J. Med. Chem. 1999, 42, 2527−2534

Potentiation of cADPR-Induced Ca^{2+}-Release by Methylxanthine Analogues[†]

Rosaria A. Cavallaro,[‡] Luigi Filocamo,*,[‡] Annamaria Galuppi,[‡] Antony Galione,[§] Mario Brufani,[‡] and Armando A. Genazzani[§,||]

Department of Biochemical Sciences, University La Sapienza, Via degli Apuli 9, 00185 Rome, Italy, and Department of Pharmacology, Mansfield Road, Oxford OX1 3QT, U.K.

Received August 10, 1998

Caffeine and other methylxanthines are known to induce Ca^{2+}-release from intracellular stores via the ryanodine receptor. In the present work, a range of caffeine analogues, in which methyl groups at the 1 and 7 positions were replaced with alkyl chains containing different functional groups (oxo, hydroxyl, propargyl, ester, and acids), were synthesized. These compounds were then screened for their ability to potentiate Ca^{2+}-release induced by cADPR (an endogenous modulator of ryanodine receptors) in sea urchin egg homogenates. Two of the synthesized methylxanthines, 1,3-dimethyl-7-(7-hydroxyoctyl)xanthine (**37**) and 3-methyl-7-(7-oxooctyl)-1-propargylxanthine (**66**), were shown to be more potent than caffeine in potentiating cADPR-induced Ca^{2+}-release, while 1,3-dimethyl-7-(5-ethylcarboxypentyl)xanthine (**14**) was shown to be more efficacious. The development of new methylxanthine analogues may lead to a better understanding of ryanodine receptor function and could possibly provide novel therapeutic agents.

Methylxanthines, such as caffeine, have important therapeutic implications for the treatment of common diseases such as asthma and migraine.[1] Although the pharmacological actions of caffeine appear to be due mainly to its ability to block adenosine receptors,[2] higher concentrations also inhibit the action of phosphodiesterases[3] and induce Ca^{2+}-release from intracellular stores.[4] A number of studies have been performed to identify structural analogues of methylxanthines which are more potent than caffeine in blocking adenosine receptors[5] and in inhibiting phosphodiesterases.[6] Surprisingly, though, few studies have dealt with structural analogues of methylxanthines able to induce or inhibit intracellular Ca^{2+}-release.[7] Although so little emphasis has been placed on the determination of more potent methylxanthine analogues on Ca^{2+}-release, caffeine is known to act as an agonist of the ryanodine receptor.[4] Ryanodine receptors, together with $InsP_3$ receptors, represent a major pathway of Ca^{2+}-release from intracellular stores and have been shown to be involved both in many physiological states, such as skeletal muscle contraction, cardiac contraction, and hormone release, and in pathological states, such as cardiac arrhythmias and malignant hyperthermia.[8]

In the present study we have synthesized a number of methylxanthines and have evaluated their capacity to modulate Ca^{2+}-release. As a model to quantify their effects we have chosen the sea urchin egg homogenate, a well-characterized, robust and reliable model to investigate Ca^{2+}-release mechanisms.[9] It has been shown that the intracellular Ca^{2+} stores of the sea urchin egg display three independent Ca^{2+}-release mechanisms.[10] Alongside the well-known $InsP_3$-sensitive mechanism, sea urchin eggs also possess ryanodine-sensitive receptors and a novel Ca^{2+}-release mechanism sensitive to the novel pyridine nucleotide, nicotinic acid adenine dinucleotide phosphate (NAADP). It was in this system that it was first shown that cADPR, a nicotinamide adenine dinucleotide (NAD^+) metabolite, was an endogenous modulator of the ryanodine receptor.[9,11] It has now been shown that this molecule is able to act as an endogenous modulator of the ryanodine receptor in many other cell types, including plant cells and mammalian tissues.[12] Lee has shown that caffeine is able to potentiate Ca^{2+}-release induced by low concentrations of cADPR, and therefore we have chosen this experimental design to investigate the methylxanthine analogues.[13]

In the present study we show that the sea urchin egg homogenate is an excellent and suitable model to investigate the modulation of Ca^{2+}-release by methylxanthines. Moreover, we have synthesized a number of methylxanthines substituted in position 1 and/or 7 and have constructed a preliminary structural requirement of methylxanthines in these positions that will allow further development of more potent and efficacious Ca^{2+}-releasing compounds. The development of new methylxanthine analogues will provide a greater understanding of ryanodine receptor function and could possibly lead to novel therapeutic agents.

Chemistry

To explore structure−activity relationships of xanthine derivatives, we have synthesized a series of methylxanthine analogues bearing on the nitrogen

[†] Abbreviations: $InsP_3$, inositol trisphosphate; cADPR, cyclic adenosine 5′-diphosphate ribose; NAADP, nicotinic acid adenine dinucleotide phosphate; ^1H NMR, proton nuclear magnetic resonance; ^{13}C NMR, carbon-13 nuclear magnetic resonance; DMF, dimethylformamide; MeOH, methanol; EtOH, ethanol; THF, tetrahydrofuran; DMSO, dimethyl sulfoxide; TsCl, tosyl chloride; Py, pyridine; PCC, pyridinium chlorochromate; $InsP_3$, inositol 1,4,5-trisphosphate.

* To whom correspondence should be addressed: Dr. Luigi Filocamo, Department of Biochemical Sciences, University “La Sapienza”, Via degli Apuli 9, 00185 Rome, Italy. E-mail: Filocamo@uniroma1.it.
[‡] University La Sapienza.
[§] Department of Pharmacology.
[||] Present address: Dept. of Pharmacology, Tennis Court Road, Cambridge CB2 1QJ, U.K.

