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STIMULATION OF CALCIUM RELEASE BY CAFFEINE ANALOGS IN PHEOCHROMOCYTOMA CELLS

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Abstract—Caffeine ($EC_{50} \sim 20$ mM) causes a maximal 400% increase in intracellular calcium ion concentration ($[Ca^{2+}]_i$) in pheochromocytoma PC12 cells. A range of caffeine analogs in which methyl groups at the 1, 3, and 7 positions were replaced with relatively nonpolar (ethyl, allyl, propyl, propargyl) or polar (CH_2COOH, CH_2CH_2OH, CH_2CN, CH_2OCH_3) substituents were tested at a 10 mM concentration. Many analogs were as efficacious or only somewhat less efficacious than 10 mM caffeine. Certain analogs with polar substituents had no effect. Disubstituted xanthines were less efficacious (theophylline, paraxanthine) than caffeine or were ineffective (theobromine). 1-Propyl-3,7-dimethylxanthine (EC_{50} 4 mM) and 1-propargyl-3,7-dimethylxanthine (EC_{50} 5 mM) were several-fold more potent than caffeine in causing elevation of $[Ca^{2+}]_i$ and the latter was at least as efficacious.

Key words: xanthines, calcium release, caffeine

The pharmacological effects of caffeine appear due primarily to blockade of adenosine receptors [1]. However, at higher concentrations caffeine also inhibits phosphodiesterases and causes release of calcium from intracellular storage sites. Caffeine remains a widely used tool for the study of a so-called calcium-sensitive intracellular pool that is distinct from an inositol-trisphosphate-sensitive pool [2–6]. In muscle, the caffeine- and calcium-sensitive pool is associated with the sarcoplasmic reticulum, whereas in nonmuscle cells it is thought to be associated with endoplasmic reticulum. In sympathetic neurons, the calcium-sensitive pool appears to be present only in cell bodies [7]. The calcium-sensitive pool has been proposed to be required for oscillations of membrane potentials [8]. Caffeine can elicit oscillations in intracellular calcium ion concentrations ($[Ca^{2+}]_i$)† in neurons [9]. Thresholds for effects of caffeine on release of intracellular calcium are at about 250 μM, while 2–20 mM concentrations are required for robust effects. The molecular site of action of caffeine appears to be a calcium-gated calcium release channel to which the alkaloid ryanodine binds with high affinity [10, 11]. A domain on this channel to which caffeine binds and thereby enhances sensitivity to activation by calcium has been proposed [10]. In addition, caffeine at millimolar concentrations has been proposed to inhibit the opening of inositol-trisphosphate-sensitive calcium channels [12, 13].

Structure–activity relationships for xanthines as calcium mobilizers are virtually unknown. At a concentration of 1.25 mM, caffeine, theophylline, theobromine, 3-isobutyl-1-methylxanthine and 3,9-dimethylxanthine all stimulate the efflux of calcium from sarcoplasmic reticulum vesicles prepared from rabbit skeletal muscle [14]. Caffeine, theophylline, paraxanthine and theobromine at 2 mM all elicit similar contractures of frog heart [15], a response probably due to the release of calcium from sarcoplasmic reticulum. 3-Isobutyl-1-methylxanthine elicits a slower response and 9-methylxanthines are inactive. Caffeine at 2 mM, presumably by release of the calcium-sensitive pool, prevents inositol trisphosphate-elicited oscillations in membrane potentials, whereas theophylline and 3-isobutyl-1-methylxanthine do not [8]. In the present study, a range of caffeine analogs were investigated for effects on mobilization of intracellular calcium in pheochromocytoma PC12 cells, using the fluorescent probe fura-2. Pheochromocytoma PC12 cells contain a caffeine-sensitive pool of calcium, which in contrast to other cell types, appears also to be sensitive to inositol trisphosphate [16]. Two xanthine analogs have now been identified that are 4- to 5-fold more potent than caffeine in mobilizing intracellular calcium in PC12 cells.

MATERIALS AND METHODS

The xanthines were commercially available from Research Biochemicals, Inc. (Natick, MA) (compounds **1**, **8**, **14**, **15**, **30–35**) or were synthesized as described [17]. Fura-2 acetoxymethylester (fura-2 AM) was from Molecular Probes, Inc. (Eugene, OR). All other chemicals and reagents were obtained from standard commercial sources.

