcium from the extracellular environment. This result is consistent with a previous study on the effects of calmidazolium on intracellular calcium in amoebae [9]. In contrast to calmidazolium, **1**, and **2**, miconazole alone caused only a transitory concentration-dependent elevation of intracellular calcium levels. The transitory elevation of intracellular calcium levels by miconazole appears to be the result of a dual effect comprised of calcium release from the IP_3-sensitive intracellular calcium stores resulting in activation of SOC channels, followed by inhibition of these channels and an associated decrease in calcium levels [13]. It seems likely that release of intracellular calcium by calmidazolium, **1**, and **2** also results from the IP_3-sensitive calcium stores associated with phosphoinositide breakdown (Table 3). The magnitude of the release was significantly less than that elicited by ATP,

perhaps reflecting a slower but more sustained generation of IP_3 with calmidazolium.

Calmidazolium, **1**, and **2** alone caused a sustained elevation in intracellular calcium levels due to influx of calcium. Part of this influx might be attributed to activation of SOC channels. However, the magnitude of the overall increase in calcium levels elicited by calmidazolium appeared greater than that which occurs when SOC channels are activated after IP_3-dependent depletion of calcium stores by ATP. Attempts to block the large calcium influx component elicited by the miconazole derivatives with a variety of calcium channel inhibitors (see Results) were unsuccessful. Miconazole caused partial inhibition of calcium levels elevated by calmidazolium and **2**, but not **1**. It is possible that partial inhibition of the calmidazolium-elicited elevation of calcium was due to a blockade of a SOC-channel component. However, other SOC channel blockers (see Results) and nickel (see below) had no effect on the calmidazolium response.

An inorganic blocker of calcium channels, namely Ni^{2+}, had no effect at 5 mM on calmidazolium-elicited influx of calcium. Nickel did block SOC channels that activate after ATP. The lack of effect of nickel also indicates that nonspecific damage to the plasma membrane and leakage of fura-2 is not responsible for the calmidazolium response, since nickel would quench any extracellular fura-2 signal. In addition, there was marked dependence on structure, with other quaternary imidazoles (**3**, **4**, **5**, **6**) being inactive.

The mechanisms underlying the elevation of intracellular calcium induced by calmidazolium, **1**, or **2** due to calcium influx are unknown. Although it is likely that calcium influx through SOC channels is involved, the magnitude of the calcium influx component elicited by these compounds appears to exceed 'normal' capacitative calcium entry. In addition, compounds such as trifluoperazine and SKF 96365, which block SOC channels [13, 17], and loperamide, which appears to enhance calcium influx through SOC channels [18], had no effect on calmidazolium-elicited influx of calcium. It is possible that the influx of calcium is associated with a relatively nonspecific increase in membrane permeability to cations due to the lipophilic nature of the molecule(s) involved [19]. However, if this were the case, then it would be expected that miconazole would have similar inhibitory effects on calmidazolium, **1**, and **2**. Furthermore, the other lipophilic imidazoles (**4**, **5**) would be expected to elicit influx, but instead were inactive.

It appears unlikely that calmodulin-dependent regulation of calcium release or influx is involved. Calmidazolium and the miconazole derivatives **1** and **2** did cause similar inhibition of a calmodulin-dependent phosphodiesterase. However, the *N*-methylmiconazole **3**, clotrimazole, the clotrimazole derivatives **4**-**6**, *N*-benzylimidazole, and the *N*-benzylimidazole derivative **7** all caused inhibition of the calmodulin-dependent phosphodiesterase but did not cause elevation of intracellular calcium levels. Furthermore, the response of HL-60 cells to calmidazolium required much higher concentrations than those required to inhibit calmodulin. In addition, other calmodulin inhibitors, such as trifluoperazine and loperamide, do not elicit a marked influx of calcium in HL-60 cells [13, 20].

In conclusion, calmidazolium, a diphenylmethyl derivative of miconazole, caused a marked elevation of intracellular calcium in HL-60 cells both alone and after depletion of the IP_3-sensitive stores by ATP. Similar effects were observed with miconazole derivatives **1** and **2**, in which the diphenylmethyl group in calmidazolium was replaced by either a 3,5-difluorobenzyl or a cyclohexylmethyl group, respectively. The elevation of intracellular calcium levels by calmidazolium, **1**, and **2** was comprised of two components, namely a calcium-release component, attributed to the generation of IP_3 resulting in the depletion of the IP_3-sensitive calcium stores, and a significant calcium influx component. The mechanism underlying the large calmidazolium-induced influx of calcium is not clear. Because of the relatively high concentrations required to elicit calcium influx, the response would not appear to involve inhibition of calmodulin. There might be a contribution from calcium influx through SOC channels activated by depletion of the intracellular calcium stores. It is perhaps that component that is inhibited by miconazole, but the major influx appears to be through an undefined cationic channel that is resistant to blockade by nickel and various calcium channel blockers. The *N*-methylated derivatives of miconazole and clotrimazole had no effect on intracellular calcium levels, indicating that either a ring charge or the presence of a hydrophobic moiety at the N-2 position prevents inhibition of SOC channels. The 3,5-difluorobenzyl and cyclohexylmethyl derivatives of clotrimazole and the 3,5-difluorobenzyl derivative of *N*-benzylimidazole did not cause an elevation of calcium, suggesting either that steric bulk at the N-1 position is not tolerated or more likely that an extended and flexible aromatic-containing N-1 substituent, as in miconazole, is required for calcium release and influx elicited by quaternary imidazoles.

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