10.1021/jm980469t CCC: $18.00 © 1999 American Chemical Society
Published on Web 06/26/1999

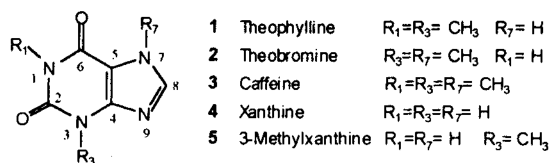


Figure 1. Chemical structures of xanthine and derivatives.

atoms N(1) and/or N(7) alkyl chains containing different functional groups with the aim to obtain compounds that preferentially release Ca^{2+} to other actions attributed to methylxanthines. This was done since it is known that xanthines containing polar moieties on N(1) and/or N(7) are poorly active on adenosine receptors.[2,5]

Theophylline **1**, theobromine **2**, and 3-methylxanthine **5** (Figure 1) served as convenient starting materials and only required the appropriate alkylating conditions to ensure N- versus O-alkylation; alkylation of xanthines with alkyl halides in basic conditions gives exclusively N-alkylation.[14]

In Scheme 1 the general alkylation procedure[5,6] for dimethylxanthines is described. To a solution of theophylline **1** or theobromine **2** in DMF was added an equivalent of K_2CO_3, and the solution was heated to 120 °C; for xanthines **8**−**14** and **16** the reaction temperature was 40−75 °C. After 1 h the appropriate alkyl halide was added to give the corresponding N(7)- or N(1)-alkylated products **6**−**16**.

The oxoalkyl xanthines **6**, **7**, and **15** were reduced by $NaBH_4$ to the corresponding alcohols **17**−**19**, and the esters **13**, **14**, and **16** were hydrolyzed to obtain the corresponding carboxylic acids **20**−**22**.

Because of the low reactivity of the chloropentanone and the low acidity of the N(1)H, the reaction between chloropentanone and theobromine failed. To achieve the analogue of **7** with the 4-oxopentyl on N(1), the alternative method to synthesize **33** was used. When the alkyl halides were not commercially available, they were prepared as described in Scheme 2. 6-Oxoheptanoic acid **23d** and 7-oxooctanoic acid **23e** were refluxed in MeOH with a catalytic amount of H_2SO_4 to give the corresponding esters **24d,e.** The esters **24a,b,d,e** were refluxed in benzene, an excess of ethylene glycol, and a catalytic amount of *p*-TsOH to give ketals **25a,b,d,e**. The alcohols **26a,b,d,e** were obtained by $LiAlH_4$ reduction of **25a,b,d,e** in dry THF, while the alcohol **26c** was prepared by ketalization, hydroboration, and oxidation of compound **24c**. Subsequent reaction with 1.4 equiv of TsCl in pyridine gave the corresponding tosyl derivates **27a**−**e**. Theophylline **1** and theobromine **2** were alkylated by **27a**−**e**, as described for the analogues **7**−**16**, and the resulting products were deprotected to yield the oxoalkylxanthines **28**−**35.**

To achieve the corresponding alcohols, the oxoalkyl xanthines **30, 31, 33, 34** were reduced by $NaBH_4$ to obtain **36**−**39** as described in Scheme 2.

The synthesis of **44** and **47** is shown in Scheme 3. Alcohols **40** and **41** were transformed into the corresponding tosyl derivatives **42** and **43** by reacting with 1.4 equiv of TsCl in pyridine. Theophylline **1** was alkylated by **42** and **43**, respectively, as described for compounds **7**−**16**; subsequently the alkene **45** was hydrated by reacting with 35% H_2SO_4, and the resulting secondary alcohol was oxidized by PCC to give the corresponding oxoalkylxanthine **47**.

The oxoalkylxanthines **55**−**57** were synthesized as shown in Scheme 4. 8-Bromooctanoyl chloride **49** was prepared from the corresponding acid **48** by reacting with $SOCl_2$. Then a Friedel−Craft reaction was performed treating anisole with, respectively, 6-bromohexanoyl chloride and acyl chloride **49** to give compounds **53**−**54**. The analogue compound **52** was obtained by a Friedel−Craft reaction between benzene and 6-bromohexanoyl chloride.Theophylline **1** was then alkylated by the alkylbromide **52**−**54** to give the oxoalkylxanthines **55**−**57**.

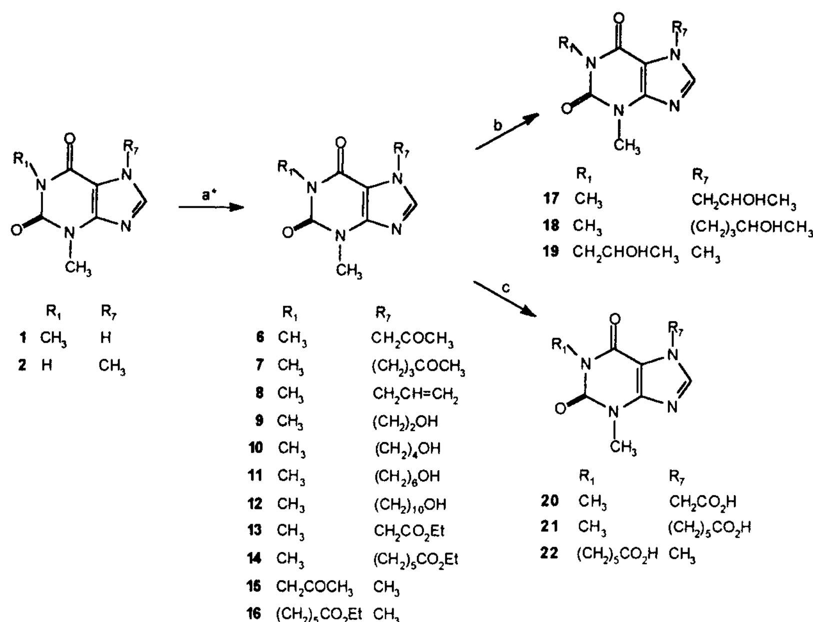
The dialkylation of 3-methylxanthine **5** was performed by reaction with 2 equiv of chloroacetone to give the xanthine **58**; the alkylation of **5** by tosyl derivative **27d** and subsequent deprotection gave the dioxoalkylxanthine **59** (Scheme 5).

To obtain alkylxanthine derivatives bearing different moieties on N(1) and N(7), sequential alkylations were performed. The nitrogen atom in N(7) is more reactive than N(1) and then it is the first position alkylated by the alkylhalide.[14] Subsequent alkylation of the N(1) position provided the desired final compounds. Thus 3-methylxanthine **5** was alkylated by 5-chloropentanone as described above to give the monoalkylated xanthine **60**. Subsequent alkylation of the N(1) position by, respectively, chloroacetone, propargylbromide, and the tosylderivative **27d** and deprotection gave the dialkylxanthines **61**−**63**, as shown in Scheme 5.

The alkylation on N(7) of 3-methylxanthine **5** with bromooctene followed by hydration with 35% H_2SO_4 and PCC oxidation provided the oxoalkyl derivative **65**. Subsequent alkylation on N(1) with propargylbromide yielded the dialkylated xanthine **66**.