The Krebs–Ringer–HEPES (KRH) buffer consisted of NaCl (125 mM), KCl (5 mM), KH_2PO_4 (1.2 mM), $MgSO_4$ (1.2 mM), $CaCl_2$ (2 mM), glucose

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† Abbreviations: $[Ca^{2+}]$, intracellular calcium ion concentration; DMEM, Dulbecco's modified Eagle's medium; EGTA, ethylene glycol-bis(β-aminoethyl ether) *N,N,N',N'*-tetraacetic acid; fura-2 AM, fura-2 acet oxymethylester; HEPES, *N*-(2-hydroxyethyl)piperazine-*N'*-(2-ethanesulfonic acid); and KRH, Krebs–Ringer–HEPES buffer.

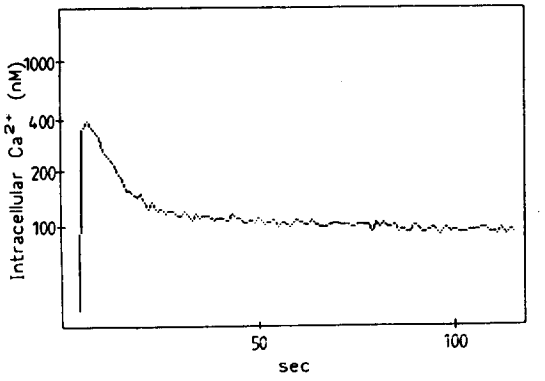

Fig. 1. Effect of caffeine on fura-2 fluorescence in PC12 cells. Caffeine (80 mM, final concentration) in 1 mL buffer was added at zero time to 2 mL of suspended fura-2-loaded cells. After 3 min, cells were lysed, F_{max} and F_{min} were determined as described in Materials and Methods, and $[Ca^{2+}]_i$ was calculated.

(6 mM), and HEPES (25 mM), adjusted to pH 7.4 with NaOH. For Ca^{2+}-free KRH buffer, $CaCl_2$ was substituted by NaCl, and 100 μM ethylene glycol-bis(β-aminoethyl ether) *N,N,N',N'*-tetraacetic acid (EGTA) was added.

The pheochromocytoma PC12 cells were provided by Dr. G. Guroff (National Institutes of Health). The cells were grown at 37° in a humidified atmosphere enriched in CO_2. The cell culture medium consisted of Dulbecco's modified Eagle's medium (DMEM) containing 7% fetal bovine serum, 7% horse serum, penicillin (100 U/mL) and streptomycin (100 μg/mL). Cells were used between passages 5 and 15. The cells were detached from the flasks by shaking gently. Cells were spun down by centrifugation at low speed for 3 min and washed with fresh cell culture medium. Cells were then resuspended in a 1:1 mixture of cell culture medium and KRH buffer at a concentration of 5×10^6 cells/mL. The viability of the cells was assessed by trypan blue staining. The 1 mM fura-2 AM in anhydrous dimethyl sulfoxide was added to obtain a final concentration of 5 μM fura-2 AM. The cells were incubated for 45 min at room temperature in the dark, washed with KRH buffer by low speed centrifugation, resuspended in KRH buffer, and kept at room temperature for no more than 2.5 hr. Before use, aliquots of cells were washed, resuspended in 2 mL Ca^{2+}-free KRH buffer at room temperature at a cell concentration of $2–5 \times 10^6$ cells/mL, and transferred to a cuvette, equipped with a magnetic stirrer. The experiment was started immediately in order to prevent Ca^{2+} depletion of the cells in the Ca^{2+}-free medium. Solutions of xanthines were prepared in Ca^{2+}-free KRH buffer. At high concentrations of xanthines (>10 mM), the solutions were made isotonic by decreasing the NaCl concentration in the buffer to prevent nonspecific osmotic effects. Since intracellular calcium release by xanthines requires high drug concentrations, and xanthines generally exhibit poor water solubility,

