Biochemical Pharmacology, Vol. 37, No. 22, pp. 4399–4403, 1988.
Printed in Great Britain.

0006-2952/88 $3.00 + 0.00

STRUCTURAL DEPENDENCY OF THE INHIBITORY ACTION OF BENZODIAZEPINES AND RELATED COMPOUNDS ON THE MITOCHONDRIAL Na^+–Ca^{2+} EXCHANGER

MICHELE CHIESI, ROLAND SCHWALLER and KURT EICHENBERGER
Research Department, Pharmaceuticals Division, Ciba-Geigy Ltd., Basel, Switzerland

(*Received* 14 *March* 1988; *accepted* 11 *June* 1988)

Abstract—Na^+-induced Ca^{2+}-release from guinea-pig heart mitochondria is inhibited by benzodiazepines such as clonazepam (compound **II**, IC_{50}: 12 μM). The capacity of various related compounds to inhibit the rapid Ca^{2+}-efflux induced by 20 mM Na^+ was examined. The potency of inhibition was found to depend on several factors, such as a 2′-halogen substitution and the presence of a secondary amido group. Very effective inhibitors were identified among the triazolo derivatives of benzodiazepines or obtained by replacing the diazepine ring by an oxazepine or a thiazepine. Some of these favourable structural modifications were compounded in the benzothiazepine 7-chloro-3,5-dihydro-5-phenyl-1*H*-4,1-benzothiazepine-2-on (compound **XVI**), which proved to be about 20 times more potent than the related compounds clonazepam and diltiazem. Compound **XVI**, which has an IC_{50} in the submicromolar range, is the most potent selective inhibitor of the mitochondrial exchanger so far reported. The structural requirements found for the inhibition of the mitochondrial Na^+–Ca^{2+} exchanger were quite distinct from those described for the binding of benzodiazepines to their central-type and peripheral-type sites.

Mitochondria from several cell types act as a Ca^{2+} buffering system of very high capacity [1, 2]. Ca^{2+} is transported against a concentration gradient into the matrix space by an electrophoretic carrier-mediated process [2]. The addition of Na^+ ions to Ca^{2+}-loaded mitochondria can induce a rapid release of Ca^{2+} ions into the medium [3]. The process operates by way of a Na^+–Ca^{2+} exchange system which is particularly developed in excitable tissues, such as heart or brain [4]. The role of mitochondria in the regulation of intracellular Ca^{2+} in healthy cells or during pathological states of Ca^{2+} or Na^+ overload is of considerable general interest, but is still poorly understood. The complexity of the inner mitochondrial membrane has precluded all attempts to identify unambiguously or to isolate the molecular components involved in the translocation of Ca^{2+} ions in and out of mitochondria. Pharmacological tools could prove to be of great help for further biochemical and physiological investigations. Several compounds are known to interfere with the mitochondrial Na^+–Ca^{2+} exchange system. However, they are neither selective nor potent enough to be used as a tool. Clonazepam is one of the best inhibitors so far reported, but is only effective in the 10^{-5} M range [5]. In this study, we investigated the activity of various benzodiazepines and related compounds and succeeded in developing an inhibitor of the mitochondrial Na^+–Ca^{2+} exchanger with a considerably higher potency.

MATERIALS AND METHODS

Mitochondria were isolated from guinea-pig hearts using the Polytron homogenization procedure essentially as described [3, 6]. Ca^{2+} fluxes were measured in a medium containing 120 mM KCl, 20 mM Tris–HCl, pH 7.2, 50 μM arsenazo III (Sigma, St. Louis, MO) and about 10 μM contaminating Ca^{2+}. Mitochondria were added to the medium at a concentration of 1 mg/ml and pre-incubated for 2 min at 37° in the presence of 5 μM rotenone to induce rapid and passive release of endogenous Ca^{2+} from the matrix space into the medium. Total Ca^{2+} in the medium was about 20 μM and comprised the endogenous Ca^{2+} liberated by the mitochondria and the contaminating Ca^{2+}. The free Ca^{2+} concentration in the suspension medium was monitored using a dual wavelength spectrophotometer (Shimadzu UV-3000). The wavelength pair was set at 675 nm and 685 nm. If required, inhibitors were added to the mitochondrial suspension during the pre-incubation period. Ca^{2+} uptake was initiated by energizing mitochondria with 5 mM succinate. Total uptake was about 15–20 nmol Ca^{2+}/mg protein. The Ca^{2+} uniporter was then selectively blocked with 1 μM Ruthenium Red to study the passive efflux of Ca^{2+} from the Ca^{2+}-loaded mitochondria. The Na^+–Ca^{2+} exchanger was then activated by addition of 20 mM NaCl, and the effects of the various compounds on the rapid Na^+-dependent efflux rate were determined. Solutions of compounds were freshly prepared in dimethylsulphoxide. The final concentration of the organic solvent present in the assay medium was kept at 1% in all experiments (this concentration had no measurable effect on the mitochondrial Ca^{2+} fluxes).

Benzodiazepines and related compounds were provided by Ciba-Geigy Ltd. (Basel, Switzerland). Other chemicals used were of the best quality commercially available.

