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Modulation of the Production of Reactive Oxygen Species by Pre-Activated Neutrophils by Aminoadamantane Derivatives

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ABSTRACT. Aminoadamantane derivatives (AAD) such as amantadine or memantine have been used for the treatment of *Morbus Parkinson* and *Morbus Alzheimer*. In this communication, we report on the immunomodulatory activities of AAD. Luminol-dependent chemiluminescence of zymosan-, *N*-formylmethionylleucylphenylalanine(FMLP)- or experimental Ca^{2+}-ionophore(A 231879)-preactivated polymorphonuclear leukocytes (PMN) was strongly enhanced by submicromolar concentrations of AAD and inhibited at higher concentrations than 0.1 mM. Light emission by phorbol-12-myristate-acetate(PMA)-preactivated cells was not further stimulated but inhibited by the elevated concentrations, just as with the other, above-mentioned activators. Ethylene formation from α-keto-methylthiobutyrate (KMB) as an indicator for production of $OH^{\cdot}$-type reactive oxygen species by the NADPH-oxidase ("respiratory burst") was augmented by AAD and completely inhibited by superoxide dismutase. In contrast, ethylene release from 1-amino-cyclopropyl-1-carboxylic acid (ACC) as relatively specific indicator for the myeloperoxidase reaction after degranulation was not influenced by AAD. As documented by several model reactions, AAD *per se* did not act as scavengers or quenchers of activated oxygen species such as superoxide, $OH^{\cdot}$-radical, hydrogen peroxide or hypochlorite. Altogether, these results suggest that submicromolar concentrations of AAD upregulate the respiratory burst, but apparently not the degranulation of prestimulated polymorphonuclear leukocytes. At higher concentrations of AAD, both respiratory burst and degranulation are inhibited, however. These effects can also be shown in complete blood samples. BIOCHEM PHARMACOL **56**;1:141–152, 1998. © 1998 Elsevier Science Inc.

KEY WORDS. aminoadamantanes; polymorphonuclear leukocytes; respiratory burst; reactive oxygen species; *Morbus Alzheimer*; *Morbus Parkinson*

MEM† and AA are AADs used as drugs for the treatment of *Morbus Alzheimer* and *Morbus Parkinson* [1, 2] and other neurodegenerative diseases [3]. Interactions with 3,4-dihydroxyphenylalanine metabolism [4, 5] or acetylcholin excess [6, 7] have been discussed as reaction mechanisms. Other hypotheses concern nonspecific membrane effects [8], interactions with ion channels [9], amelioration of synaptic transmissions [10]) or influences on secondary messenger systems [11]. AAD might also act as noncompetitive NMDA-receptor antagonists, thus influencing the glutaminergic system [12]. Because ROS have been discussed in context with neuronal cell death [13], we investigated AAD (Fig. 1) as to their antioxidative potential or their activity as singlet oxygen quenchers similar to DABCO (Fig. 1). We found, however, that AAD acted neither as free radical scavengers nor as singlet oxygen quenchers like DABCO. However, AAD modulated leukocyte activities, i.e. oxygen activation by PMN after their preactivation by certain compounds such as zymosan, FMLP or A 23187. These effects could be measured both with isolated PMN and in complete blood.

MATERIALS AND METHODS

Materials

AAD were a generous gift from Dr. Quack, Merz & Co. All other chemicals and enzymes were of the highest analytical quality commercially available and obtained from Boehringer Mannheim (CAT E.C.11.1.6., peroxidase E.C.11.1.7., XOD E.C.2.3.2., DIA E.C.8.1.4.), Sigma (ACC, A 23187,

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† *Abbreviations:* AA, 1-adamantanamine; AAD, aminoadamantane derivatives; ACC, 1-aminocyclopropane-1-carboxylic acid; ASC, ascorbate; A 23187, experimental Ca^{++}-ionophore; CAT, catalase; α1-PI, a_1-proteinase inhibitor; CL, chemiluminescence; DABCO, diazabicyclo[2.2.2]octane; DIA, diaphorase; FMLP, formyl-met-leu-phe = leukotactic tripeptide; His, histidine; HRP, horseradish peroxidase; IAA, indolyl acetic acid; KMB, α-keto-γ-methiolbutyric acid; LIN, linolenic acid; MEM, memantine; Met, methionine; MPO, myeloperoxidase; NMDA, *N*-methyl-D-aspartate; PMA, phorbol-12-myristate acetate; PMN, polymorphonuclear leukocytes; RB, rose bengal; RLU, relative light units; ROS, reactive oxygen species; SOD, superoxide dismutase; TBARS, thiobarbituric acid reactive substances; and XOD, xanthine oxidase.

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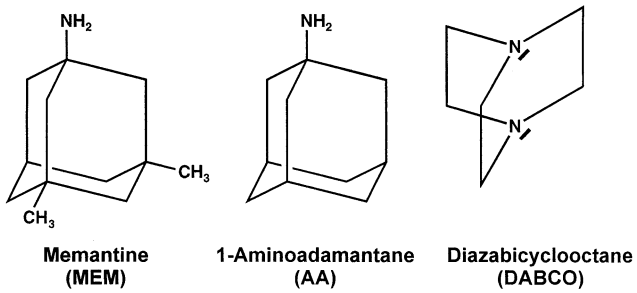

FIG. 1. Chemical structures of the different aminoadamantane derivatives (AAD) tested in comparison to diazabicyclo[2.2.2]octane (DABCO).

FMLP, DABCO, indole acetic acid, KMB, LIN, MPO, PMA, RB, SOD), or from Merck.

Methods

The following model reactions were conducted in order to evaluate the "radical scavenging" or "activated state-quenching" (singlet oxygen, $^1O_2$) activities:

Photodynamic reactions with riboflavin or RB as photoactivators were tested as ethylene release from KMB determined gas chromatographically as recently described [14] or as bleaching of the carotenoid, crocin [15], followed photometrically as a decrease of extinction at 440 nm. Two mL of the test system contained: 0.1 M of phosphate buffer (pH 7.4), 0.1 μM of riboflavin or 5 μM of RB, 1.5 mM of KMB and the substances to be tested. The test mixture was incubated under illumination (600 mE m^{-2} s^{-1}) for 30 min at 37°.

