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DIMETHYL SULFOXIDE: INHIBITION OF ACETYLCHOLINESTERASE IN THE MAMMALIAN HEART

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Abstract—The heart rate of the isolated, perfused, working rat heart was significantly and equally depressed by 1×10^{-6} M acetylcholine (ACh) and by 6×10^{-5} M 4-ketoamyltrimethylammonium (4K), a cholinomimetic agonist. Dimethyl sulfoxide (DMSO) (10 μl/ml, 140 mM) strongly potentiated the effect of ACh but did not alter the effect of 4K. DMSO (10 μl/ml, 140 mM final concentration) alone had no significant effect upon heart rate when added to the perfusate in incremental additions of 1 μl·(ml perfusate)$^{-1}$·min^{-1} over a 10-min period. The specific activity of atrial homogenate cholinesterase was 48.8 ± 3.46 nmoles·min^{-1}·(mg protein)$^{-1}$ (mean ± S.E.M.), 38.2 ± 1.60 for butyrylcholinesterase, and 11.2 ± 0.86 for acetylcholinesterase (AChE). True AChE activity (measured in the presence of a maximally effective concentration of tetraisopropylpyrophosphoramide) had a V_{max} of 13.4 ± 0.17 nmoles·min^{-1}·(mg protein)$^{-1}$ and an apparent K_m value of 1×10^{-4} M acetylthiocholine. At this K_m substrate concentration, DMSO inhibited atrial AChE activity ($I_{50} = 9$ μl/ml). At the concentration tested, DMSO inhibited atrial AChE and potentiated ACh effects.

Dimethyl sulfoxide (DMSO) has received attention for its ability to penetrate through the skin [1] and its analgesic and anti-inflammatory effects [2, 3]. DMSO, however, has other pharmacological actions (reviewed in Ref. 4). Sams and Carroll [5] and Sams *et al.* [6] suggested that DMSO inhibits cholinesterase (ChE), but few studies have tested this hypothesis further.

We have demonstrated recently that DMSO inhibits acetylcholinesterase (AChE) in several muscle systems of invertebrate animals [4]. This conclusion is founded upon both pharmacological and enzymatic data. DMSO is a competitive inhibitor of AChE with respect to substrate acetylthiocholine as determined by enzymatic assays of purified eel preparations and homogenates of oyster hearts [4]. In addition, the concentration of DMSO required for AChE inhibition in oyster hearts is the same as that required for the potentiation of the acetylcholine (ACh) effects observed in pharmacological experiments [4]. In the present report, we have tested a mammalian heart in order to determine if DMSO inhibits AChE.

Using mammalian cardiovascular systems, some effects of DMSO have been described. These effects, however, have been described in studies *in vivo* where the mechanisms of action of DMSO were not fully tested [7, 8], or in studies *in vitro* which had pharmacological data only [5, 6] or which used concentrations of DMSO that acted without the addition of ACh or nervous activity [9–11]. It is necessary to test in the mammalian heart for the interaction of DMSO with exogenously applied ACh, using a concentration of DMSO which has no action when added alone.

Cholinergic effects through nerve stimulation are enhanced by DMSO in isolated atria [5, 6], skeletal muscle [12], stomach smooth muscle [5, 6], and the *Aplysia* central nervous system [13]. In addition, the action of ACh, but not of carbachol, is potentiated in skeletal muscle [12]. Biochemical evidence, however, is lacking in most of these systems.

Direct enzymatic measurements of cholinesterase (ChE) inhibition by DMSO have been made in erythrocytes [5], diaphragm muscle [6], chick skeletal muscle [12], brain [14] and atria [11]. Kinetic information on the conditions of assay, however, was not well described in any of these studies. In addition, a distinction between "true" AChE and "pseudo" or butyrylcholinesterase (BuChE) was not made. Thus, previous comparisons between enzymatic data and pharmacological effects of DMSO on muscle contraction have not been precise.

We first describe the pharmacological effect of relatively low concentrations of DMSO on cholinergic regulation of the isolated rat heart. These concentrations had no measurable action, alone, on heart rate. We then demonstrate that these same concentrations of DMSO inhibited the AChE activity of atrial homogenates. These experiments test the hypothesis that DMSO inhibits AChE in the mammalian heart. A preliminary report of these findings has been presented to the American Physiological Society [15].

METHODS

Heart perfusions. Hearts were isolated from heparinized (2–5 mg heparin injected intraperitoneally), pentobarbital-anesthetized (30 mg, i.p.) Sprague–

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Dawley rats (250–400 g). All hearts were perfused for 10 min (nonrecirculating) at 60 mm Hg aortic pressure (Langendorff procedure) followed by the working heart perfusion (recirculating buffer) method described by Neely and Rovetto [16]; the left atrial filling pressure was 10 cm H_2O and the hydrostatic afterload was 60 mm Hg. Heart rate was recorded with a Statham pressure transducer (Gould) and an ink writing oscillograph (Grass, model 79D).