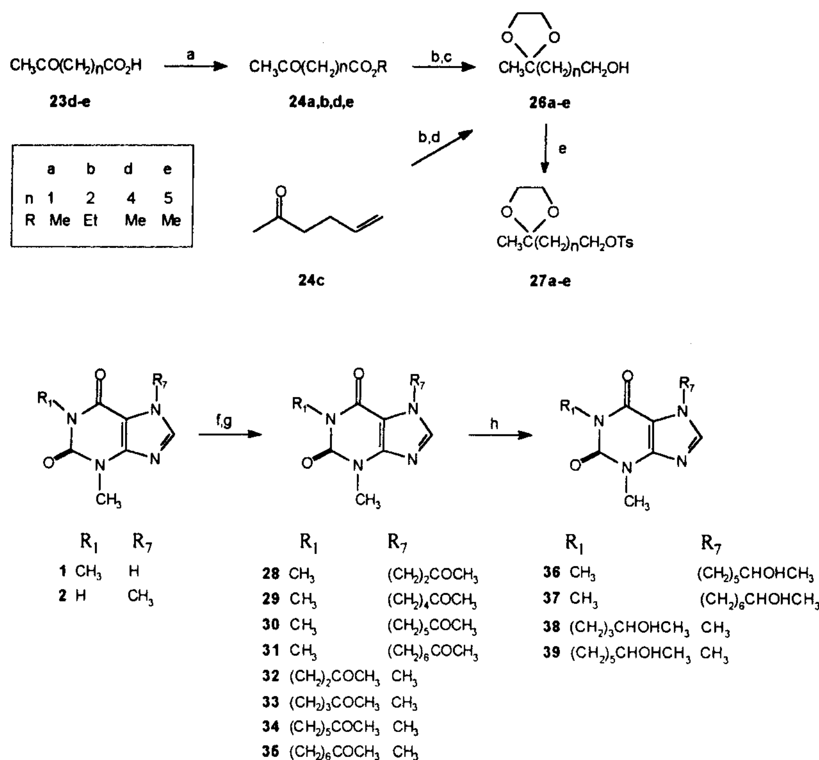
Ca^{2+}-Release Assays. In the present experiments, cADPR released Ca^{2+} with an EC_{50} of 40−60 nM on different experimental days (Figure 2). As previously shown by Lee, the addition of 1 mM caffeine was able to shift the concentration−response curve to the left[13] (Figure 2). In the presence of caffeine, cADPR released Ca^{2+} with an EC_{50} of 10−20 nM on different experimental days. It was therefore decided that to test the activity of the newly synthesized methylxanthines, the ability of this class of compounds to potentiate cADPR-induced Ca^{2+}-release could be used. During each experiment, a concentration−response curve of cADPR was performed, and a concentration of cADPR that induced only a slight Ca^{2+}-release was chosen. Methylxanthine analogues were added 1 min prior to cADPR at the chosen concentration, and caffeine was tested daily to standardize results. Although our protocol does not directly measure methylxanthine-induced Ca^{2+}-release but rather the ability of methylxanthines to potentiate cADPR-induced Ca^{2+}-release, it provides a good indication of the activity of these compounds on ryanodine receptors. The advantages of using the sea urchin egg homogenate are that large amounts of eggs can be collected, the model is robust and well characterized, and it is also reasonably economical. The advantages of measuring potentiation of cADPR-induced Ca^{2+}-release instead of Ca^{2+}-release directly is that lower concentrations of methylxanthines are needed, which is important since these compounds are poorly soluble in water.

Scheme 1[a]



[a] Reagents: (a) RX, DMF, K_2CO_3; (b) $NaBH_4$, MeOH/Et_2O 1:1; (c) HCl 1 N, reflux. Compound **6** was synthesized by (1) NaOH, H_2O/EtOH 4:6; (2) DMF, CH_3COCH_2Cl, 60 °C.

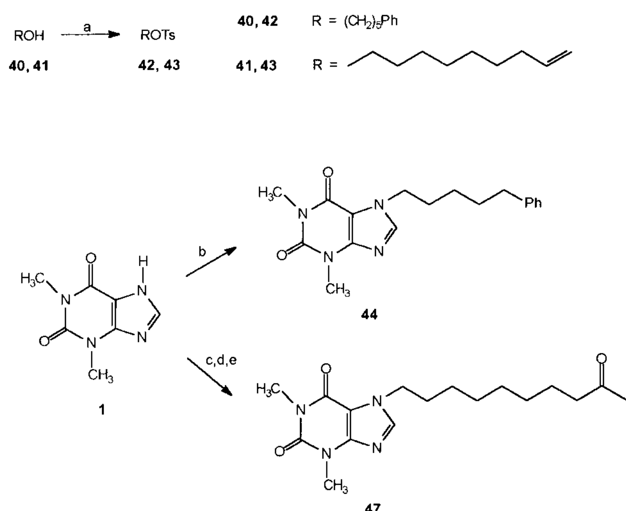
Scheme 2[a]



[a] Reagents: (a) MeOH, H_2SO_4; (b) ethylene glycol, *p*-TsOH, benzene; (c) $LiAlH_4$, THF; (d) (1) BH_3/THF, (2) H_2O_2/OH^-; (c) TsCl, Py; (f) **27a**−**e**, K_2CO_3, DMF; (g) MeOH/HCl 1 N 9:1; (h) $NaBH_4$, MeOH/Et_2O 1:1.