The XOD reaction was supplemented either with KMB as test for production of OH·-radical types or with nitroblue tetrazolium (NBT reduction) as test for superoxide ($O_2^{\cdot-}$) formation. Two mL of the KMB test system contained: 0.1 M of phosphate buffer (pH 7.4), 0.5 mM of xanthine, 1.5 mM of KMB and 0.08 U of XOD and the substances to be tested. The reaction was conducted for 30 min at 37° in the dark. Two mL of the NBT system contained: 50 mM of phosphate buffer (pH 7.8), 1.5 mM of EDTA, 0.5 mL of gelatine, 0.65 mM of phenazonium methosulfate (PMS), 1.36 mM of NBT, 6.0 mM of xanthine and the substances to be tested.

Diaphorase-catalyzed oxygen activation with NADH as electron donor in the presence of the naphthoquinone, juglone, as electron acceptor and catalyst of redox cycling was tested as ethylene release from KMB as described above. Two mL of the KMB test system contained: 0.1 M of phosphate buffer pH 7.4, 0.375 mM of NADH, 0.05 mM of juglone, 1 U of diaphorase (Sigma, pig heart; E.C.1.8.1.4), 1.5 mM of KMB. Incubation was carried out for 30 min at 37° in the dark.

IAA oxidation by HRP was followed by a test system essentially described in [16] as modified by [17]. Two mL of the test system contained: 150 mM of citrate-phosphate buffer (pH 5.6), 0.3 mM of IAA, 5 μM of vanillin, 0.3 U of HRP. Incubation was performed for 30 min at 37° in the dark. Nonoxidized IAA was determined photometrically at 530 nm after this incubation. For this purpose, 500 μL of the test reaction was treated for 14 min in the dark with 500 μL of the $FeCl_3$-H_2SO_4 reagent [18].

Ethylene formation from ACC driven by hypochlorite was followed as described in [14]. Two mL of the test system contained: 0.1 M of phosphate buffer (pH 7.4), 100 μM of NaOCl, 1.5 mM of ACC and the AAD to be tested.

The MPO-catalysis was followed either via ethylene formation from ACC [14] or by detection of CL during the reaction [19]. Two mL of the ACC test system contained: 0.1 M of phosphate buffer (pH 5.7), 150 mM of NaCl, 25 μM of H_2O_2, 1.5 mM of ACC, 0.1 U of MPO (E.C.11.1.7.) and the AAD to be tested. The reaction was conducted for 30 min at 37° in the dark. The determination of CL during the MPO reaction was performed as follows: 250-mL test solution contained 0.1 mL of phosphate buffer (0.2 M, pH 5.7), 150 mM of NaCl, 25 μM of H_2O_2, 80 μM of luminol, 0.05 U of MPO and the AAD to be tested. The CL was followed for 3 min with an ML 3000 Microtiter Plateluminometer (Dynatech Labs).

In the elastase/α_1-PI-inhibitor system, α_1-PI prevents tissue destruction by leukocyte-derived elastase. However, α_1-PI can be inactivated by hypochlorite [20], thus permitting the proteolytic and destructive activities of elastase. Potential prevention of α_1-PI destruction by OCl^- was followed essentially as described in [21]. The result of this test system must be seen in the context of the result obtained with the ACC-OCl^- system described above.

Lipidperoxidation was measured as ethane release, TBARS (thiobarbituric acid reactive substance) or CL from peroxidized a-linolenic acid. Reactive oxygen species were produced either by XOD-xanthine (see above) or by RB in the light. the CL SYSTEM (250 μL) contained: 0.1 M of phosphate buffer (pH 7.4), 0.5 mM of xanthine, 80 μM of luminol, 0.08 U of XOD, 2.8 mM of α-LIN and the AAD to be tested. The reaction was followed for 3 min.

The test system for ethane release from 3.55 mM of α-LIN was identical to the one just mentioned except that 1 mM of Fe^{2+} was included instead of 80 μM of luminol. Ethane was determined gas chromatographically as described [22, 23] after a 30-min reaction at 37° in the dark.

TBARS was determined after incubation of α-linolenic acid either in the XOD or in the RB system (see above). After the incubation, TBARS was detected photometrically at 535 nm after incubation for 30 min at 95° of 500 μL of the corresponding reaction mix with 1 mL of the TBA mix.

Neutrophils were isolated from the blood of sacrificed pigs [24].

Whole Blood Experiments

Experiments with complete blood contained 1 mL of blood sample (pig) in PBS buffer containing the described additions in 2 mL. Five hundred mL of blood was withdrawn

TABLE 1. Photodynamic ethylene release from KMB and crocin bleaching

System % Reaction	pmol Ethylene KMB/RF	%	pmol Ethylene KMB/RB	%	E_{440nm} Crocin/RB	%
Control	4505 (232)	100	66283 (2718)	100	0.244 (0.01)	100
+MEM	3720 (174)	82	54461 (3835)	82	0.248 (0.013)	102
+AA	3901 (234)	86	58278 (2334)	88	0.243 (0.01)	99
+DABCO	4732 (283)	105	37461 (5724)	57	0.118 (0.002)	48
+ASC	9261 (118)	206	7478 (348)	11	0.353 (0.014)	145
+MET	3447 (92)	77	13462 (867)	20	0.310 (0.009)	127
+His	5968 (124)	132	16796 (2288)	25	0.177 (0.007)	73
+SOD	2135 (25)	47	28245 (1745)	43	0.161 (0.001)	66
+CAT	1958 (23)	44	36333 (2329)	55	0.256 (0.022)	105
+SOD + CAT	1453 (25)	32	20450 (2562)	31	0.165 (0.002)	68

Additions: 10^{-3} M or 100 U. Deviations: in brackets.

from freshly sacrificed pigs and mixed with 20 mL of 2.5% EDTA and used within 5 hr after storage at 5°. The following test systems were used: a) CL with luminol for the determination of "overall" activation of PMN; b) KMB fragmentation for the determination of superoxide-based ROS produced by the respiratory burst; and c) ACC fragmentation for the determination of MPO activity [14].

The following incubations with complete blood were conducted in order to evaluate the different activities of PMN. The CL test system (250 μL) contained: 150 μL of complete blood, 1 mg of zymosan (or 2 μM of PMA), 120 μM of luminol, different concentrations of AAD and PBS buffer (pH 7.4). CL was followed for 3 min with an ML 3000 Microtiter Photoluminometer (Dynatech Labs).

Two mL of the KMB test system contained 1 mL of complete blood, PBS buffer (pH 7.4) and 2 mL, 3 mM of ACC, different concentrations of the AAD to be tested, and 2.5 mg of nonopsonized zymosan. The reaction was conducted for the times indicated in the individual figures at 37° in the dark.

