# Inhibition of $Na^+$-dependent $Ca^{2+}$ efflux from heart mitochondria by amiloride analogues

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The $Na^+$-induced release of accumulated $Ca^{2+}$ from heart mitochondria is inhibited by amiloride, benzamil and several other amiloride analogues. These drugs do not affect uptake or release of $Ca^{2+}$ mediated by the ruthenium red-sensitive uniporter and their effects, like those of diltiazem and other $Ca^{2+}$-antagonists, appear to be localized principally at the $Na^+/Ca^{2+}$ antiporter of the mitochondrion. Benzamil inhibits $Na^+/Ca^{2+}$ antiport non-competitively with respect to $[Na^+]$ with a $K_i$ of 167 $\mu$M. In the presence of 1.5 mM $P_i$ the $K_i$ for benzamil inhibition of this reaction is decreased to 87 $\mu$M.

*Mitochondrial $Na^+/Ca^{2+}$ antiporter* *Amiloride* *Benzamil* *Diltiazem* *$Ca^{2+}$ antagonist*

## 1. INTRODUCTION

Diltiazem and other $Ca^{2+}$ antagonists selectively inhibit the $Na^+$-induced release of $Ca^{2+}$ from heart mitochondria [1]. This $Ca^{2+}$ efflux is thought to balance the electrophoretic uptake of $Ca^{2+}$ on the mitochondrial $Ca^{2+}$ uniporter and to result from the combined activity of a $Na^+/Ca^{2+}$ antiporter and the extrusion of $Na^+$ by $Na^+/H^+$ exchange (review [2]). Despite the intense current interest in the uptake and release of $Ca^{2+}$ by mitochondria, little is known of the molecular properties of the components responsible for $Na^+$-dependent $Ca^{2+}$ extrusion. We report that $Na^+$-dependent $Ca^{2+}$ release from heart mitochondria is inhibited by benzamil and other analogues of amiloride, a widely used probe of $Na^+$ transport [3,4].

## 2. METHODS

Beef heart mitochondria prepared using Nagarse and EGTA [5] were suspended at 0.66 mg protein/ml in a medium of KCl (125 mM), the $K^+$ salt of *N*-2-hydroxyethylpiperazine-*N'*-2 ethane sulfonic acid (HEPES, 4 mM, pH 7.0), $K^+$-malate (5 mM), $K^+$-glutamate (5 mM) and antipyralazo III (50 $\mu$M). The temperature was maintainedat 37°C and the uptake of $Ca^{2+}$ (14 $\mu$M) followed at 720–790 nm in an Aminco DW-2 spectrophotometer. Ruthenium red (0.8 $\mu$M) was added and the release of $Ca^{2+}$ initiated by the addition of NaCl (10 mM, or other concentrations as indicated).

## 3. RESULTS

Beef heart mitochondria, respiring with glutamate–malate in a KCl medium, release accumulated $Ca^{2+}$ in a ruthenium red-insensitive reaction when challenged with $Na^+$ (fig.1). In agreement with [1], this reaction is strongly inhibited by diltiazem with a $K_i$ of about 13 $\mu$M. The $Na^+$-dependent efflux of $Ca^{2+}$ is also inhibited by the amiloride analogue benzamil (fig.1) with a $K_i$ of 167 $\mu$M and by amiloride at somewhat higher concentrations ($K_i$ about 350 $\mu$M, fig.2). Benzamil at $<500$ $\mu$M has no effect on the rate of $Ca^{2+}$ uptake via the ruthenium red-sensitive uniport (not shown). In this concentration range benzamil also does not alter the rate of efflux of $Ca^{2+}$ from mitochondria treated with an uncoupler. Under the conditions of fig.1 the loss of accumulated



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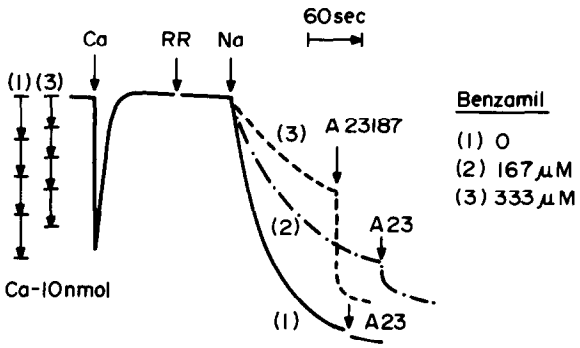


Fig.1. Inhibition of $Na^+$-dependent release of accumulated $Ca^{2+}$ from beef heart mitochondria by benzamil. The release of $Ca^{2+}$ was initiated by addition of NaCl to 10 mM. The effect on $Ca^{2+}$ release of benzamil (167 and 333 μM) present from the start of the incubation is shown. Benzamil changes the response of antipyralazo III to four consecutive additions of 10 nmol $Ca^{2+}$ as shown. All rates reported in this communication are based on such $Ca^{2+}$ titration curves. The ionophore A23187 (1 μM) releases all accumulated $Ca^{2+}$ in the presence of benzamil.