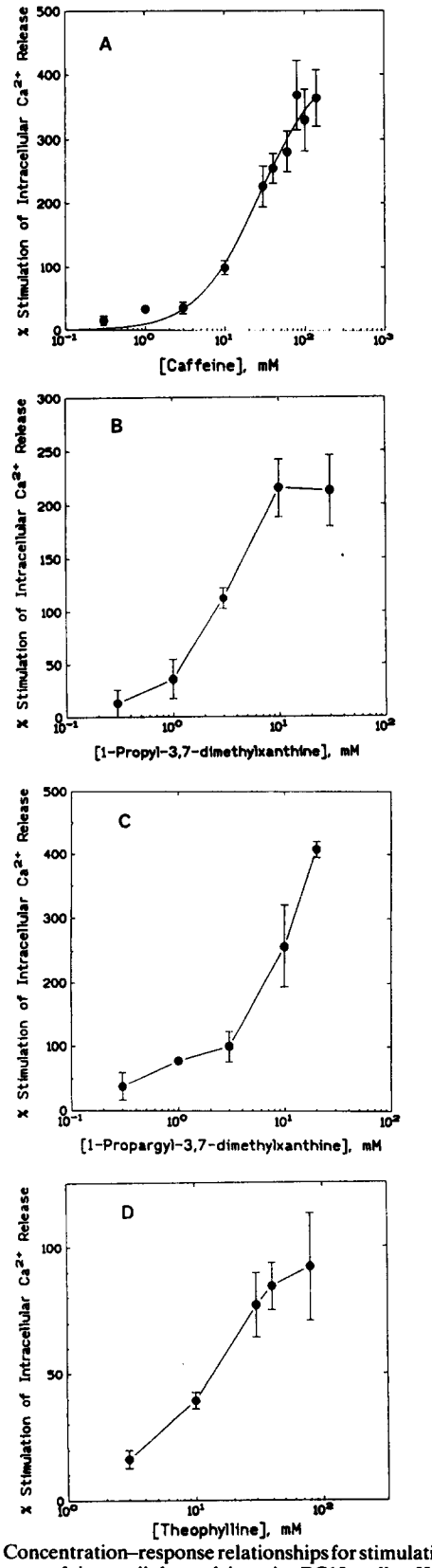

Fig. 2. Concentration–response relationships for stimulation of release of intracellular calcium in PC12 cells. Key: (A) caffeine, (B) 1-propyl-3,7-dimethylxanthine, (C) 1-propargyl-3,7-dimethylxanthine, and (D) theophylline. Values are means ± SEM (N = 3).

Table 1. Activity of xanthines as stimulants of intracellular calcium release in PC12 cells

Compound number	Xanthine substituent(s)	Increase in $[Ca^{2+}]_i$ relative to caffeine set equal to 100
1-Substituted 3,7-dimethylxanthines		
1	1-CH_3 (caffeine)	100 (N = 24)
2	1-$CH_2CH_2CH_3$	221 ± 19
3	1-$CH_2CH_2CH_2CH_2C(O)CH_3$ (pentoxyfylline)	93 ± 12
4	1-CH_2COOH	No effect (N = 1)
5	1-CH_2COOEt	86 ± 19 (N = 2)
6	1-CH_2OCH_3	No effect
7	1-CH_2CN	110 ± 3 (N = 2)
8	1-$CH_2C{\equiv}CH$	285 ± 9
9	1-$CH_2CH{=}CH_2$	144 ± 19
3-Substituted 1,7-dimethylxanthines		
10	3-$CH_2CH_2CH_3$	107 ± 11
11	3-$CH_2CH(CH_3)_2$	105 ± 17
12	3-$CH_2C{\equiv}CH$	205 ± 30
7-Substituted 1,3-dimethylxanthines		
13	7-$CH_2CH_2CH_3$	134 ± 9
14	7-CH_2CH_2Cl	No effect
15	7-CH_2CH_2OH	No effect
16	7-$CH_2CH_2NH_2$	No effect
17	7-$CH_2C(O)CH_3$	70 ± 9
18	7-CH_2COOH	50 ± 9
19	7-CH_2COOEt	76 ± 4
20	7-$CH_2CH_2OC(O)CH_3$	69 ± 11
21	7-CH_2CN	50 ± 15
Further trisubstituted xanthines		
22	1,3-Diethyl-7-methyl	96 ± 11
23	1,7-Diethyl-3-methyl	65 ± 3 (N = 2)
24	1,3-Diallyl-7-methyl	103 ± 14
25	1,3,7-Triethyl	90 ± 13 (N = 2)
26	1,3,7-Tripropargyl (2 mM)	115 ± 18
27	1,3,9-Trimethyl (isocaffeine)	No effect
Disubstituted xanthines		
28	1,3-Dimethyl (theophylline)	40 ± 2
29	1,3-Diallyl	56 ± 10
30	3-Isobutyl-1-methyl (1 mM)	No effect
31	3,7-Dimethyl (theobromine) (5 mM)	No effect
32	1,7-Dimethyl (paraxanthine)	64 ± 1
33	3,9-Dimethyl	No effect
34	7,9-Dimethyl	No effect
Monosubstituted xanthines		
35	3-Propyl	No effect
36	9-Methyl	No effect
8-Substituted xanthines		
37	8-Cyclopentyltheophylline (0.1 mM)	No effect
38	8-Cyclopentyl-1,3-dipropyl (0.1 mM)	No effect