Two mL of the ACC test system contained 1 mL of complete blood, PBS buffer (2 mL, pH 7.4), 3 mM of ACC, different concentrations of the AAD to be tested and 2.5 mg of nonopsonized zymosan. The reaction was conducted for the times indicated in the individual figures at 37° in the dark.

TABLE 2. KMB fragmentation by XOD or DIA

System % Reaction	pmol Ethylene KMB/XOD	%	pmol Ethylene KMB/DIA	%
Control	8907 (223)	100	9878 (492)	100
+MEM	7499 (224)	84	8168 (516)	83
+AA	7273 (316)	82	8450 (596)	86
+DABCO	7524 (374)	85	7838 (194)	79
+ASC	28581 (2074)	321	11958 (568)	121
+MET	4566 (324)	51	7130 (171)	72
+His	5595 (219)	63	5534 (40)	56
+SOD	155 (23)	2	3059 (218)	31
+CAT	1750 (125)	20	2757 (271)	28
+SOD + CAT	125 (6)	1	561 (52)	0.5

Additions: 10^{-3} M or 100 U. Deviations: in brackets.

For more details, see [25] and details below. All experiments were repeated twice at least in triplicate and standard deviations are expressed as $s_{(n-1)}$.

RESULTS

Antioxidative Properties of AAD

In order to test for the potentially antioxidative properties of AAD, MEM and AA were investigated in different more or less well-known test systems [26] and as described in more detail in [18, 27]. In all test systems, concentrations of MEM, AA or DABCO between 10^{-7} M and 10^{-3} M were tested. Because all concentrations below 10^{-4} M were without effect, only the data for 1-mM concentrations are shown.

Photodynamic Reactions

As described in Methods, photodynamic ethylene release from KMB or crocin bleaching was driven by either riboflavin or RB in the light. The effects of MEM, AA and DABCO (all 1 mM) on these reactions were compared with several known inhibitors (also 1 mM or 100 U) as shown in Table 1. It is evident from the table that in the KMB/riboflavin system only SOD and CAT significantly inhibited the reaction, whereas in the KMB/RB system,

TABLE 3. MPO-catalyzed ACC fragmentation and light emission

System % Reaction	pmol Ethylene MPO/ACC	%	RLU MPO/CL	%
Control	856 (26)	100	8.31 (0.55)	100
+MEM	846 (25)	99	8.20 (0.6)	99
+DABCO	261 (9)	30	6.94 (0.5)	84
+ASC	225 (28)	26	0.13 (0.01)	2
+MET	39 (1)	5	0.20 (0.1)	2
+His	383 (16)	45	2.62 (0.21)	32
+SOD	701 (47)	82	8.13 (0.73)	98
+CAT	0 (0)	0	0.13 (0.0)	2
+SOD + CAT	0 (0)	0	0.07 (0.0)	1

Additions: 10^{-3} M or 100 U. Deviation: in brackets.

TABLE 4. Effects of MEM and several antioxidants on LIN oxidation by XOD and Fe^{2+}

System % Reaction	Ethane (pmol)	%	TBARS (E_{530})(E_{530})	%	CL (RLU)	%
Control	1836 (55)	100	0.709 (0.05)	100	4.80 (0.32)	100
+MEM	2572 (67)	140	0.861 (0.05)	121	6.75 (0.31)	127
+ASC	1485 (119)	81	0.905 (0.08)	128	0.00 (0)	0
+MET	2271 (160)	124	0.694 (0.06)	98	2.27 (021)	47
+His	1468 (57)	80	0.642 (0.05)	91	2.62 (24)	55
+SOD	3201 (51)	174	0.537 (0.04)	76	0.17 (0.01)	4
+CAT	2266 (206)	123	0.730 (0.07)	102	0.97 (0.02)	20
+SOD + CAT	2641 (218)	144	0.621 (0.06)	88	0.15 (0.01)	3

Additions: 10^{-3} M or 100 U. Deviations: in brackets. RLU after 22.5 sec reaction.

ASC, Met and His were good inhibitors and DABCO, SOD and CAT inhibited by 43%, 57% and 45%, respectively. Crocin bleaching by RB was best inhibited by DABCO (52%) and SOD (34%). In all systems, MEM and AA exhibited little (up to 18%) or no effect. Because RB and riboflavin catalyze photodynamic reactions of both type I and II to different degrees [28], the differential effects of SOD and of other antioxidants may thus reflect different degrees of production of different oxidizing species. The effect of DABCO, which is a better $^1O_2$ generator than riboflavin, in the RB system can be explained as quenching of $^1O_2$ produced by RB catalysis.

XOD- and DIA-Catalyzed Reactions

XOD and DIA in the presence of appropriate autoxidizable electron acceptors produce both superoxide and hydrogen peroxide and, perhaps unspecifically via bound or free (contaminated) iron ions, $OH^{\cdot}$- radicals [29, 28]. We tested the influence of certain antioxidants in comparison to MEM and AA (Table 2). Ethylene release from KMB was stimulated by ascorbic acid and strongly inhibited by SOD, CAT and even more strongly by SOD and CAT. Met and His inhibited MEM by less than 50% and AA by less than 20%.

IAA Oxidation by HRP

In this test system, only MEM, AA and DABCO were compared. Neither of these compounds had an influence on IAA oxidation by HRP (data not shown).

Ethylene Formation from ACC and α_1-PI Inactivation by Hypochlorous Acid

ACC fragmentation by OCl^--forming ethylene was not influenced by MEM, AA or DABCO (data not shown). Since, however, amino groups can form reactive chloramines which also might form ethylene from ACC (Schempp H and Elstner EF, unpublished results), effects of AAD might have masking effects on ACC fragmentation. We therefore investigated a1-protease inactivation as an ACC-independent detector system for the protective effects of AAD and thus their reaction with HOCl. Neither the elastase reaction per se nor the inhibition of α_1-PI was influenced by either MEM, AA or DABCO (data not shown).

The MPO Reaction

The effect of MEM on MPO-catalyzed ACC fragmentation and on CL (after a 22.5-min reaction) was compared with the effects of several antioxidants (Table 3). As shown in Table 3, MEM had no influence on either ACC fragmentation or on CL via the MPO system. The strongest inhibition of the ACC system was documented for CAT (which is more or less trivial, because hydrogen peroxide was added to the test system) and Met. DABCO, ASC and His inhibited by 70%, 74%, and 55%, respectively. In the CL system, Met and ASC were especially strong inhibitors whereas the effect of CAT was again trivial.