The control perfusion fluid consisted of Krebs–Henseleit bicarbonate buffer equilibrated with 95% O_2–5% CO_2 (37°, pH 7.4). This buffer contained NaCl, 118 mM; KCl, 4.7 mM; $CaCl_2$, 2.5 mM; $MgSO_4$, 1.2 mM; KH_2PO_4, 1.2 mM; EDTA, 0.5 mM; $NaHCO_3$, 25 mM; and dextrose, 13 mM. Acetylcholine iodide (Sigma) was added at 1×10^{-6} M final concentration, 4-ketoamyltrimethylammonium iodide (4K) (ICN) at 6×10^{-5} M and dimethyl sulfoxide (DMSO) (Sigma) to a final concentration of 10 μl DMSO/ml of perfusate (140 mM). DMSO was added to the perfusate in increments of 1 μl/ml of perfusate each min for 10 min to obtain the final concentration (10 μl/ml). This cumulative addition was designed to minimize nonspecific osmotic effects of DMSO on heart beat (see discussion for references). Since DMSO is a liquid at room temperature, volumetric doses were administered. The dose concentrations are presented as microliters of pure DMSO per milliliter of perfusate. To convert: 1 μl/ml = 14 mM.

In experiment 1, each of eight hearts were subjected to the following sequence of treatments: 5 min of control perfusion, 3 min with ACh, 5 min of control, 10 min of DMSO, 3 min of DMSO plus ACh, 5 min of control, and 3 min of ACh. Spontaneous heart rates (not electrically paced) were determined at the end of each of the treatments. In experiment 2, 4K was substituted for ACh in this procedure. In preliminary experiments, it was determined that these treatment times allowed for equilibration within the perfusion system (100 ml total volume) and yielded stable and maximum observable drug effects. In addition, the washout periods resulted in complete reversal of the treatment effects.

Cholinesterase determination. The rats were killed with a blow to the head, and the hearts were removed and placed in 0.1 M phosphate buffer (pH 8.0; 4°). The atria from three hearts were removed, minced, and thoroughly rinsed to remove blood. The pooled atria were then homogenized (six atria/5 ml of 0.1 M phosphate buffer) using a Polytron homogenizer (setting 6 for 30 sec).

Homogenate cholinesterase activity was assayed by the method of Ellman *et al.* [17] as previously described [18]. The reaction mixtures contained 0.1 ml of homogenate (protein = 3.2 ± 0.41 mg/ml, mean ± S.E.M., N = 3), 2.9 ml of 0.1 M phosphate buffer (pH 8.0), and 0.1 ml of 0.1 M 5,5-dithiobis-(2-nitrobenzoic acid) (DTNB) in 0.1 M phosphate buffer (pH 7.0). The reaction was initiated at 23° by the addition of 20 μl of acetylthiocholine iodine (AThCh) (Sigma) substrate (1×10^{-3} M final concentration), a concentration of substrate which gave maximum activation of atrial ChE in preliminary experiments. The enzyme blanks contained no substrate. The reaction rates were recorded using a double beam spectrophotometer (Beckman, model 25) at 412 nm, as free SH groups exposed by enzymatic hydrolysis react with DTNB. The extinction units were converted to micromoles of substrate hydrolyzed by comparison with a reduced glutathione standard curve [19]. The reactions were linear with respect to time and protein under the conditions employed. The inhibitors tested were tetraisopropylpyrophosphoramide (isoOMPA) (Koch & Light), 1,5-bis (4-allyldimethylammoniumphenyl) pentan-3-one dibromide (BW284c51) (Burroughs Wellcome), and eserine sulfate (Sigma). The inhibitors were present for 5 min prior to the initiation of the reaction to ensure that full inhibition would occur.

Homogenate acetylcholinesterase (AChE, EC 3.1.1.7) was determined by a modification of the assay procedure. The reaction vessels contained 0.1 ml of homogenate (protein = 1.4 ± 0.08 mg/ml, mean ± S.E.M., N = 3), 0.2 ml of isoOMPA (1×10^{-4} M final concentration), and 0.86 ml of 0.1 M phosphate buffer (pH 8.0). IsoOMPA was included in the reaction to inhibit butyrylcholinesterase and to allow the measurement of "true" AChE activity. Reactions were initiated at 23° with the addition of 0.02 ml of AThCh (appropriate concentrations with each figure). The reactions were terminated with the addition of eserine (1×10^{-4} M final concentration) and reaction blanks received substrate after the addition of eserine. Preliminary experiments showed that this concentration of eserine completely inhibited the homogenate AChE. The addition of 0.03 ml of DTNB, as before, resulted in color development which was measured (412 nm) with the spectrophotometer, and the blank values were subtracted to determine enzyme activity. The reactions were determined to be linear with time (30 min) and protein concentration under these conditions. When DMSO was used, it was added to both reaction and blank tubes 5 min before the reactions were initiated.

