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SHORT COMMUNICATIONS

Effect of piperidines and fire ant venom on ATPase activities from brain homogenate fractions and characterization of Na^+-K^+ ATPase inhibition*

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We reported the presence of an ATPase inhibitor(s) in the abdomen of the fire ant, *Solenopsis richteri* (Forel), and demonstrated that pure samples of C_{15}- and $C_{15:1}$-2,6-methyl piperidine derivatives produced similar inhibitory effects [1–3]. During our initial studies, we believed that the piperidine derivatives of fire ant venom were not the inhibitory components [1, 2]. An investigation of the effects of pure piperidine (at 1.0 mM in the reaction mixture) on a fire ant head (FAH) homogenate showed little or no inhibition of the ATPase activities. Also, the fact that the inhibitor sedimented with the 13,000 *g* fraction of a 0.32 M sucrose homogenate of fire ant abdomens led to our initial false conclusion. However, further study of a pure venom collected by the method of Blum *et al.*[4] and of synthetically prepared samples of C_{15} and $C_{15:1}$ piperidine derivatives† (samples were gifts from Dr. Murray S. Blum, University of Georgia) showed that our initial findings were indeed due to the piperidine derivatives [3].

Our initial false conclusion, however, did lead us to a further investigation of piperidine, piperidine derivatives and the fire ant venom to determine the importance of the hydrocarbon side chain in ATPase inhibition. Brain homogenates were prepared from chickens, catfish and fire ants (the latter was a whole head homogenate) according to the procedure reported earlier [3]. A cyclohexane extract of crushed whole fire ants, used at a concentration shown to give essentially the same inhibition of ATPase activities as 3×10^{-6} M synthetic derivatives [3], was used as the source of fire ant venom inhibitor.

In earlier studies, piperidine inhibition was investigated at a maximum concentration of 1.0 mM. From Table 2 it is evident that even at 20 mM, piperidine had little effect on FAH ATPase activities. Substantial inhibition required the addition of 40 mM piperidine. A comparison of a FAH preparation with chicken and catfish brain preparations using piperidine and *N*-methyl piperidine (see Table 2) showed that, in general, the FAH ATPase activities were least sensitive to the inhibitors. This was also true for propyl piperidine when comparing FAH with catfish preparation (Table 1). The reverse was true for the whole fire ant (WFA) cyclohexane extract preparation (Table 1).

In general, there was very little inhibition of ATPase activities from the three types of brain tissue at the lower concentrations of piperidine and *N*-methyl piperidine (Table 2). Some differences in inhibition occurred at the higher concentration as mentioned above, but they were not consistent enough to indicate that methylation of the ring nitrogen had strong adverse or enhancing effects on ATPase inhibition by piperidine. However, it is obvious from Tables I and 2 that the addition of even a short hydrocarbon chain to a ring carbon of piperidine had a profound effect on the inhibition of the ATPase activities. The extension of the non-polar hydrocarbon chain, as is found in the fire ant venom [5], produced an even greater enhancement of ATPase inhibition by piperidine (Table 2). The sensitivity of the ATPase activities from the FAH fraction was greater than 1,000- and 10,000-fold more sensitive to WFA extract and C_{15} piperidine derivatives (Table 1 and Ref. 3) than to propyl piperidine (Table 1) and piperidine and *N*-methyl piperidine (Table 2) respectively.

It has been reported that interference with nerve function caused by fire ant venom may be due to one of the hydrocarbon derivatives present therein [8]. To further understand the inhibitory action of the venom, studies were conducted on the Na^+-K^+ ATPase in isolated plasma membranes from rat brain.‡ Preparation, properties and characterization of the rat brain preparation have been described [9–11]. The synthetically prepared venom components, C_{15} and $C_{15:1}$ piperidine derivatives (from M. S. Blum), were used to further define the sites of action of fire ant venom on some of the various partial reactions of the Na^+-K^+ ATPase. These studies included the overall Na^+-K^+ ATPase activity, the Na^+-dependent phosphoenzyme formation, the Na^+-dependent ADP–ATP exchange, and the K^+-dependent *p*-nitrophenyl phosphatase activity.

Tris salts of the substrates ATP and *p*-nitrophenyl phosphate were prepared by chromatography on a 2.5 cm × 30 cm column containing the Tris form of AG-50 × 8 Dowex resin. Sucrose and Tris were of an ultrapure grade obtained from Schwartz-Mann, New York. [γ-^{32}P]ATP was prepared and purified as described previously [12, 13]. EDTA was dissolved in Tris base and chromatographed on the Dowex AG-50 × 8 (Tris form) column. NaCl, KCl and $MgCl_2$ were spectroscopically pure. All stock solutions were stored frozen.