Lipidperoxidation

Lipidperoxidation was studied with LIN where different products and indicators were determined.

TABLE 5. Ethane and TBARS formation during peroxidation of LIN by RB in the light

System % Reaction	Ethane (pmol)	%	TBARS (E_{530})	%
Control	335 (17)	100	1.039 (0.02)	100
+MEM	600 (27)	180	1.285 (0.04)	124
+ASC	4692 (336)	1400	0.928 (0.04)	89
+MET	316 (21)	94	0.879 (0.03)	85
+His	234 (14)	80	0.823 (0.04)	79
+SOD	237 (19)	70	0.723 (0.07)	70
+CAT	319 (26)	95	0.957 (0.05)	92
+SOD + CAT	222 (26)	43	0.805 (0.04)	78

Reaction conditions: 10^7 cells in 1 mL of PBS buffer supplemented with 2.5 mg of opsonized zymosan and with 1.5 mM of KMB; reaction for 15 min at 37° in the dark. Additions: 10^{-3} M or 100 U. Deviations: in brackets.

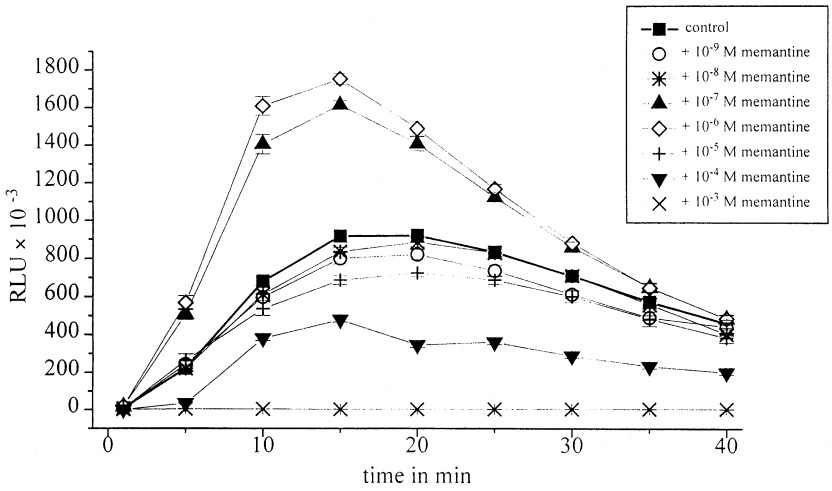

FIG. 2. Effects of different concentrations of memantine on chemiluminescence of isolated zymosan-stimulated neutrophils. For experimental conditions see Materials and Methods.

XOD AS OXYGEN ACTIVATOR. Oxygen activation by XOD in the presence of LIN and Fe^{2+} (1 mM) yields ethane, TBARS and CL. The activity of MEM and several antioxidants were studied in these systems (Table 4). This result indicates that lipid peroxidation, measured by three different parameters, was enhanced by 1 mM of MEM. The other compounds tested demonstrated that the effects on ethane, TBARS and CL were hardly comparable as to their generation from linolenic acid, because one and the same antioxidant may have a scarcely predictable influence. This was especially true of ASC, SOD and CAT, which stimulated the generation of one product and inhibited the production of another at the same time. This indicates that their generation from LIN was based on different mechanisms although the oxidative initiation was identical.

RB AS OXYGEN ACTIVATOR IN THE LIGHT. The production of ethane and TBARS as indicators of lipid peroxidation was tested in the RB system (Table 5). Ethane and TBARS formation from linolenic acid was again stimulated by MEM; ASC stimulated ethane formation but not TBARS. All other antioxidants exhibited little influence on either ethane or TBARS. The results obtained in this section clearly indicate that MEM (and likewise AA) did not act as antioxidants and scarcely interfered with redox reactions.

Modulation of Leukocyte Activities by AAD

Respiratory burst and degranulation of isolated PMN was initiated by several compounds such as opsonized zymosan, FMLP, A 23187 or PMA. During this process of oxygen reduction, superoxide and hydrogen peroxide were produced. $OH^{\cdot}$- radical and hypochlorous acid were secondary products due to the catalytic activities of traces of iron ions and secreted MPO, respectively. Effects of AAD on the production of ROS by zymosan-, FMLP-, A 23187- or PMA-prestimulated PMN were monitored by CL quantification and by ethylene formation from either ACC or KMB.

LUMINOL-ENHANCED CL OF ZYMOSAN-STIMULATED PMN. As shown in Fig. 2, low concentrations (10^{-7} M to 10^{-6} M) of MEM strongly enhanced zymosan-elicited CL,

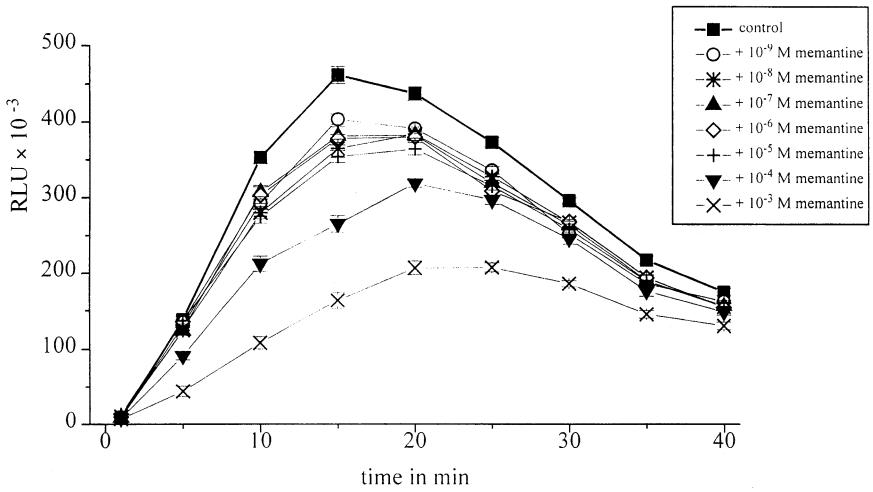

FIG. 3. Effects of different concentrations of memantine on chemiluminescence of isolated phorbol ester-stimulated neutrophils. For experimental conditions see Materials and Methods.