Protein was determined by the method of Lowry *et al.* [20] using a bovine serum albumin standard (Sigma).

Statistical analysis. The effects of treatments on heart rates were analyzed by two-way ANOVA without replication [21] to remove variance due to differing innate heart rates. Individual treatment effects were tested within each experiment by planned comparisons as follows. Experiment 1: comparisons among control groups (C_1, C_2, and C_3) and between ACh treatments (ACh_1 and ACh_2) showed that there were no significant differences within these treatments. Therefore, the control data were combined, as were the ACh data for further analyses. Comparisons were also made between controls and DMSO, controls and ACh, controls and DMSO plus ACh, and ACh and DMSO plus ACh. Experiment 2: the control groups (C_1, C_2, and C_3) were not significantly different, nor were the two 4K groups ($4K_1$ and $4K_2$). Therefore, these data were again combined within these treatments and comparisons were made between controls and DMSO, controls and 4K, controls and DMSO plus 4K, and 4K and DMSO plus 4K. For the analysis of inhibitor effects on ChE activity the one-way ANOVA test was per-

Table 1. Effects of DMSO, ACh and 4K on the spontaneous heart rate of isolated perfused rat hearts*

	Spontaneous heart rate (beats/min)						
				Experiment 1			
	C_1	ACh	C_2	DMSO	DMSO + ACh	C_3	ACh
$\bar{X}$	273†	240‡	279†	287†	156§	273†	253‡
S.E.M.	14.3	14.5	12.0	12.1	12.6	13.8	11.7
				Experiment 2			
	C_1	4K	C_2	DMSO	DMSO + 4K	C_3	4K
$\bar{X}$	272†	242‡	275†	281†	244‡	274†	238‡
S.E.M.	4.1	3.4	5.3	7.1	6.1	7.9	7.0

* In Experiment 1, the sequence of treatments (N = 8, 37°) was 5 min control perfusion (C_1), 3 min acetylcholine (ACh, 1×10^{-6} M), 5 min control (C_2), 10 min dimethyl sulfoxide (DMSO, 10 μl/ml, 140 mM), 3 min DMSO plus ACh (concentrations as before), 5 min control (C_3), and 3 min ACh (1×10^{-6} M). In Experiment 2, 4 ketoamyltrimethylammonium iodide (4K, 6×10^{-5} M) was substituted for ACh.

†‡§ Within each experiment treatments with different superscripts are significantly different ($P < 0.01$).

formed and the Student–Neuman–Keuls test [21] was used to test for differences among treatments.

RESULTS

Pharmacology of DMSO. The spontaneous heart rates observed in the three control groups before and after treatments were not significantly different in either experiment 1 or 2 (Table 1, $F = 0.11$, $F = 0.08$; $df = 2, 42$). Thus, all treatment effects were fully reversible. These control data were combined within each experiment for further analysis. When added alone, DMSO did not significantly alter heart rate in either experiment ($F = 0.95$, $F = 2.60$, $df = 1, 42$). Also, contractility of the hearts as measured by pressure development was not changed significantly by DMSO. ACh (1×10^{-6} M) significantly depressed the heart rate ($F = 9.16$, $df = 1,42$). When ACh was added in the presence of DMSO, the heart rate decreased further (control vs DMSO + ACh $F = 101.55$, ACh vs DMSO + ACh $F = 52.50$, $df = 1,42$). Thus, the effect of ACh was markedly potentiated by DMSO. Treatment with 4K (6×10^{-5} M) resulted in a depression of heart rate ($F = 77.81$, $df = 1,42$) equal to that observed with ACh. In contrast to ACh, when 4K was added in the presence of DMSO, no significant potentiation of the 4K effect was observed ($F = 0.54$, $df = 1,42$). Since ACh is hydrolyzed by AChE while 4K is not, these data support the hypothesis that AChE inhibition had occurred at this concentration of DMSO (10 μl/ml, 140 mM).