$Ca^{2+}$ from uncoupled mitochondria is strongly inhibited by ruthenium red and appears to occur by backflow through the uniport [6]. Benzamil (100–600 μM) does not inhibit either $Ca^{2+}$-stimulated, uncoupler-stimulated or state 3 respiration under the conditions of fig.1 (not shown). The drug also does not increase state 4 respiration in this concentration range. Benzamil (⩾300 μM) shows a weak inhibition of the swelling of heart mitochondria in $Na^+$ acetate (100 mM), a reaction thought to reflect $Na^+/H^+$ exchange activity (review [7]). The effects of these drugs on mitochondrial swelling are complex and will be discussed in detail elsewhere.

Analysis of the kinetics of $Na^+$-induced $Ca^{2+}$ release under the conditions of fig.1 shows that the initial rate of $Ca^{2+}$ loss is a sigmoidal function of $[Na^+]$ (Hill coefficient of 2.0–2.2). Plots of $1/v$ *vs* $1/[Na^+]^2$ are linear (fig.3) and show benzamil to be a non-competitive inhibitor with respect to $[Na^+]$. A similar plot for diltiazem also shows non-competitive inhibition (not shown). In the presence of 1.5 mM $P_i$ there is no change in the $V_{max}$ for $Na^+$-induced $Ca^{2+}$ release (38 nmol.mg$^{-1}$.min$^{-1}$ in the presence or absence of $P_i$) but the $K_m$ for $Na^+$ decreased from 6.5–2.7 mM. Benzamil is a more effective inhibitor in the presence of $P_i$, showing a $K_i$ of 87 μM.

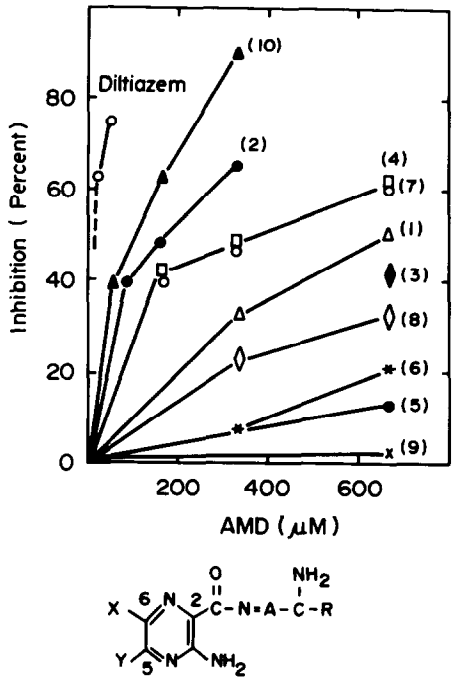


Fig.2. Inhibition of $Na^+$-dependent release of accumulated $Ca^{2+}$ from beef heart mitochondria by analogues of amiloride. Rates of $Na^+$-dependent $Ca^{2+}$ efflux were evaluated under the conditions of fig.1 in the presence of the indicated concentrations of the following analogues (identified by the number in parentheses):

| Compound no. | X | Y | A | R |
|---|---|---|---|---|
| 1 | Br | $NH_2$ | – | $NH_2$ |
| 2 | Cl | $NH_2$ | – | $NHCH_2C_6H_5$ (Benzamil) |
| 3 | F | $NH_2$ | – | $NH_2$ |
| 4 | Cl | $N(CH_3)_2$ | – | $NH_2$ |
| 5 | Cl | $NHCH(CH_3)_2$ | – | $N(CH_3)_2$ |
| 6 | Cl | H | – | $NH_2$ |
| 7 | Cl | $NH_2$ | – | $NH_2$ (Amiloride) |
| 8 | Cl | $NH_2$ | – | $NHCONH_2$ |
| 9 | Cl | $NH_2$ | NH | $NH_2$ |
| 10 | Cl | $NH_2$ | – | $NHCH_2C_6H_4F$ |

A number of analogues of amiloride [8,9] have been used in structure–function studies of electrolyte excretion in the rat and $Na^+$ conductance in frog skin [3,4]. The relative effectiveness of these

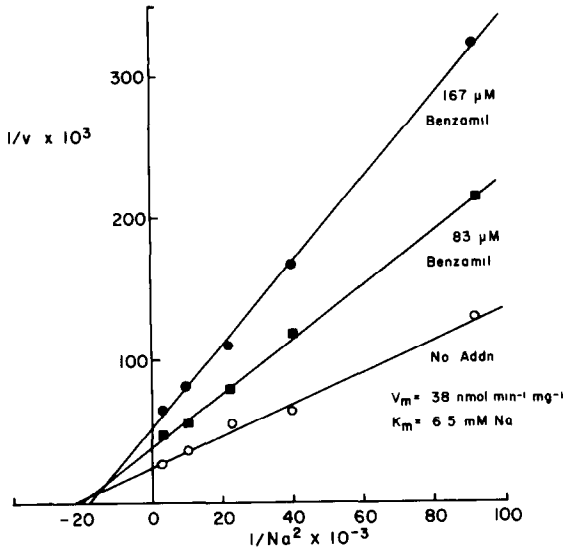


Fig.3. Effects of benzamil on the kinetics of $Na^+$-induced $Ca^{2+}$ efflux. The experimental conditions were as for fig.1 with the addition of 0, 83 or 167 $\mu$M benzamil and varying amounts of $Na^+$ to initiate $Ca^{2+}$ release. The $V$ (least squares) for the 3 plots shown is 38, 24 and 19 nmol.$mg^{-1}$.$min^{-1}$ and the $K_m$ is 6.5, 6.8 and 7.4 mM $Na^+$. The Hill coefficient of 2.0–2.2 does not change in the presence of benzamil.