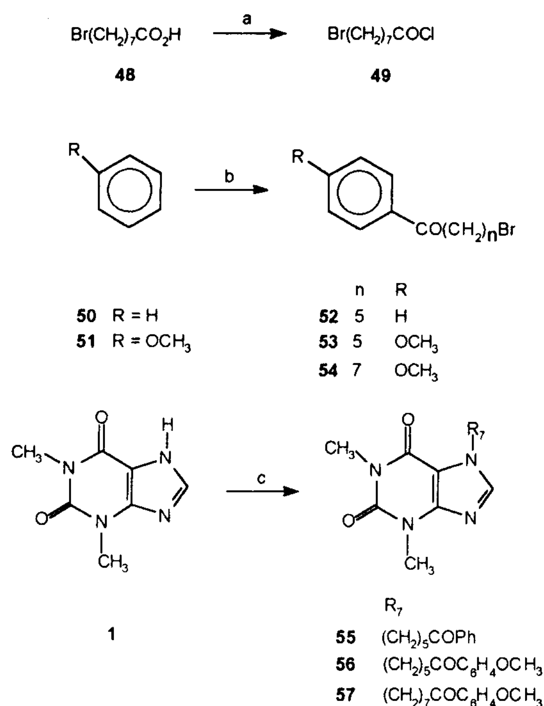
Initially, commercially available compounds were used to identify the general motifs necessary for methylxanthines to potentiate cADPR-induced Ca^{2+}-release. Xanthine at a concentration of 2 mM did enhance cADPR-induced Ca^{2+}-release but only to a minor extent (Table 1). 1,3,9-Trimethylxanthine (isocaffeine), which differs from caffeine by methylation in position 9 instead of position 7, also potentiated cADPR-induced Ca^{2+}-release, but caffeine again proved to be more potent (Table 1). This is consistent with a report by Lee, in which it is shown that isocaffeine is less potent than caffeine.[13] This is also consistent with reports that isocaffeine is inactive or poorly active in inducing Ca^{2+}-release in PC12 cells, where it has been attributed to the high polar nature of this xanthine.[7] 1,3-Dimethylxanthine (theophylline) was as efficacious as caffeine when tested at a concentration of 2 mM (Table 1), although when tested at 1 mM the cADPR-induced Ca^{2+}-release potentiation was significantly lower compared to caffeine (data not shown). These preliminary

Scheme 3[a]



[a] Reagents: (a) TsCl, Py; (b) **42**, DMF, K_2CO_3; (c) **43**, DMF, K_2CO_3; (d) H_2SO_4 35%; (e) PCC, CH_2Cl_2.

Scheme 4[a]



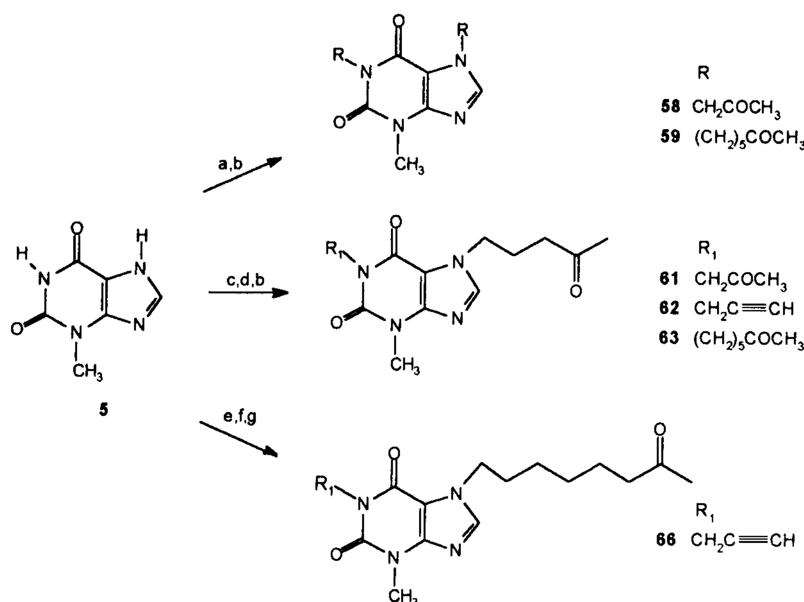
[a] Reagents: (a) $SOCl_2$, CCl_4, DMF; (b) $Br(CH_2)_nCOCl$, $AlCl_3$, CH_2Cl_2; (c) **52**–**54**, DMF, K_2CO_3.

data suggest that the methyl groups on the xanthine enhance the effect of these compounds on cADPR-induced Ca^{2+}-release and that the presence of a methyl in position 9 diminishes the potency of methylxanthines.

Since caffeine appeared to be more potent than theophylline, we decided to investigate substitutions at position 7. All synthesized compounds were initially tested at a concentration of 2 mM, unless the solubility of the methylxanthines did not allow it. When a short carbonyl chain was present at position 7 (compounds **6, 7, 28, 29**), the methylxanthine appeared to lose activity compared to theophylline (Table 2). Increasing the length of the carbonyl chain restored the activity of the molecules. However, when the carbonyl chain was too long the solubility of the compounds decreased, as shown by the inability to test compound **47** at 2 mM. When secondary alcohols (compounds **17**, **18**, **36**, **37**) were prepared from the 7-carbonyl-1,3-dimethylxanthines, the ability to potentiate cADPR-induced Ca^{2+}-release did not appear to be modified in respect to the initial compounds (Table 2). Therefore, increasing the length of the chain increased the activity of the compounds. These compounds also appeared to be more soluble in water than the initial compounds. However, it appears that the carbonyl group at the end of the chain is not responsible for the increased activity since it can be substituted with an –OH group. A concentration–response curve was performed on compound **37** (Figure 3), and it was shown that this compound was approximately 2-fold more potent than caffeine in potentiating cADPR-induced Ca^{2+}-release, although it appeared to possess the same efficacy. Next, we decided to synthesize and test 7-substituted alcohols linked to the 7 position through $-CH_2$ groups (compounds **9**–**12**) (Table 2). Once again, the presence of the substitution too close to the xanthine hampered the potentiation of cADPR-induced Ca^{2+}-release, while longer chains allowed the novel methylxanthines to be as potent and efficacious as caffeine. Substitution of position 7 with ethyl esters was also attempted, and it was found that a $-CH_2CO_2Et$ group (cf. **13**) did not significantly alter the capacity of the compound to potentiate cADPR-induced Ca^{2+}-release compared to caffeine. Strikingly, though, when the ethyl ester group was placed further from the xanthine (cf. **14**), the new molecule was more efficacious than caffeine (Table 2). A concentration–response curve was performed, and it was shown that caffeine and **14** did not significantly differ in potency (**37** being slightly more potent) but did in efficacy (Figure 3). To our knowledge this is the first evidence of a compound more efficacious than caffeine, and development of this compound could help functional studies of ryanodine receptor-induced Ca^{2+}-release in many systems. Surprisingly, the respective acids (**20, 21**) of compounds **13** and **14** were inactive (Table 2), suggesting that the ester group is important for the conformation of these new active molecules. Compounds **20** and **21** were also tested for a possible antagonistic effect on caffeine-induced potentiation of cADPR-induced Ca^{2+}-release by adding to the homogenate either **20** or **21**, then caffeine, and then cADPR. These two compounds, at a concentration of 2 mM, were devoid of antagonistic activity, suggesting that their affinity for the caffeine binding site is extremely low. Other substitutions tested in position 7 were an allyl group (cf. **8**), a chain with a terminal phenyl group (**44**), and chains with terminal benzoyl or *p*-metoxybenzoyl groups (**55**–**57**), and while the first molecule did not appear to significantly differ from caffeine, the others were less potent and less soluble in water (Table 2).