Preparation and measurement of Na^+-dependent [^{32}P]phosphoenzyme have been described in detail [11]. The Na^+-dependent ADP–ATP exchange reaction was measured as described earlier [14]. The Na^+, K^+-ATPase activity and K^+-dependent *p*-nitrophenyl phosphatase activity were assayed according to Ahmed *et al.* [9]. In all of these assays, the reaction medium was prepared to accommodate the final concentrations of 5 μM piperidine compounds and 0.5% ethanol. These agents were added to the reaction medium prior to the enzyme and substrate, and immediately the tubes were vortexed vigorously. Suitable controls containing the same amount of ethanol were included throughout. Protein was estimated by the method of Lowry *et al.* [15].

The effects of 5 μM piperidine derivatives on the reactions of the Na^+, K^+-ATPase are shown in Table 3. The C_{15} derivative inhibited the ATPase reaction by about 13 per cent and only slightly stimulated the ADP–ATP exchange (7 per cent). The $C_{15:1}$ derivative inhibited the ATPase reaction by 34 per cent and stimulated the exchange by 62 per cent. There was no significant effect of either agent on the other two partial reactions, i.e. the Na^+-dependent phosphoenzyme formation and the K^+-dependent *p*-nitrophenyl phosphatase activity.

The mechanism of operation of the Na^+, K^+-ATPase

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† C_{15} = *cis*-2-methyl-6-*n*-pentadecylpiperidine; $C_{15:1}$ = *cis*-2-methyl-6-(*cis*-6′-*n*′pentadecenyl) piperidine.

‡ The rat brain preparation was selected for these studies because it was readily available and its Na^+-K^+ ATPase well characterized.

Table 1. Effect of propyl piperidine and whole fire ant extract on brain ATPase activities*

Inhibitor and tissue source	ATPase† (per cent inhibition) Na^+-K^+	Oligomycin-sensitive Mg^{2+}	Oligomycin-insensitive Mg^{2+}
Propyl piperidine (5 μl)‡			
Fire ant head	12.9	9.4	15.7
Catfish	9.2	62.9	30.8
Whole fire ant extract (2 μl)§			
Fire ant head	75.0	74.0	40.0
Catfish	53.6	88.8	47.6
Chicken	66.4	55.5	36.0

* ATPase activity was measured essentially according to the method of Pullman *et al.* [6] and Fritz and Hamrick [7]. A 1-ml reaction mixture contained: 4.3 mM ATP, 5 mM Mg^{2+}, 100 mM Na^+, 20 mM K^+, 135 mM imidazole–Cl buffer (pH 7.5), 0.2 mM NADH, 0.5 mM phosphoenol pyruvate, 0.02% bovine serum albumin, approximately 9 units pyruvate kinase and 12 units lactic acid dehydrogenase. Fifty-μl aliquots of the tissue preparations were used with a protein content of 20–30 μg. Absorbance changes in the reaction mixture were measured at 340 nm using a Gilford 2400 recording spectrophotometer with temperature controlled at 37°. The change in absorbance at 340 nm over a period of 10 min was used in calculating the specific activity. Enzyme activities were expressed as μmoles P_i mg^{-1} protein hr^{-1}.

† The data obtained were based on, for the most part, triplicate determinations. The variation in the replicate values was less than 10 per cent of the average.

‡ Coniine·HBr (2-propyl piperidine·HBr) in 0.2 ml H_2O was adjusted to pH 10.0 with NaOH and then extracted with cyclohexane. The volume of the cyclohexane extract was adjusted to 0.2 ml. Assuming 100 per cent recovery of coniine, a 5-μl aliquot would yield 1.7 mM in the reaction mixture.

§ WFA cyclohexane extract (2 μl) was shown to inhibit FAH ATPase activities to the same level as 3×10^{-6} M $C_{15:1}$ and C_{15} piperidine derivatives (see Ref. 3).

is believed to involve a series of sequential steps as illustrated previously [11]. Several of these steps may be kinetically isolated and observed separately as in the present studies. In the presence of free enzyme, Na^+, ATP and Mg^{2+}, an E_1-phosphoenzyme complex is formed which is sensitive to breakdown by ADP but not K^+. This complex undergoes a change in the enzyme conformation to form an E_2-phosphoenzyme complex which is sensitive to hydrolysis by K^+ but not ADP [16].