Stimulations by xanthines are reported relative to the stimulation elicited by 10 mM caffeine in that experiment, which was set equal to 100. Concentrations were 10 mM, unless otherwise noted. Results are means ± SEM from three separate experiments (N = 3) performed in duplicate, unless otherwise indicated. Where N = 2, values are means ± range.

dissolution of the compounds was a major problem in the present study, and the experiments were limited by the solubility of several compounds. Compounds were tested in at least three separate experiments in duplicate, unless otherwise noted in some cases, due to the lack of sufficient compound. None of the xanthines reported appeared to interfere with the fura-2 assay. The fura-2 fluorescence was recorded on a Spex Fluorolog 1680 spectrometer (Glen Creston Instruments Ltd., Stanmore, Middx, U.K.) with excitation at 340 nm and emission at 510 nm. To 2 mL of cell suspension, 1 mL of drug solution was added and the fluorescence was recorded over a period of 3 min. The ratios of maximum and minimum fluorescence (F_{max} and F_{min}) were then determined by the addition in small volumes of first 0.1% Triton X-100 and 3 mM $CaCl_2$ to obtain F_{max}, and then 6 mM EGTA in 30 mM Tris base to obtain F_{min}. Basal $[Ca^{2+}]_i$ was determined by addition of 1 mL Ca^{2+}-free KRH buffer to the cell suspension

in parallel experiments. It was observed that xanthine stimulation resulted in a rapid transient increase in $[Ca^{2+}]_i$. After 90 sec or less, the $[Ca^{2+}]_i$ had returned to the basal level as measured in the parallel experiments. Therefore, the $[Ca^{2+}]_i$ at a time greater than 90 sec after stimulation was taken as the basal value in each experiment. The $[Ca^{2+}]_i$ was calculated from the fluorescence, F_{max} and F_{min}, as described using a dissociation constant for the fura-2–Ca^{2+} complex of 224 nM and the equation $[Ca^{2+}]_i = (F - F_{min}/F_{max} - F) \times K_D$ [18]. Basal $[Ca^{2+}]_i$ in PC12 cells was found to be 110 ± 5 nM in the present experiments.

RESULTS AND DISCUSSION

Caffeine at a concentration of 10 mM elicited a rapid elevation of $[Ca^{2+}]_i$ in pheochromocytoma PC12 cells of 112 ± 7% (N = 24), i.e. basal levels increased on an average from 110 to 233 nM. The maximal response to caffeine was 404 ± 44%, which occurred at 80 mM caffeine. Caffeine induced a rapid, transient increase in $[Ca^{2+}]_i$ which returned to basal levels in about 30 sec (Fig. 1). Rapid transient increases also occurred with most caffeine analogs including 1-propyl-3,7-dimethylxanthine (**2**) and 1-propargyl-3,7-dimethylxanthine (**8**) (data not shown). The 1,3-disubstituted xanthines, such as theophylline (**28**), 1,3-diallylxanthine (**29**) as paraxanthine (**32**), elicited a somewhat slower and more sustained increase in $[Ca^{2+}]_i$ (data not shown); whether this is due to slower penetration into cells or other factors is not known. Such xanthines appeared to be "partial agonists" compared with caffeine, but this may be due to the somewhat slower and sustained release. The EC_{50} for caffeine was 19 ± 2.6 mM (see Table 2). Concentration–response curves for caffeine and three other xanthines are shown in Fig. 2.