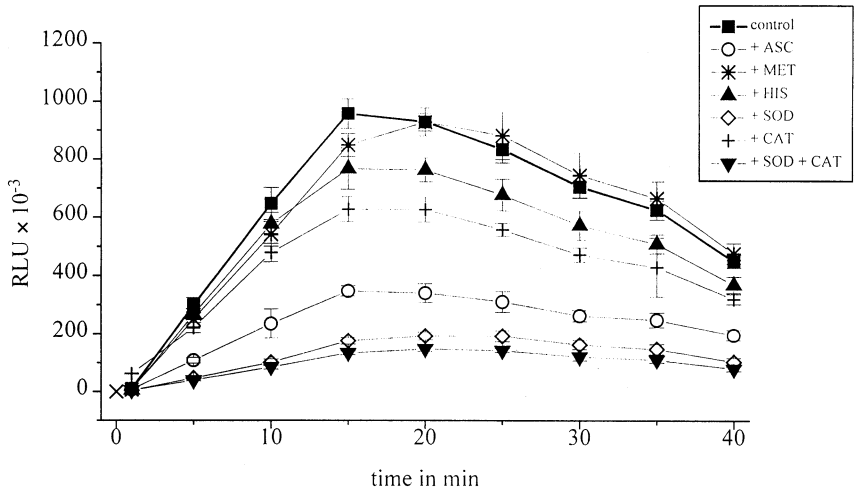

FIG. 4. Effects of different antioxidants on chemiluminescence of isolated zymosan-stimulated neutrophils. For experimental conditions see Materials and Methods.

whereas higher concentrations (10^{-4} M to 10^{-3} M) were inhibitory. The maximum CL was visible after 15 min. At this point, the reaction was stimulated by 1 mM of MEM by 91% and inhibition by 1 mM of MEM was practically 100%. DABCO in different concentrations from 0.1 mM up to 1 mM had no influence on CL after 15 min. At longer times (20 to 40 min), only 1 mM of DABCO stimulated CL by approximately 40%. The AAD (see Fig. 1) exhibited almost identical properties as MEM (data not shown). Prestimulation by FMLP or A 23187 yielded equivalent results to those observed with zymosan-stimulated PMN. The effects of AAD on CL by PMA-prestimulated PMN were different, however, because no enhancement but only inhibition by increasing AAD concentrations was visible (Fig. 3). Luminol-enhanced CL was inhibited by 1 mM of ASC or by 100 U of SOD by 74% and 81%, respectively, while Met or His had little effect (Fig. 4).

Lucigenin-enhanced CL by prestimulated PMN. Lucigenin as enhancer of CL yielded very similar results as compared to luminol-enhanced CL by prestimulated cells. The effects of AAD were also equivalent to the luminol data reported above. One difference, however, was that SOD inhibited lucigenin-enhanced CL by 100% and not by 74% as reported above (data not shown).

TABLE 6. Effects of MEM, AA and DABCO on ethylene release from KMB by zymosan-stimulated PMN

Additions (M)	Ethylene formed from KMB (pmol/15 min)					
	MEM	%	AA	%	DABCO	%
0 (Control)			306			100
10^{-9}	326 (4)	107	334 (11)	109	299 (5)	98
10^{-7}	410 (22)	134	398 (24)	131	304 (10)	99
10^{-6}	392 (26)	130	384 (16)	126	298 (8)	97
10^{-5}	315 (23)	103	339 (18)	111	309 (10)	101
10^{-3}	120 (7)	39	132 (9)	43	186 (4)	61

Ethylene Formation from KMB

Activated PMN (10^7 cells, in the presence of 2.5 mg of opsonized zymosan and 1.5 mM of KMB in 1 mL of PBS buffer) oxidized KMB in a time-dependent manner, linearly producing approximately 1,600 pmol of ethylene within 1 hr. After this time, there was no further reaction. If we compare the rates of ethylene formation after a 15-min reaction time, 10^{-7} M and 10^{-6} M of either added MEM or AA enhanced the reaction, whereas 10^{-3} M of these compounds were inhibitory (Table 6).

In comparison to MEM or AA, several antioxidants were tested in terms of their effects on KMB fragmentation by PMN in another experiment. As shown in Table 7, this reaction was strongly inhibited by 1 mM of MEM, AA, ASC or His, or by 100 U of SOD.

Ethylene Formation from ACC by Isolated PMNs

After appropriate stimulation by zymosan, isolated PMN produce HOCl which rapidly react with ACC to form ethylene [14]. When an identical experiment as described for Table 7 was conducted in the presence of 1.5 mM of

TABLE 7. Effects of several antioxidants on ethylene formation from KMB

Additions (1 mM or 100 U)	Ethylene (pmol/15 min)	%
None (Control)	305 (12)	100
MEM	120 (7)	39
AA	132 (9)	43
DABCO	253 (4)	83
ASC	26 (1)	8
MET	200 (5)	66
His	152 (6)	50
SOD (100U)	0 (0)	0
CAT (100U)	428 (11)	140
SOD + CAT	0 (0)	0

Reaction conditions were identical to those decribed in Table 6.

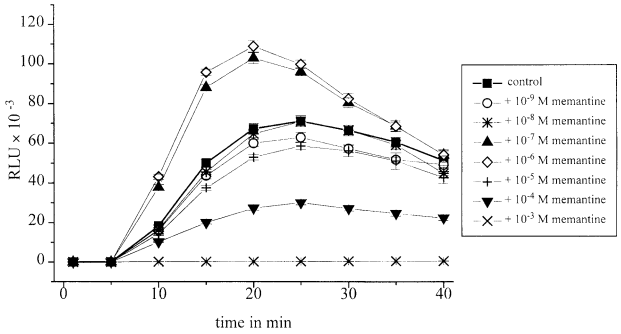

FIG. 5. Effects of different concentrations of memantine on luminol-enhanced chemiluminescence of zymosan-stimulated neutrophils in complete blood. For experimental conditions see Materials and Methods.

ACC instead of KMB, approximately 550 pmol of ethylene were formed linearly within 15 min. After 15 min, this activity continued for a further 60 min at approximately one third of this initial velocity. Neither MEM, AA nor DABCO had the slightest effect on this reaction at a concentration range between 10^{-9} M and 10^{-4} M (data not shown). With 1 mM of MEM or AA, a 60% inhibition was observed, while 1 mM of DABCO only showed a negligible effect of nearly 17% inhibition. When 1 mM of concentrations of several antioxidants or 100 U of SOD or CAT were tested in this ACC system, only His had a strong inhibitory effect (85% inhibition), whereas ascorbate (35% inhibition) and CAT (46% inhibition) were less active and SOD was without effect.