In order for an agent to fail to inhibit either the first step, represented by Na^+-dependent phosphoenzyme formation and the ADP–ATP exchange, or the terminal step, represented by the K^+-dependent *p*-nitrophenyl phosphatase reaction [17, 18], but still inhibit the ATPase reaction,

Table 2. Effect of piperidine and *N*-methyl piperidine on brain ATPase activities

Inhibitor and tissue source	ATPase* (per cent inhibition) Na^+-K^+	Oligomycin-sensitive Mg^{2+}	Oligomycin-insensitive Mg^{2+}
Pure piperidine† (20 mM)			
Fire ant head	16	8	0
Chicken brain	20	0	7
Catfish brain	17	18	0
Pure piperidine† (40 mM)			
Fire ant head	86	52	31
Chicken brain	34	45	51
Catfish brain	78	100	80
N-methyl piperidine† (20.5 mM)			
Fire ant head	7	16	0
Chicken brain	23	0	0
Catfish brain	4	15	0
N-methyl piperidine† (41 mM)			
Fire ant head	16	8	0
Chicken brain	100	100	20
Catfish brain	84	100	71
Specific activities‡			
Fire ant head	8.4	8.6	7.4
Chicken brain	14.1	5.0	15.0
Catfish brain	16.6	5.1	11.2

* The data obtained were based on, for the most part, triplicate determinations. The variation in the replicate values was less than 10 per cent on the average.

† Piperidine and *N*-methyl piperidine solutions were prepared by appropriate dilutions with deionized water to require 5 μl additions to obtain indicated concentrations.

‡ For reaction conditions and specific activity units see Table 1.

Table 3. Effect of aliphatic piperidines on reactions of the Na^+, K^+-ATPase of rat brain*

Experimental	Na^+, K^+-ATPase (μM P_i/mg/hr)	K^+-*p*-NPPase (μM pNPP/mg/hr)	Phosphoenzyme (pmoles ^{32}P/mg)	ADP–ATP exchange $\left(\frac{\text{cpm ATP}}{\text{cpm ADP}} \times 100\right)$
Control	85.57	14.26	93.49	3.57
C_{15} piperidine	74.87	14.15	98.03	3.82
$C_{15:1}$ piperidine	56.82	14.79	98.29	5.79

* Each activity was assayed as described in the text. The piperidine compounds were present in a final concentration of 5 μM. The values are the mean of two experiments, with less than 5 per cent variation in the replicates.

it must of necessity act at an intermediate step which may include a conformational change of the enzyme. Similar effects on the partial reactions of the Na^+, K^+-ATPase have been observed for oligomycin and related types of antibiotics [19, 20]. Therefore, it appears that the aliphatic derivatives of piperidine may be oligomycin-type inhibitors of the Na^+, K^+-ATPase. This similarity of action may not be related to common structural features but perhaps to their solubility in the membrane lipids. It is interesting that the degree of inhibition by aliphatic piperidines [3] and fatty acids [21] is related to the length of the hydrocarbon chain. In conclusion, the aliphatic derivatives of piperidine represent a new class of toxins of the Na^+, K^+-ATPase that appear to interfere with a conformational transition of the enzyme from an E_1 state to an E_2 state. Thus, this cation transport enzyme is a likely candidate as one locus of action of these toxins *in vivo*.

However, the fire ant venom has a broader action on the ATPase system in that unlike oligomycin it also has an uncoupling action on oxidative phosphorylation [22]. It also has an unusual effect on oligomycin-insensitive Mg^{2+} ATPase activity. At low concentrations, fire ant venom and the $C_{15:1}$ piperidine derivative were equally inhibitory to the three types of ATPase activity (see Table 4 of Ref. 3). But at higher concentrations of the above components, where Na^+-K^+ ATPase and mitochondrial Mg^{2+} ATPase were completely inhibited, the oligomycin-insensitive Mg^{2+} ATPase activity showed strong stimulation (>200 per cent [3]).

It seems clear that the length of the C_6-hydrocarbon side chain of piperidine plays an important role in enhancing piperidine inhibiton of the ATPase activities. Because of the lipoprotein nature of the ATPase, we propose that the nonpolar hydrocarbon side chain assists the more polar piperidine moiety in obtaining access to an inhibitory site on the enzyme complex. Additional work is in progress to isolate and purify the *cis* $C_{13:1}$ and C_{11} piperidine derivatives from WFA extracts. The latter two compounds are the more prevalent piperidine derivatives in the black fire ant, *S. richteri*, venom [5]. This will enable a direct comparison to be made of more closely related C_6 derivatives.

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