A variety of analogs in which the 1-methyl, 3-methyl, or 7-methyl caffeine was replaced with larger substituents were tested at a single concentration of 10 mM in comparison with caffeine at 10 mM. A few analogs in which more than one methyl group of caffeine was replaced and a few disubstituted and monosubstituted xanthines were also tested. The results indicate that most analogs of caffeine were at 10 mM equally efficacious to caffeine in stimulating calcium release (Table 1). Only a few analogs with polar substituents had no effect (compounds **4**, **6**, **14**, **15**, and **16**). In addition, most analogs with a polar 7-substituent (compounds **17–21**) were less efficacious than caffeine. Lack of penetration in cells might play a role in the low activity for analogs with polar substituents. Also, the lack of effect of 9-substituted xanthines, such as isocaffeine (compound **27**) and compounds **33**, **34** and **36** may be due to the highly polar character of such xanthines. Isocaffeine and 1,9-dimethylxanthine had been reported previously [14] to be ineffective in causing release of calcium from sarcoplasmic reticulum vesicles. 3,9-Dimethylxanthine was reported to be active in contrast to the lack of effect of this analog (compound **33**) in pheochromocytoma cells. The disubstituted xanthines theophylline, 1,3-diallylxanthine, and paraxanthine (compounds **28**, **29**, and **32**) at 10 mM were less efficacious than caffeine, while 3-isobutyl-1-methylxanthine at 1 mM and theobromine at 5 mM were ineffective. These were the highest concentrations that could be tested because of solubility. In contrast, at a 1.25 mM concentration, caffeine, theophylline, 3-isobutyl-1-methylxanthine and theobromine were reported to cause a similar release of calcium from rabbit sarcoplasmic reticulum vesicles [14].

The responses to certain xanthines exceeded that of caffeine at the 10 mM concentration. These are two compounds (**2** and **8**) in which the 1-methyl of caffeine is replaced with either *n*-propyl or propargyl and one compound (**12**) in which the 3-methyl of caffeine is replaced with propargyl. The 1,3,7-tripropargylxanthine (compound **26**) also elicited a much larger response than caffeine, but could only be tested at 2 mM because of solubility. At that concentration the release of calcium was several-fold greater than that evoked by 2 mM caffeine.

Two of the three very active compounds, namely 1-propyl-3,7-dimethylxanthine (**2**) and 1-propargyl-3,7-dimethylxanthine (**8**), were available in sufficient quantities for concentration–response studies. Both proved several-fold more potent than caffeine in stimulating release of intracellular calcium. One

Table 2. Potency and efficacy of xanthines as stimulants of intracellular calcium release in PC12 cells

Compound	EC_{50} (mM)	Efficacy relative to caffeine
Caffeine (**1**)	19 ± 2.6	100
1-Propyl-3,7-dimethylxanthine (**2**)	3.6 ± 0.7	58
1-Propargyl-3,7-dimethylxanthine (**8**)	5.0 ± 0.4*	100*
7-(β-Hydroxyethyl)theophylline (**15**)	29 ± 4.0	40
Theophylline (**28**)	14 ± 1.3	25

The EC_{50} values are presented as means ± SEM (N = 3). Data are from concentration–response curves (Fig. 2A–D and data not shown), analyzed by the Graphpad program.

* Solubility precluded determination of effects at concentrations > 20 mM. The EC_{50} and efficacy are based on the assumption that the response of a 400% stimulation at 20 mM is maximal as was a 404% response to 80 mM caffeine.

(compound 2) was somewhat less efficacious, whereas the other (compound 8) was fully as efficacious as caffeine (Table 2). Theophylline (28) and the 7-(β-hydroxyethyl)theophylline (15) were similar in potency or somewhat less potent, respectively, compared with caffeine, but both were less efficacious.

Little is known at present of the site or the nature of the interaction of caffeine with the so-called calcium-sensitive storage pool(s) of intracellular calcium. The present study defines caffeine analogs, in particular 1-propargyl-3,7-dimethylxanthine (compound 8), that because of a much higher potency may be useful in studying this site. It should be noted that compound 8 also has activity as an adenosine receptor antagonist [17, 19] and as a phosphodiesterase inhibitor [19]. 1-Propargyl-3,7-dimethylxanthine (compound 8) has a K_i at A_1-adenosine receptors of 45 μM, comparable to that of caffeine (44 μM), and a K_i at A_{2a}-adenosine receptors of 16 μM, somewhat less than that of caffeine (45 μM) [17]. It is only somewhat more potent than caffeine, as an inhibitor of calcium-dependent and -independent phosphodiesterases [19]. It is somewhat more potent than caffeine as a behavioral stimulant [20, 21] and as a tracheal relaxant [22].

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