Modulation of Leukocyte Activities in Complete Blood by AAD

Respiratory burst and degranulation of isolated PMN was initiated by several compounds such as opsonized zymosan, FMLP, A 23187 or PMA. During this process of oxygen reduction, superoxide and hydrogen peroxide were produced; $OH^{\cdot}$-radical and hypochlorous acid were secondary products due to the catalytic activities of traces of iron ions and secreted myeloperoxidase, respectively. Effects of AAD on the production of reactive oxygen species by zymosan-, FMLP-, A23187- or PMA-prestimulated PMN were monitored by CL quantification and by ethylene formation from either ACC or KMB.

Luminol-enhanced CL of zymosan-stimulated PMN. As shown in Fig. 5, low concentrations (10^{-7} M to 10^{-6} M) of MEM strongly enhanced zymosan-elicited CL with luminol (Fig. 5) as CL enhancer. Higher concentrations (10^{-4} M to 10^{-3} M) were inhibitory. The maximum CL was visible after 20 min. At this point, the reaction was stimulated by 0.1 mM and 1 mM of MEM by approximately 80%, and inhibition by 1 mM of MEM was practically 100% in both luminol- and lucigenin-enhanced CL systems.

DABCO at various concentrations from 0.1 mM to 1 mM had no influence on CL after 15 min. At longer periods of time (20 to 40 min), only 1 mM of DABCO stimulated CL (data not shown). Prestimulation by FMLP or A23187 yielded no significant CL enhancement. The effects of MEM on CL by PMA-prestimulated PMN were different, however, because no enhancement but only inhibition by increasing MEM concentrations was visible (data not shown).

Ethylene Formation from KMB

Activated PMN (10^7 isolated PMN cells in the presence of 2.5 mg of opsonized zymosan and 1.5 mM of KMB in 1 mL of PBS buffer) oxidized KMB in a time-dependent manner, linearly producing approximately 1,600 pmol of ethylene within one hour. If the same reaction was performed with 1 mL of complete blood in 1 mL of PBS buffer with 2.5 mg of nonopsonized zymosan and 3 mM of KMB, a biphasic rate

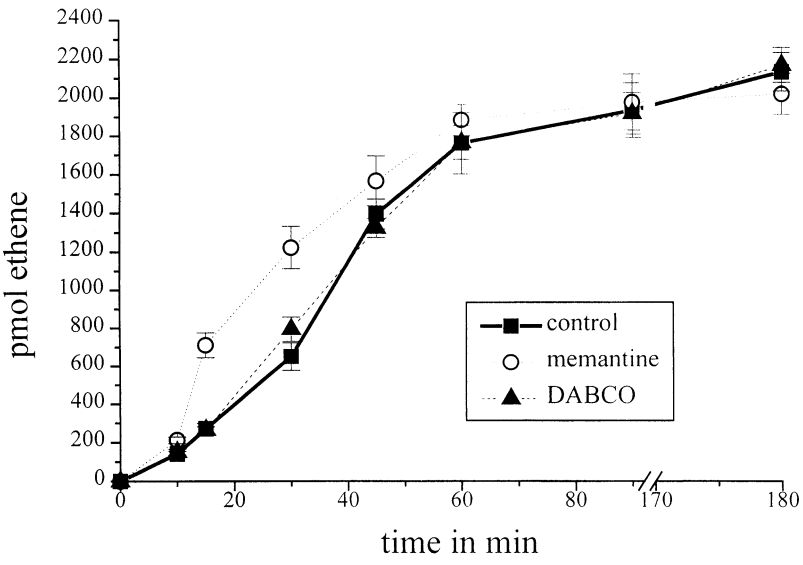

FIG. 6. Kinetics of 10^{-6} M of memantine or DABCO on KMB fragmentation in the presence of 10^{-3} M of Fe^{3+} by zymosan-stimulated neutrophils in complete blood. For experimental conditions see Materials and Methods.