As part of the initial characterization of the structure–activity relationship of methylxanthines on potentiation of cADPR-induced Ca^{2+}-release, we also synthesized a number of 1-substituted-3,7-methylxanthines along the same lines as the 7-substituted-1,3-methylxanthines. When carbonyl groups were substituted on position 1 of the methylxanthine, the compounds (**15**, **32**–**35**) appeared to lose activity, in a similar way to the 7-substituted ones (Table 3). 3,7-Dimethylxanthine

Scheme 5[a]



[a] Reagents: (a) RX (X = Cl, OTs), DMF, K_2CO_3; (b) MeOH/1 N HCl 9:1; (c) $Cl(CH_2)_3COCH_3$, DMF, K_2CO_3; (d) R_1X (X = Cl, Br, TsO), DMF, K_2CO_3; (e) 1-bromo-7-octene, DMF, K_2CO_3, 70 °C; (f) (1) H_2SO_4 35%; (2) PCC, CH_2Cl_2; (g) propargyl bromide, DMF, K_2CO_3.

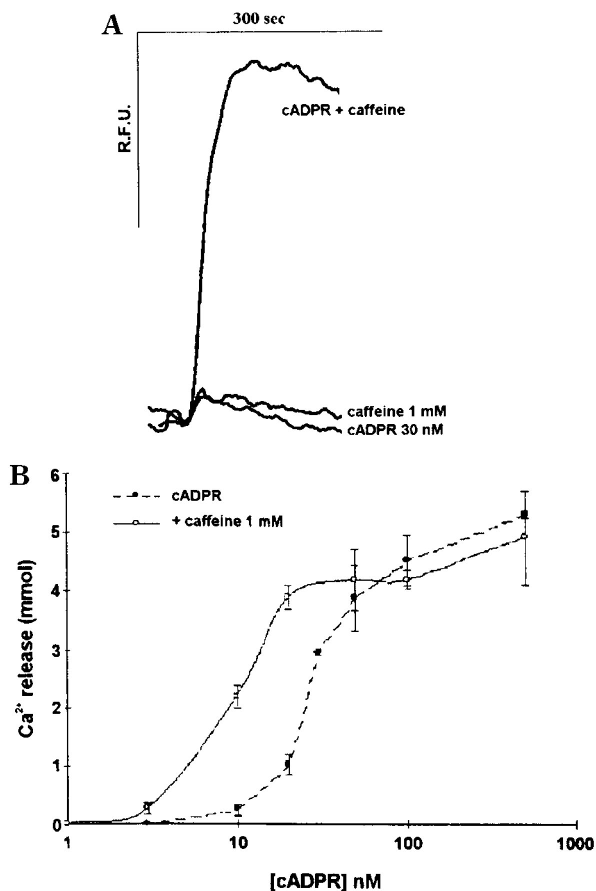


Figure 2. (A) Representative fluorimetric traces demonstrating the caffeine-induced potentiation of cADPR-induced Ca^{2+}-release; (B) concentration–response curves of cADPR-induced Ca^{2+}-release in the absence or presence of caffeine. Values are mean ± SEM of six to nine determinations.

Table 1. Effect of Some Commercially Available Compounds Structurally Similar to Caffeine[a]

compds	concn (mM)	% of caffeine potentiation
caffeine	1	100 ± 5.5
xanthine	2	41.0 ± 2.7
isocaffeine	2	28.7 ± 10
theophylline	2	95.3 ± 8.0

[a] Values represent mean ± SEM of 3–7 determinations. Compounds were dissolved in hot distilled water; 5 μL was added to a final volume of 500 μL.

(theobromine) could not be used as a control because of its poor solubility in water. Once again, elongation of the carbonyl chain increased the activity of these compounds, although they appeared always less active than caffeine. The secondary alcohols (**19**, **38**, **39**) derived from the 1-substituted compounds did not alter significantly the activity of these compounds, as was the case with the 7-substituted secondary alcohols (Table 3). Substitution with ethyl esters was also performed on position 1. The activity of compound **16** did not significantly differ from caffeine, while its respective acid **22** was less active although some activity was retained, unlike the acids derived from 7-substituted ethyl esters where the activity was lost (Table 3).

7-Substitutions were also performed in a previous report by Daly et al., and some methylxanthines overlap in the two studies.[7] In accord with our data, compounds **6**, **9**, **13**, and **20** (Table 2), which were tested for their capacity to release Ca^{2+} at a concentration of 10 mM in PC12 cells, were less efficacious than caffeine.

In an attempt to understand the relationship between position 1 and position 7, we decided to perform dual substitutions. In view of further development of compounds with Ca^{2+}-releasing activity, this is an important step because it could lead to information on the possibilities for substitutions for the development of more potent and efficacious compounds. When short carbonyl chains were substituted at both positions, the compounds lost activity (cf. **58**, **61**) (Table 4). The compounds slightly regained activity when the carbonyl chains were longer, although not to the full extent (cf. **59**, **63**). These compounds were also tested for possible antagonistic activity, but they did not interfere with caffeine-induced