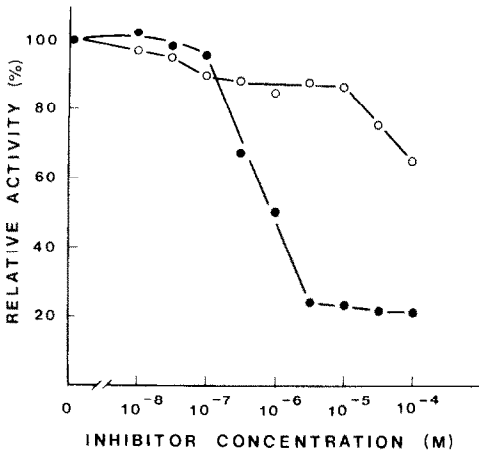

Fig. 1. Effects of the specific inhibitors isoOMPA (●) and BW284c51 (○) on atrial homogenate ChE activity. The enzyme was preincubated with the inhibitor for 5 min prior to the addition of the substrate AThCh (1×10^{-3} M). Each value represents the mean of two replicate assays.

Effects of inhibitors on ChE activity. IsoOMPA, a specific BuChE inhibitor [22], maximally inhibited ChE activity at concentrations of 5×10^{-6} M or greater (Fig. 1). BW284c51, a specific AChE antagonist [23, 24], maximally inhibited AChE activity at concentrations between 5×10^{-7} M and 1×10^{-5} M, while greater concentrations resulted in additional inhibition. This phenomenon of partial inhibition of BuChE with higher concentrations of the AChE antagonist has been described previously [23].

Atrial homogenate esterase activity in the presence of AThCh substrate (48.8 ± 3.46 nmoles·min^{-1}·mg^{-1}, mean ± S.E.M., N = 3) was totally abolished by eserine (1×10^{-4} M), indicating that all of the activity was due to ChE enzymes. IsoOMPA (1×10^{-4} M) significantly inhibited ChE activity (11.2 ± 0.86, $P < 0.01$), while BW284c51 (3×10^{-5} M) also significantly inhibited ChE activity (38.2 ± 1.60, $P < 0.01$). Thus, the atrial homogenate ChE activity was comprised of approximately 22% specific AChE activity and some 78% BuChE.

Effects of DMSO on AChE activity. To examine the effects of DMSO on the AChE activity which is primarily associated with the pacemaker system

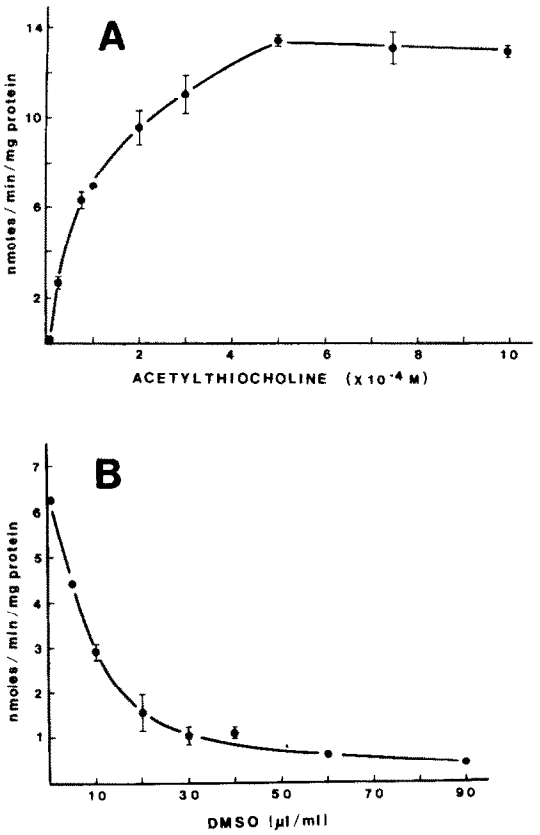

Fig. 2. Atrial homogenate AChE. Each reaction mixture contained 1×10^{-4} M isoOMPA to inhibit BuChE. Mean ± S.E.M. for three separate enzyme preparations. (A) Substate saturation of AChE. (B) Effect of DMSO on AChE activity in the presence of substrate at apparent K_m concentration of substrate (1×10^{-4} M). One µl DMSO/ml = 14 mM.

of the heart, isoOMPA (1×10^{-4} M) was included in all further reactions to inhibit BuChE. Maximum rates of hydrolysis by AChE (13.4 ± 0.17 nmoles·min^{-1}·mg^{-1}, mean ± S.E.M., N = 3) occurred with 5×10^{-4} M AThCh (Fig. 2A). The apparent K_m for substrate activation was 1×10^{-4} M. We recently showed in purified eel AChE and in oyster heart homogenate AChE that DMSO is a competitive inhibitor of AChE activity [4]. Thus, we examined the effects of DMSO on AChE activity under nonsaturating conditions (apparent K_m) (Fig. 2B). DMSO caused strong inhibition of atrial AChE activity with 50% inhibition (I_{50}) having occurred at 9 µl/ml (126 mM).