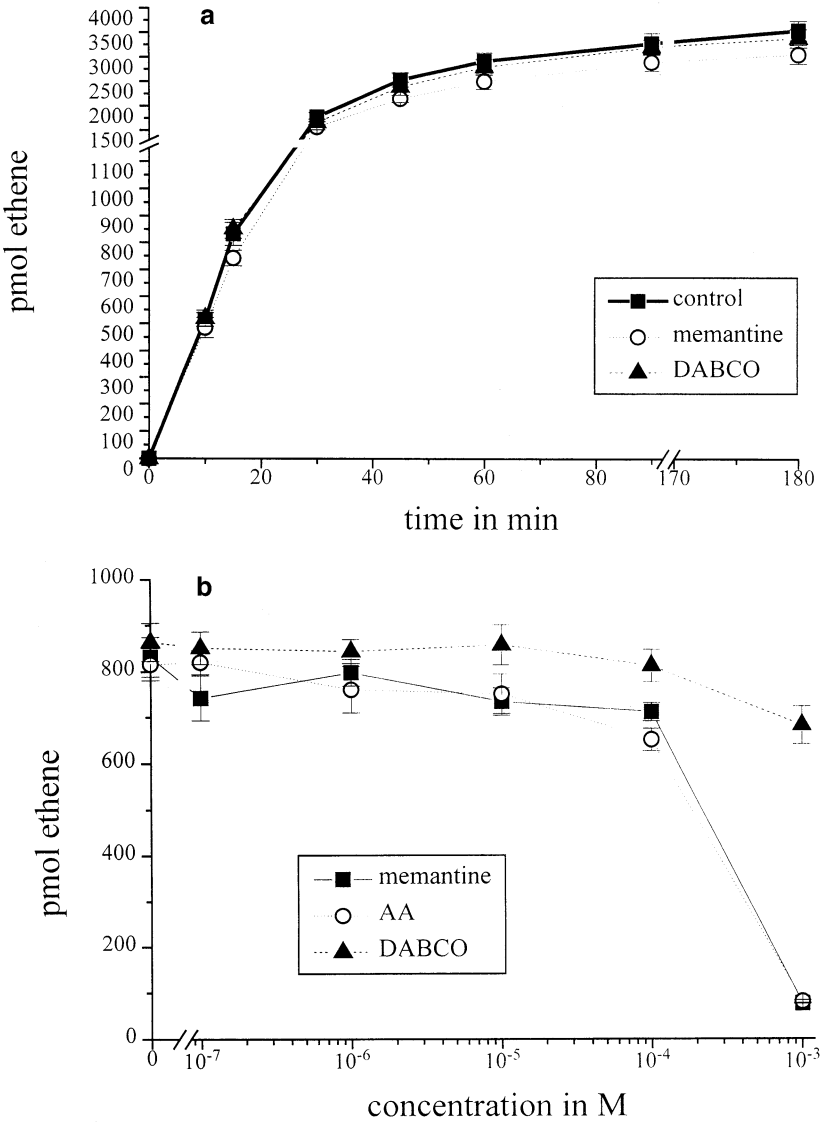

FIG. 7. (a) Kinetics of 10^{-6} M memantine or DABCO on ACC fragmentation by zymosan-stimulated neutrophils in complete blood; (b) effect of different concentrations of memantine, AA and DABCO on ACC fragmentation by zymosan-stimulated neutrophils in complete blood after a 15-min incubation. For experimental conditions see Materials and Methods.

of ethene production was observed, lasting at least 3 hr. The presence of 1 mM of MEM or DABCO had no influence on this reaction (data not shown). If Fe^{3+}-ions were added to this incubation mixture, an enhancement of ethene formation at Fe concentrations higher than 500 μM was observed (data not shown). Under these conditions (presence of 1 mM of Fe^{3+}-ions), a stimulating effect of 1 mM of MEM but not of DABCO was measurable between 10 and 45 min of incubation, where the strongest enhancement was visible at 15 and 30 min (Fig. 6).

Ethylene Formation from ACC

After stimulation by zymosan, isolated PMN produce HOCl, which rapidly reacts with ACC to form ethylene [14]. If similar experiments as described for isolated PMN were conducted in the presence of complete blood and 3 mM of ACC instead of KMB, approximately 1700 pmol of ethene were formed quasi-linearly within 30 min. After 30 min, this activity continued for a further 150 min at a greatly reduced velocity. Neither MEM nor DABCO had any effect on this reaction (Fig. 7a). In contrast to the KMB system, addition of Fe^{3+} ions had no effect on ethene release from ACC. Likewise, increasing concentrations of MEM or AA had no stimulatory effect at ca. of 1 mM; only a higher concentration than 100 μM inhibited the reaction both in the absence or in the presence of Fe^{3+} ions as compared to the DABCO control (Fig. 7b).

DISCUSSION

Neurodegenerative diseases, especially those affecting elderly people, appear to be increasing in industrialized countries, undoubtedly connected with increasing life spans. *Morbus Parkinson* and *Morbus Alzheimer* [30] are the most important and widespread diseases in this respect [31]. Therapeutic possibilities, however, are limited to amelioration of the symptoms, definitive cures not being available at

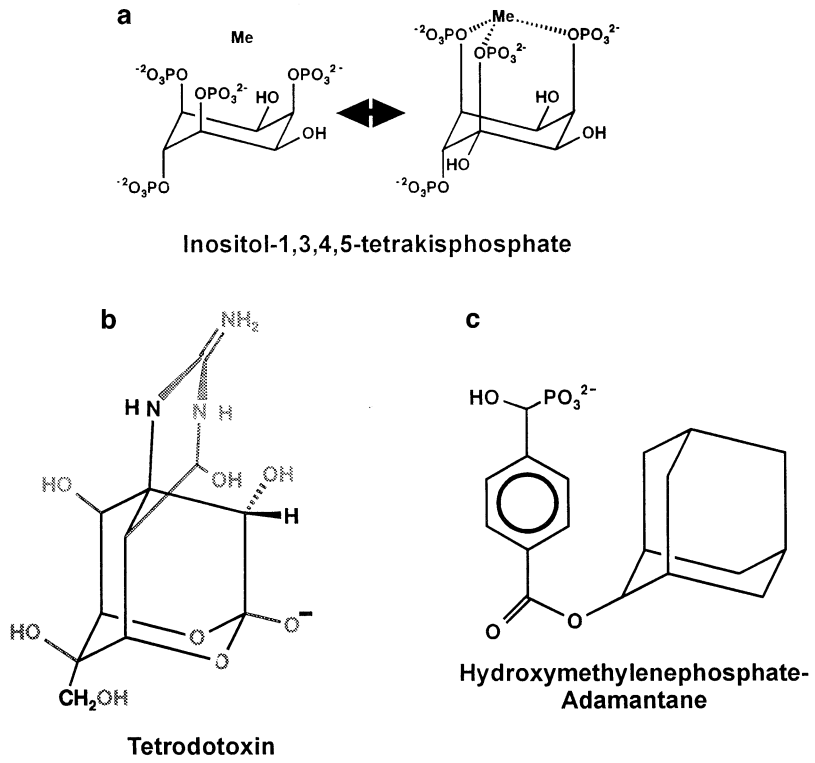

FIG. 8. Chemical structures of three different compounds acting as inhibitors or effectors in signal transduction. (a) Metal chelate of inositol-polyphosphate; (b) tetrodotoxin; (c) derivative of hydroxymethylenephosphonate.

present. MEM as a therapeutic drug (Akatinol®) may act as a noncompetitive NMDA receptor antagonist yielding positive results in both disease areas. This is thought to be due to the limited access of glutamate to this receptor, thus reducing harmful calcium influx into neurons [32]. There are several indications that pro-oxidative conditions in brain areas might be involved in the provocation of these disease symptoms [33, 34]. Very recently, nitrotyrosin as an indicator for peroxynitrite breakdown was identified in neurofibrillary tangles from Alzheimer patients [35]. This marker of oxidative protein damage is derived from the interaction of superoxide with NO, both stemming from activated neutrophils, macrophages or microglia. It was of utmost interest, therefore, to investigate possible antioxidative activities of aminoadamantane derivatives which might partially contribute to the ameliorating effects of these drugs. Because a superficial look at the chemical structures of aminoadamantanes suggests a certain similarity with the well-known singlet oxygen quencher, DABCO, the latter has been used as a "reference" molecule in the investigation of general antioxidative properties of AAD. For this purpose, several radical-generating as well as relevant radical-indicator systems have been tested in this context. The potential effects of DABCO were compared to MEM and AA as representative AAD (see Fig. 1). As outlined above, photodynamic reactions with RB as photoactivator and HOCl or enzymic oxygen-activating reactions (HOCl, XOD, DIA, HRP, MPO) were used in the presence of several, very differently responding indicators for ROS activity or ROS damage such as KMB, ACC, crocin, indole acetate, a1-PI, linolenic acid peroxidation (TBARS) and CL. In addition, AAD and DABCO were compared to well-known antioxidants such as ASC, His, Met, SOD and CAT. As demonstrated above, there was no indication that one of the tested AAD as compared to DABCO and other more or less specific radical scavengers, activated state quenchers or antioxidants acted as singlet quenchers, radical scavengers or generally as antioxidants. The test systems studied cannot comprise the whole scale of possible biologic scenarios concerning oxygen activation and protection against the deleterious effects of these reactive species. They nevertheless cover a representative range of standard activating and indicator reactions with relevant connections to *in vivo* situations. It can be concluded from these results with great certainty that AAD may not act as antioxidants in protecting brain cells from the deleterious influences of ROS produced at glutamate-activated receptor sites.