Table 2. 7-Substituted 1,3-Dimethylxanthines: Activity on Ca^{2+} release[a]

compd	R_7	concn (mM)	% of caffeine 1 mM effect
1	H	2	106 ± 13
3	CH_3	1	100 ± 2.3
	Carbonyl Groups		
6	CH_2COCH_3	2	65 ± 18
28	$(CH_2)_2COCH_3$	2	97 ± 1.5
7	$(CH_2)_3COCH_3$	2	60 ± 16
29	$(CH_2)_4COCH_3$	2	65 ± 9.0
30	$(CH_2)_5COCH_3$	2	107 ± 3.1
31	$(CH_2)_6COCH_3$	2	112 ± 14
47	$(CH_2)_8COCH_3$	1	62 ± 7.1
	Secondary Alcohols		
17	$CH_2CHOHCH_3$	2	47 ± 8.5
18	$(CH_2)_3CHOHCH_3$	2	82 ± 15
36	$(CH_2)_5CHOHCH_3$	2	105 ± 8.8
37	$(CH_2)_6CHOHCH_3$	1	98 ± 4.5
	Primary Alcohols		
9	$(CH_2)_2OH$	2	43 ± 6.3
10	$(CH_2)_4OH$	2	81 ± 3.2
11	$(CH_2)_6OH$	1	97 ± 1.7
12	$(CH_2)_{10}OH$	1	113 ± 11
	Ethyl Esters and Respective Acids		
13	CH_2CO_2Et	1	87 ± 17
14	$(CH_2)_5CO_2Et$	1	145 ± 8.1
20	CH_2CO_2H	1	1.4 ± 0.9
21	$(CH_2)_5CO_2H$	1	9.4 ± 0.1
	Others		
8	$CH_2CH=CH_2$	2	100 ± 13
44	$(CH_2)_5Ph$	2	70 ± 7.1

[a] Values represent mean ± SEM of 3−7 determinations. Compounds were dissolved in hot distilled water; 5 μL was added to a final volume of 500 μL.

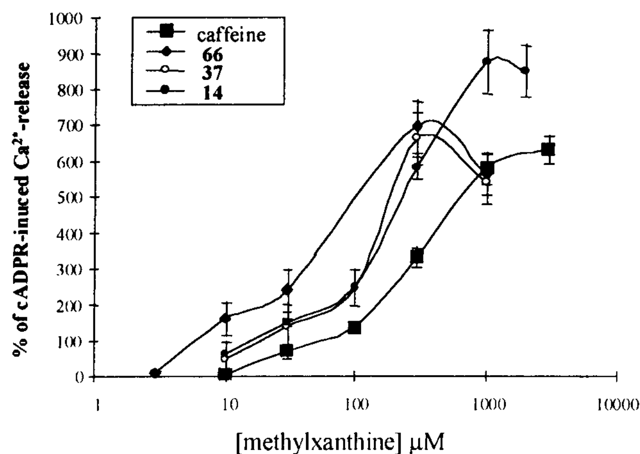


Figure 3. Concentration−response curve of methylxanthine analogues that were shown to be more potent and/or efficacious than caffeine. cADPR was used at concentration of 20 nM. Values are ± SEM of three to nine determinations.

potentiation of cADPR-induced Ca^{2+}-release. This would suggest that 1,7-dicarbonyl-3-methylxanthines have a very low affinity for the caffeine binding site on the ryanodine receptor. Propargyl groups were then substituted at position 1, and the length of the carbonyl substitution on position 7 changed (cf. **62**, **66**) (Table 4). Compared to the 1,7-dicarbonyl-3-methylxanthines, these new compounds were much more potent. Indeed, when a concentration−response curve was performed with compound **66** (Figure 3), it was shown that this novel compound was approximately 4-fold more potent than caffeine itself, while possessing the same efficacy.

Table 3. 1-Substituted-3,7-dimethylxanthines: Activity on Ca^{2+} Release[a]

compd	R_1	concn (mM)	% of caffeine 1 mM effect
3	CH_3	1	100 ± 3.1
	Carbonyl Groups		
15	CH_2COCH_3	2	43 ± 7.6
32	$(CH_2)_2COCH_3$	2	46 ± 0.32
33	$(CH_2)_3COCH_3$	2	37 ± 1.6
34	$(CH_2)_5COCH_3$	2	96 ± 1.9
35	$(CH_2)_6COCH_3$	2	76 ± 1.8
	Secondary Alcohols		
19	$CH_2CHOHCH_3$	2	83 ± 4.0
38	$(CH_2)_3CHOHCH_3$	2	59 ± 2.8
39	$(CH_2)_5CHOHCH_3$	2	54 ± 1.3
	Ethyl Esters and Respective Acids		
16	$(CH_2)_5CO_2Et$	1	103 ± 11
22	$(CH_2)_5CO_2H$	1	61 ± 7.6

[a] Values represent mean ± SEM of 3−7 determinations. Compounds were dissolved in hot distilled water; 5 μL was added to a final volume of 500 μL.

Table 4. 1,7-Disubstituted-3-methylxanthines: Activity on Ca^{2+} Release[a]

compd	R_1	R_7	concn (mM)	% of caffeine 1 mM effect
3	CH_3	CH_3	1	100 ± 0.91
58	CH_2COCH_3	CH_2COCH_3	2	17 ± 2.2
61	CH_2COCH_3	$(CH_2)_3COCH_3$	2	6.7 ± 0.75
63	$(CH_2)_5COCH_3$	$(CH_2)_3COCH_3$	2	38 ± 2.4
59	$(CH_2)_5COCH_3$	$(CH_2)_5COCH_3$	2	54 ± 4.5
62	Propargyl	$(CH_2)_3COCH_3$	2	81 ± 1.8
66	Propargyl	$(CH_2)_6COCH_3$	1	98 ± 14

[a] Values represent mean ± SEM of 3−7 determinations. Compounds were dissolved in hot distilled water; 5 μL was added to a final volume of 500 μL.

This is in agreement with the work previously published by Daly et al.[7] In this report the authors showed that 1-propargyl-3,7-dimethylxanthine was more potent than caffeine (285 ± 9% of the caffeine response) in releasing Ca^{2+} from PC12 cells. This result is encouraging, since it suggests that substitutions on both positions 1 and 7 can lead to compounds that are active.

In the present work, we demonstrate that the sea urchin egg homogenate model can be used as preliminary screening model for methylxanthines. A simple and convenient procedure has been developed which could also be automated by the use of microplate readers, as previously suggested for the measurement of cADPR levels.[15] The sea urchin egg homogenate model is suited for the study of the direct effect of methylxanthines on the ryanodine receptor since it is a broken-cell system. Once this system has highlighted a number of potentially interesting molecules, a whole cell system, such as the one described by Muller and Daly,[7] will be needed to assess the ability of the newly synthesized methylxanthines to cross the plasma membrane and reach their intracellular target.