DISCUSSION

Here we have shown that DMSO (10 µl/ml, 140 mM) potentiated the negative chronotropic effects of ACh on the isolated, perfused, working rat heart. In contrast, the effects of 4K were not potentiated by DMSO. 4K is an analog of ACh that lacks the ester linkage and is, therefore, not cleaved by AChE. Furthermore, we found that the same concentration of DMSO that potentiated the pharmacological effects of ACh inhibited the activity of AChE (I_{50} = 9 µl/ml) when measured in the presence of an inhibitor of BuChE (isoOMPA). We have shown previously that the action of acetylcholine (ACh) is potentiated by low concentrations of DMSO (1–5 µl/ml) in invertebrate hearts and smooth muscles which possess strong AChE activity, while the effects of 4-ketoamyltrimethylammonium (4K) are not potentiated by DMSO in these systems. In contrast to the oysters, the action of ACh is not potentiated by DMSO in clam hearts which have only butyrylcholinesterase with low activity toward ACh [4]. Other workers have shown that the vagal threshold of isolated guinea pig atria is reduced by 6 µl/ml DMSO [5, 6]. Moreover, the negative chronotropic effect of DMSO when added alone to rabbit atria is blocked by atropine, which suggested that the effect was from accumulation of endogenous ACh due to AChE inhibition [11]. These data demonstrate that the potentiation by DMSO of the effects of ACh in *in vitro* preparations was due to inhibition of AChE. Infusion of DMSO into the circulatory system of cats [7] and rabbits [8] results in sinus bradycardia and hypotension. However, the *in vivo* mode of action of DMSO on the cardiovascular system is, as yet, unclear.

Direct enzymatic measurements have been made in a few systems. Sams and Carroll [5] reported the inhibition of erythrocyte ChE by DMSO. The concentration which inhibited 50% of the enzyme activity (I_{50}) was 45 µl/ml (630 mM). Sams *et al.* [6] reported that 7.8, 39, and 78 µl/ml DMSO inhibits the ChE of diaphragm muscle by 16, 44 and 85%, respectively (I_{50} = 40–50 µl/ml, 560–700 mM). DMSO has also been reported to inhibit ChE in chick skeletal muscle [12] (58% inhibition with 50 µl/ml, 700 mM), rabbit brain [14] (100 µl/ml, 1.4 M, completely inhibited ChE), and rabbit atrial preparations [11] (I_{50} = 60 µl/ml, 840 mM). While not stating kinetic information, these studies were done with high substrate conditions (5×10^{-4} M or greater) which probably saturated the enzymes.

Recently, we showed that DMSO is a competitive inhibitor of AChE [4]. Using nonsaturating conditions of enzyme activity, which are probably more physiological [25], the concentration of DMSO which inhibits AChE correlates well with the lower concentration required for specific pharmacological effects (I_{50} = 10 µl/ml, 140 mM or less) ([4], present data). In addition, in the present studies on mammalian heart, we measured the activity of the true AChE that is associated with pacemaker areas, where workers previously had not distinguished among ChE, BuChE and AChE activities. In the adult mammalian heart, the SA and AV nodes contain high concentrations of AChE activity, while the muscle cells contain BuChE activity [26–28]. Since the major action of ACh is on the heart rate whether by nerve stimulation or exogenous application [29, 30], the pacemaker AChE is most pertinent.

Sawada and Sato [13] suggest that relatively low concentrations of DMSO (1–10 µl/ml, 14–140 mM) inhibit AChE, while higher concentrations directly block ionic conductance, interact with the acetylcholine receptor, and inhibit AChE in the *Aplysia* central nervous system, thus producing mixed

results. Both positive and negative inotropic responses have been described for the effects of DMSO when added to isolated cardiac tissue (reviewed in Ref. 9). However, the concentrations of DMSO used in those studies were remarkably high, generally from 0.42 M to 2.1 M, and these variable effects were probably due to osmotically induced changes in cellular hydration [9, 10, 31, 32]. The concentration of DMSO used in the present experiments ($10\,\mu l$/ml, added at $1\,\mu l \cdot (\text{ml perfusate})^{-1} \cdot \text{min}^{-1}$, 0.14 M) was below these concentrations.

Our findings substantiate the notion that DMSO inhibits AChE and, thereby, potentiates cholinergic action. This information should be taken into consideration in the increasing popularity of DMSO treatments.

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