On the other hand, as shown in Results, AAD can operate as immunomodulators upregulating the respiratory burst in PMN at micromolar concentrations and downregulating this response at concentrations in the millimolar range. Upregulation only concerns PMN prestimulated by activators other than PMA. Upregulation can be shown with either luminol- or lucigenin-enhanced CL or with KMB as indicator for ROS other than HOCl. With ACC as a relatively specific indicator for HOCl production [14], no AAD enhancement of PMN activation could be seen. This likely indicates that no further enhancement of myeloperoxidase extrusion via degranulation by the white blood cells occurs in the presence of AAD. Thus, only the respiratory burst and not degranulation seems to be upregulated by 0.1

μM of AAD. PMN, in addition to macrophages, represent one important component of the blood cell-intrinsic immune system responsible for the ingestion of various xenobiotic particles. Acute inflammations cause massive local accumulation of PMN [36] which, after activation by antigens, produce ROS via the respiratory burst [37, 38] and release oxidizing and degradative enzymes such as myeloperoxidase, lysozyme, acid hydrolases and elastase [39].

The effects described in Results can also be shown for activated neutrophils in whole blood. As described above, luminol-enhanced CL was enhanced in whole blood samples by 10^{-7} and 10^{-6} M of MEM (Fig. 5). Following the effects on the "respiratory burst" with the KMB method, MEM stimulated ethene release (in the presence of 10^{-3} M of Fe^{3+} in the blood samples) in the time course between 15 and 30 min. This stimulating effect was lost after 50 min of incubation (Fig. 6). In contrast, MEM had no stimulating effect on ethene release from ACC as an indicator for degranulation (Fig. 7a). A higher concentration than 10^{-4} M of MEM (data not shown) or AA inhibited the ACC reaction (Fig. 7b), however. These results are in agreement with the observations made with isolated PMN. The results shown above represent a new possibility for a rapid assay of neutrophil activation in whole blood.

Activated PMN seem to play an important role in inflammatory processes in the brain after traumatic injury [40] and after reperfusion of cerebral ischemia [41]. Very recently, immune reactions have been suggested to be involved in neuronal destruction in Morbus Parkinson [42], and chronic brain ischemia seems to be characteristic for M. *Alzheimer* [43], because ca. 60% of Alzheimer patients were shown to exhibit ischemic atrophia [44].

In the light of the present results, it is of interest to note that besides PMN certain brain cell types are involved both in the production of and the protection from deleterious effects of ROS: microglia cells as well as PMN stem from promonocytes [45] and exhibit phagocytosis under production of ROS after activation by xenobiotic particles [46, 47]. During the pathogenesis of M. *Alzheimer*, a chronic inflammatory process is discussed [48], possibly initiated by activated microglia cells [49]. On the other hand, astrocytes are known to: a) produce glutamate [50]; b) modulate NMDA receptors [51]; and c) have neuroprotective functions against deleterious ROS effects [52]. Effects of AAD on astrocytes have not been investigated, however.

How can the effects of AAD on PMN be explained in the light of their positive activity in neurodegenerative diseases? First, it is important to stress that AAD stimulate PMN respiratory burst (CL; KMB fragmentation), but apparently not degranulation (ACC fragmentation) at low concentrations (at ca. 10^{-6} M), but inhibit both the respiratory burst and degranulation at higher concentrations (approximately millimolar). AAD, like MEM, seem to interact with membranes and accumulate intracellularly after multiple applications. This conclusion can be drawn on the basis of the observations that: a) antiviral activity is apparently due to the interaction during "enveloping" inside animal cells [53]; and b) MEM concentrations accumulated 200-fold after multiple administration, whereas the application of a single dose was reversible [54]. These observations allow the speculation that stimulation of preactivated peripheral blood cells by AAD is likely to occur, because MEM concentrations in the micromolar range are obtained in the peripheral blood stream. Inhibition of activated, tissue-bound cells such as microglia may be achieved by accumulation of MEM after multiple administration. Thus, both down- and up-regulation of ROS formation by preactivated immunoreactive cells has to be envisaged.

What is the possible pathway of signal transmission by AAD? This question may be answered by combining several reports and reasonable assumptions:

1. AAD exhibit common features, with three distinct compounds known to act in signal transducing pathways or inhibitors: the metal chelate of inositol polyphosphate, the adamantane derivative of benzoic acid *p*-hydroxymethylenphosphonate and tetrodotoxin, the poison of the "ball-fish" *Spheroides porphyreus* (Fig. 8).
2. Inositolphosphates play a crucial role in cellular signal transduction [55, 56]. Ignoring electrical charges, the elementary structure of the metal chelate of inositoltetraphosphate [57] is indeed very similar to the AAD skeleton.
3. An adamantane derivative of benzoic acid-*p*-hydroxymethylene phosphonate, (such as Li^{+}-ions; [58]), is a strong inhibitor of inositol monophosphatase with an inhibition constant of 6.3 mM [59], which is exactly in the range of values reported for upregulation of prestimulated PMN in this communication. This property is thought to play a role in the treatment of neuropathological disorders [60, 61].
4. Tetrodotoxin blocks Na^{+}-ion channels and has neuroprotective properties [62]; Na^{+}-neurotoxicity has to be seen in the context of Ca^{++}-influx and ATP depletion and thus cell death.

Although no conclusive answer can be given as to the mechanisms operating during up- and down-regulation of the activity of prestimulated PMN by AAD, it seems to be clear that scavenging or quenching of ROS by AAD can be excluded, and an interaction with intracellular signal transduction seems to be indicated.

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