To test the appropriateness of the method, we have started a preliminary screening of 1-substituted, 7-substituted, and 1,7-disubstituted methylxanthines and have highlighted one compound which is 4-fold more potent than caffeine in potentiating cADPR-induced Ca^{2+}-release (cf. **66**), one compound which is 2-fold more potent than caffeine (cf. **37**), and one compound which is more efficacious than caffeine in this paradigm (cf. **14**) (Figure 3). These data complement a previous

report[7] that showed that 1-propargyl-3,7-dimethylxanthine and 1-propyl-3,7-dimethylxanthine were more potent than caffeine in releasing Ca^{2+} from PC12 cells.

These findings provide novel tools for basic research and suggest possibilities to improve both the potency and the efficacy of caffeine. In the future, it is possible to envisage that parent compounds developed through the use of this information could be used for the treatment of disorders where it is known that intracellular Ca^{2+} homeostasis is altered. Further work is needed to dissect potent and efficacious molecules that possess Ca^{2+}-releasing activity but are devoid of activity on adenosine receptors and phosphodiesterases.

Experimental Section

Chemistry. Meltings points were determined using a Reichert Thermovar apparatus and are uncorrected. NMR spectra were recorded on a Varian Gemini 200 MHz; the chemical shifts are expressed in δ values (parts per million) relative to tetramethylsilane as internal standard. Thin-layer chromatography (TLC) was performed on silica gel 60F-254 glass-supported plates with 0.25 mm thickness (Macherey-Nagel Reagents). Spots were visualized by either ultraviolet light, exposure to iodine, or spraying with an acid solution of dinitrophenylhydrazine (DNF) in ethanol. Preparative thin-layer chromatography was performed on silica gel 60F-254 glass-supported plates with 2 mm thickness (Macherey-Nagel Reagents). Merck silica gel 60 (70−230 mesh) was used for column chromatography. All the reactions were monitored by TLC. The physical−chemical data and purification methods of the compounds are available as Supporting Information.

1,3-Dimethyl-7-(2-oxopropyl)xanthine, 6. Xanthine **6** was synthesized in two steps. The first step was performed refluxing theophylline **1** (2.5 mmol) in a mixture of H_2O (15 mL), EtOH (25 mL), and NaOH (2.5 mmol) for 25 min. The mixture was evaporated and the residue dried in a vacuum at 80 °C for 2 h. To the resulting sodium salt in DMF (15 mL) a 5-fold excess of $ClCH_2COCH_3$ was added, and the reaction mixture was heated for 24 h at 60 °C. Solvent was removed in a vacuum, and then the residue was dissolved in saturated $NaHCO_3$, extracted with $CHCl_3$, dried over Na_2SO_4, and evaporated.

General Alkylation Procedure for Dialkylxanthines. A stirred suspension of dialkylxanthine (2 mmol) and anhydrous K_2CO_3 (2 mmol) in dry DMF was brought to 120 °C (for xanthines **8**−**14** and **16** the reaction temperature was 40−75 °C) for 1 h; then the appropriate alkyl halide or tosyl derivative (2.2 mmol) was added dropwise, and the mixture was stirred for 1−18 h. The reaction mixture was cooled at room temperature, H_2O was added, and it was neutralized by adding 0.1 N HCl. The mixture was extracted with $CHCl_3$; after drying over Na_2SO_4, the organic layer was evaporated in vacuo to give crude products. The alkylation with tosyl derivatives was followed by deprotection of the correspondig ketals stirring in MeOH/HCl 1 N 9:1 at room temperature for 3−14 h. Compounds **7**−**16**, **28**−**35**, **44**, **45**, **55**−**57**, **61**−**63**, and **66** were obtained.

Selective Monoalkylation of 3-Methylxanthine 5. 3-Methylxanthine **5** was alkylated as described above using equimolar amounts of alkylating agent at 70 °C for 2 h. Compounds **60** and **64** were obtained.

Dialkylation of 3-Methylxanthine 5. 3-Methylxanthine **5** (1 mmol) was dialkylated as described above, using 2 equiv of alkylating agent and K_2CO_3. The alkylation with tosyl derivative was followed by deprotection of the corresponding ketal stirring in MeOH/HCl 1 N 9:1 at room temperature for 3−14 h. Compounds **58**−**59** were obtained.

Preparation of the Esters 24d−e. Compounds **24d** and **24e** were synthesized by refluxing the acids **23d**−**e** (20 mmol) in MeOH (20 mL) with some drops of concentrated H_2SO_4 for 1.5 h. The reaction mixture was cooled and neutralized by adding saturated $NaHCO_3$, and MeOH was evaporated. Then H_2O was added to the residue, and it was extracted with Et_2O, dried over Na_2SO_4, and evaporated to give the esters **24d**−**e** (95−98% yield) that were used without further purification.

Preparation of the Ketals 25a−e. The carbonylic groups were protected by refluxing in a Dean−Stark apparatus **24a**−**e** (10−20 mmol) in dry benzene (50 mL) with an excess of dry ethylene glycol (3 mL) and a catalytic amount of *p*-toluenesulfonic acid for 2 h. Then Et_2O was added, and the mixture was neutralized and extracted with Et_2O. The organic layer was dried over Na_2SO_4 and then evaporated to give the ketals **25a**−**e** (90−95% yield), which were used without further purification.

Preparation of the Alcohols 26a,b,d,e. The esters **25a,b,d,e** (8−18 mmol) in dry THF (3−6 mL) were added to a solution of 3 equiv of $LiAlH_4$ in dry THF (30−40 mL), stirring the mixture at room temperature for 2−4 h. Then Et_2O/HCl 1 N was added to the mixture and it was filtered over Celite; the organic layer was evaporated to give crude products.

6-Hydroxy-2-hexanone Ethylene Acetal, 26c. Ketal **25c** was hydroborated (10.6 mmol) in dry THF (15 mL), and 0.33 equiv of BH_3 was added dropwise under nitrogen atmosphere at 0 °C. To the mixture, after stirring at room temperature for 2 h, was added dropwise a solution of 2.25 equiv of NaOH 3 M and 0.1 mL of H_2O_2 40% for each mmol of **25c**. Then the temperature was brought 50 °C for 2−6 h. Et_2O was added, and the mixture was neutralized by adding 0.1 N HCl and extracted with Et_2O. The organic layer was dried over Na_2SO_4 and evaporated to give the crude product, which was purified by chromatography on a silica gel column eluted with $CHCl_3$/MeOH 95:5 to provide the alcohol **26c** as an oil.