analogues as inhibitors of mitochondrial $Na^+/Ca^{2+}$ exchange is shown in fig.2. Introduction of a benzyl group on the terminal guanidino nitrogen of amiloride (analogue 7 in fig.2) to produce benzamil (analogue 2, fig.2) enhances the inhibition of mitochondrial $Na^+/Ca^{2+}$ exchange. The *p*-F-derivative of benzamil is even more effective in this regard (analogue 10 in fig.2; $K_i$ = 100 $\mu$M). Substitution of bromine for chlorine at position 6 (analogue 1 in fig.2) reduces inhibitor effectiveness to some extent, as does substitution of F (analogue 3; fig.2). Introduction of two methyl groups on the 5-amino nitrogen does not alter the inhibition (analogue 4 *vs* 7 in fig.2). Replacement of the 5-$NH_2$ group by H greatly reduces inhibitor effectiveness (analogue 6, fig.2). Analogue 6 is also a poor inhibitor of $Na^+$ flux in frog skin [3]. The introduction of a carbamoyl group on the terminal guanidino group of amiloride (analogue 8) produces a decrease in activity and introduction of a NH group between the carbonyl and the guanidino groups (analogue 9) produces the least effective inhibitor of the analogues tested (fig.2). Analogue 5 which bears an isopropyl substituent on the 5-amino nitrogen and two methyl groups on the terminal guanidino nitrogen is also an ineffective inhibitor (fig.2).

## 4. DISCUSSION

These studies establish that benzamil and several other amiloride analogues are inhibitors of the $Na^+$-dependent efflux of $Ca^{2+}$ from heart mitochondria. Half-maximal inhibition of the reaction exchange falls from 100–400 $\mu$M for the effective analogues. The inhibition of $Na^+$-dependent $Ca^{2+}$ loss by levels of benzamil that do not affect swelling of heart mitochondria in $Na^+$ acetate suggests that the primary effect of the drug is at the level of the $Na^+/Ca^{2+}$ rather than the $Na^+/H^+$ exchange.

Amiloride-sensitive $Na^+$-dependent processes can be divided into two categories: high affinity reactions, with $K_i$ of $\leqslant 1$ $\mu$M; and a more insensitive group of processes with half-maximal inhibition above this value [3]. Conductive $Na^+$ entry in most systems falls in the high affinity group, whereas the low amiloride affinity is more characteristic of $Na^+/H^+$ exchange reactions. Many, but not all, changes in molecular structure that increase or decrease the effectiveness of amiloride on a high-affinity reaction, such as $Na^+$ conductance by frog skin [3], produce a similar alteration in inhibitor effectiveness for the low-affinity mitochondrial exchange reaction.

In addition to diltiazem [1] and benzamil and the other amiloride analogues (fig.2), several other reagents have been reported to inhibit $Na^+$-induced $Ca^{2+}$ release in heart mitochondria. These include dibucaine ($K_i$ 120 $\mu$M) and trifluoroperazine ($K_i$ 20 $\mu$M) as reported [10] and verapamil which is effective only in the presence of $P_i$ [11]. Benzamil inhibits the $Na^+/Ca^{2+}$ exchange more effectively in the presence of $P_i$, but does not require $P_i$ addition (see fig.1).

These studies also point out a number of inconsistencies in the recent literature concerning the kinetics of $Na^+$-dependent $Ca^{2+}$ efflux: Wolkowicz et al. [11] report a sigmoid dependency on $Na^+$ (Hill coefficient of 3) for dog heart mitochondria prepared using a Polytron when measured in the presence (but not the absence) of $P_i$; Nagarse mitochondria from the same hearts showed only

hyperbolic response to $Na^+$ [11]; Hayat and Crompton [12] also found a Hill coefficient of 3 with rat heart mitochondria (Polytron) but did not add $P_i$. The nagarse beef heart mitochondria used in the present study show a Hill coefficient of 2 in the absence of added $P_i$. The linear plot of $1/v$ *vs* $1/[Na^+]^2$ (fig.3) closely resembles that in [13] (rat heart, Polytron, no $P_i$). Some of these discrepancies could be species related, but it appears more likely that differences in experimental conditions may result in variable activation of a regulatory site [12] or alterations in the association of antiporter subunits. Further study will be necessary to clarify this point.

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