Preparation of the Tosylderivatives 27a−e, 42, 43. Compounds **26a**−**e**, **40**, and **41** (4−8 mmol) were tosylated in pyridine (2−5 mL), 1.4 equiv of *p*-TsCl was added, and the mixture was stirred at room temperature for 18 h. Then H_2O was added and, after 10 min, Et_2O was added to the mixture. The organic layer was separated and washed several times with H_2O/HCl 1 N, dried over Na_2SO_4, and evaporated to give the crude product. Compounds **27a**−**e**, **42**, **43** were obtained in 50−60% yields and used without further purification.

1,3-Dimethyl-7-(9-oxodecyl)xanthine, 47, and 3-Methyl-7-(7-oxooctyl)xanthine, 65. Compounds **45** and **64** (0.5 mmol) were hydrated with H_2SO_4 35% (4 mL) at 50 °C for 2 h, under nitrogen atmosphere. The mixtures were then cooled in the refrigerator, neutralized with NaOH 5 N, extracted with $CHCl_3$, dried over Na_2SO_4, and evaporated to give the corresponding secondary alcohols in quantitative yields. The crude products, used without further purification, were treated with PCC (0.8 mmol) in dry CH_2Cl_2 (5 mL) at room temperature for 3 h. Then the mixtures were filtered over SiO_2 and evaporated.

General Procedure for Reduction of Oxoalkyldimethylxanthines. Compounds **6, 7, 15, 30, 31, 33**, and **34** (0.2−0.65 mmol) were reduced in a mixture of MeOH/Et_2O 1:1 (3−6 mL) by adding 10 mg of $NaBH_4$, each time monitoring the reaction by TLC ($CHCl_3$/MeOH 9:1) until it was completed. Then the mixture was neutralized by adding 0.1 N HCl, extracted with $CHCl_3$, dried over Na_2SO_4, and evaporated to give crude products.

General Procedure for Esters Hydrolysis. The esters **13**, **14**, and **16** (0.6 mmol) were heated to boiling with 1 N HCl (5 mL) for 1 h. The mixtures were then allowed to cool, producing microneedles of desired products **20**, **21**, and **22** as white solids.

Preparation of 8-Bromooctanoyl Chloride, 49. 8-Bromooctanoyl chloride was prepared by adding $SOCl_2$ (10.5 mmol) to 8-bromooctanoic acid (10.3 mmol) in CCl_4 (10 mL) and some drops of DMF, stirring at 100 °C overnight. The solvent was evaporated to provide **49** in quantitative yield, and it was used without further purification.

Preparation of Alkyl Halides 52−54. A mixture of the aromatic starting material **50** or **51** (benzene or anisolo, 10 mmol), dry CH_2Cl_2 (20 mL), and $AlCl_3$ (6 mmol) was stirred at −10 °C under nitrogen atmosphere. Then the appropriate acylhalide (6-bromohexanoyl chloride or **49**, 7.8 mmol) in dry

CH_2Cl_2 was added, and stirring was continued for 1−2 h. Then the reaction mixture was poured with stirring in crushed ice (20 g) and concentrated HCl (2.8 mL) for 20 min until the temperature was raised to room temperature. Then the mixture was extracted with CH_2Cl_2, dried over Na_2SO_4, and evaporated.

Biological Activity Assays. (1) Collection of Sea Urchin Eggs and Preparation of Homogenates. Eggs were obtained by stimulating ovulation of female *Lytechinus pictus* with a intracoelomic injection of 0.5 M KCl. Eggs were collected in artificial seawater (ASW), consisting of 435 mM NaCl; 40 mM $MgCl_2 \cdot 6H_2O$; 15 mM $MgSO_4 \cdot 7H_2O$; 11 mM $CaCl_2$; 10 mM KCl; 2.5 mM $NaHCO_3$, pH 8.0; and 1 mM EDTA.

(2) Homogenate Preparation. Homogenates (2.5%) of *Lytechinus pictus* eggs were prepared as described previously.[16a] In brief, eggs were dejellied in ASW by filtering through 85 μm Nitex mesh and then washed twice in EGTA-Ca^{2+}-free ASW and twice in Ca^{2+}-free ASW, each containing 470 mM NaCl; 27 mM $MgCl_2 \cdot 6H_2O$; 28 mM $MgSO_4 \cdot 7H_2O$; 10 mM KCl; and 2.5 mM $NaHCO_3$ (pH 8.0), plus 1 mM EGTA for the first two washes. Finally, eggs were washed with medium (Glu-IM) consisting of 250 mM potassium gluconate; 250 mM *N*-methylglucamine; 20 mM Hepes (pH 7.2), and 1 mM $MgCl_2$. Eggs were then homogenized in Glu-IM plus 2 mM ATP; 2000 U/mL creatine phosphokinase; 2 M phosphocreatine; 50 μg/mL Leupeptin; 20 μg/mL Aprotinin; 100 μg/mL SBTI, using a dounce glass tissue homogenizer, size "A" pestle, and cortical granules removed by centrifugation at 13000*g*, 4 °C. Homogenates were stored at −70 °C, until used for Ca^{2+}-release studies.

Ca^{2+}-Release Assays. The 2.5% homogenates were prepared from 50% stocks as described previously.[16b] Ca^{2+}-loading was achieved by incubation at room temperature for 3 h in Glu-IM, plus 0.5 mM ATP; 10 mM phosphocreatine; 10 U/mL creatine phosphokinase; 1 mg/mL oligomycin; 1 mg/mL antimycin; 1 mM sodium azide; 3 μM fluo-3. Free Ca^{2+} concentration was measured by monitoring fluorescence intensity at excitation and emission wavelengths of 490 and 535 nm, respectively. Fluorimetry was performed at 17 °C using 500 μL of homogenate in a Perkin-Elmer LS-50B fluorimeter. Additions were made in 5 μL volume, and all chemicals were added in Glu-IM. Basal concentrations of Ca^{2+} were typically between 100 and 150 nM. Sequestered Ca^{2+} was determined by monitoring the decrease in fluo-3 fluorescence during microsomal loading and by measuring Ca^{2+} release in response to ionomycin (5 μM) and was constant between experiments. Methylxanthines were added 1 min prior to cADPR.

Supporting Information Available: Physical−chemical data and purification methods of the compounds. This material is available free of charge via the Internet at http://pubs.acs.org.

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JM980469T