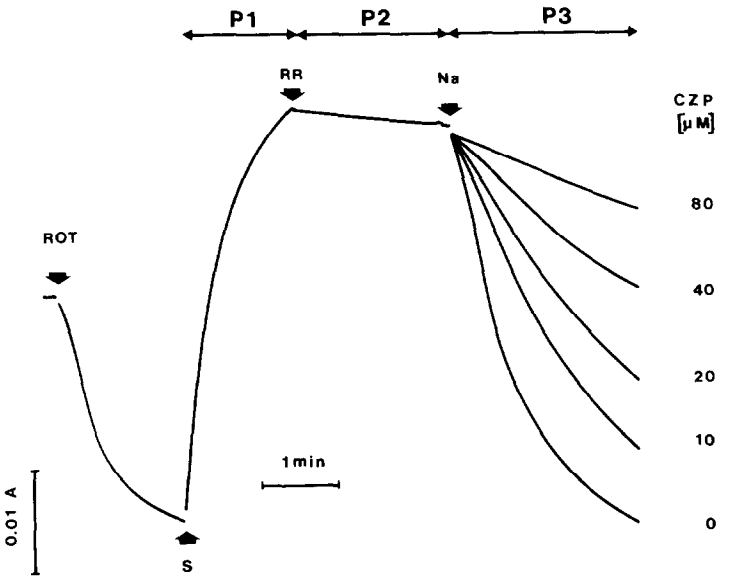

Fig. 1. Measurement of Ca^{2+} fluxes across heart mitochondrial membranes. The capacity of mitochondria to take up and to release Ca^{2+} ions was investigated with the metallochromic Ca^{2+}-indicator arsenazo III as described in the Materials and Methods section. Phase 1 (P1): active Ca^{2+} uptake. Phase 2 (P2): passive Na^+-independent Ca^{2+} release. Phase 3 (P3): Na^+-induced Ca^{2+}-release. Abbreviations: rotenone (ROT); succinate (S); Ruthenium Red (RR); clonazepam (CZP).

RESULTS

The passive permeability of mitochondria to Ca^{2+} ions was measured according to a standard procedure using the metallochromic Ca^{2+}-indicator arsenazo III [7, 8]. Figure 1 shows a typical signal recording of the measurement of Ca^{2+}-fluxes across the inner mitochondrial membrane. Despite the various washing steps in the presence of EGTA included in the preparative procedure [6], heart mitochondria still contained some endogenous Ca^{2+}, which could be rapidly released in the presence of rotenone (see Fig. 1). Rotenone prevents the energization of mitochondria by endogenous NAD-linked substrates. After rotenone treatment, respiration was restored by the addition of a substrate such as succinate to induce a rapid reaccumulation of Ca^{2+} ions. The Ca^{2+}-uptake mechanism was then selectively blocked by low concentrations of Ruthenium Red, and the kinetics of the passive Ca^{2+}-efflux from the Ca^{2+}-loaded mitochondria in the presence or absence of Na^+ ions in the medium were studied.

Following this protocol, the kinetics of active Ca^{2+} accumulation, of passive Ca^{2+} permeability and of Na^+–Ca^{2+} exchange activity of mitochondria were investigated (corresponding to phases 1, 2, and 3 in Fig. 1, respectively). In agreement with a previous report [5], clonazepam (compound **II**) was found to abolish selectively Na^+-induced stimulation of Ca^{2+} release (see Fig. 1) with an IC_{50} of 12 μM. Neither the active Ca^{2+} uptake, nor the passive, Na^+-independent Ca^{2+}-efflux rates were affected by clonazepam (compound **II**) in concentrations as high as 100 μM (Fig. 1). The inhibitory characteristics of a number of related compounds were investigated to gain an insight into the structure–function relation. Figure 2 summarizes the results obtained and shows that the potency of inhibition depended on a few interesting structural features. A halogen substitution in the 2′-position improved 10–20-fold the capacity of benzodiazepines to inhibit the exchanger (see, for instance, the difference between nitrazepam (**I**) and oxazepam (**III**) and their 2′-chloro derivatives clonazepam (**II**) and lorazepam (**IV**), respectively). Triazolo derivatives were also found to be active (see, for instance, compounds **V**, **VI**, **VII**, **VIII**, and **IX**). Some of them were even superior to the non-triazolo parent compound (compare, for instance, derivatives **IX** and **X**). The presence of a secondary amido group on the triazolo ring is essential for a good activity (see, for instance, compound **IX**). *N*-methylation of the secondary amido group reduced it considerably (compare compounds **VIII** and **IX**). A secondary amide also proved superior to a tertiary amide in all the non-triazolo benzodiazepines which we investigated (compare, for instance, compounds **X** and **XI**, and **II** and **XII**). Augmented inhibitory potency was likewise obtained by substituting an oxazepine ring for the diazepine ring (see, for instance compounds **VII** and **XIII**). A replacement of oxazepine by thiazepine further enhanced the inhibitory potency (see the series of compounds **X**, **XIV** and **XV**). Taking advantage of these observations, we then synthesized compound **XVI** which showed an inhibitory activity in the submicromolar range (0.8 μM). In Fig. 3 the inhibitory potency of compound **XVI** is compared with that of the various inhibitors of the mitochondrial exchanger reported in the literature, such as diltiazem [8, 9], amiloride derivatives [10] and bepridil [11]. Compound **XVI** is 10–20 times more potent than both the related diltiazem and clonazepam and than bepridil. Na^+-

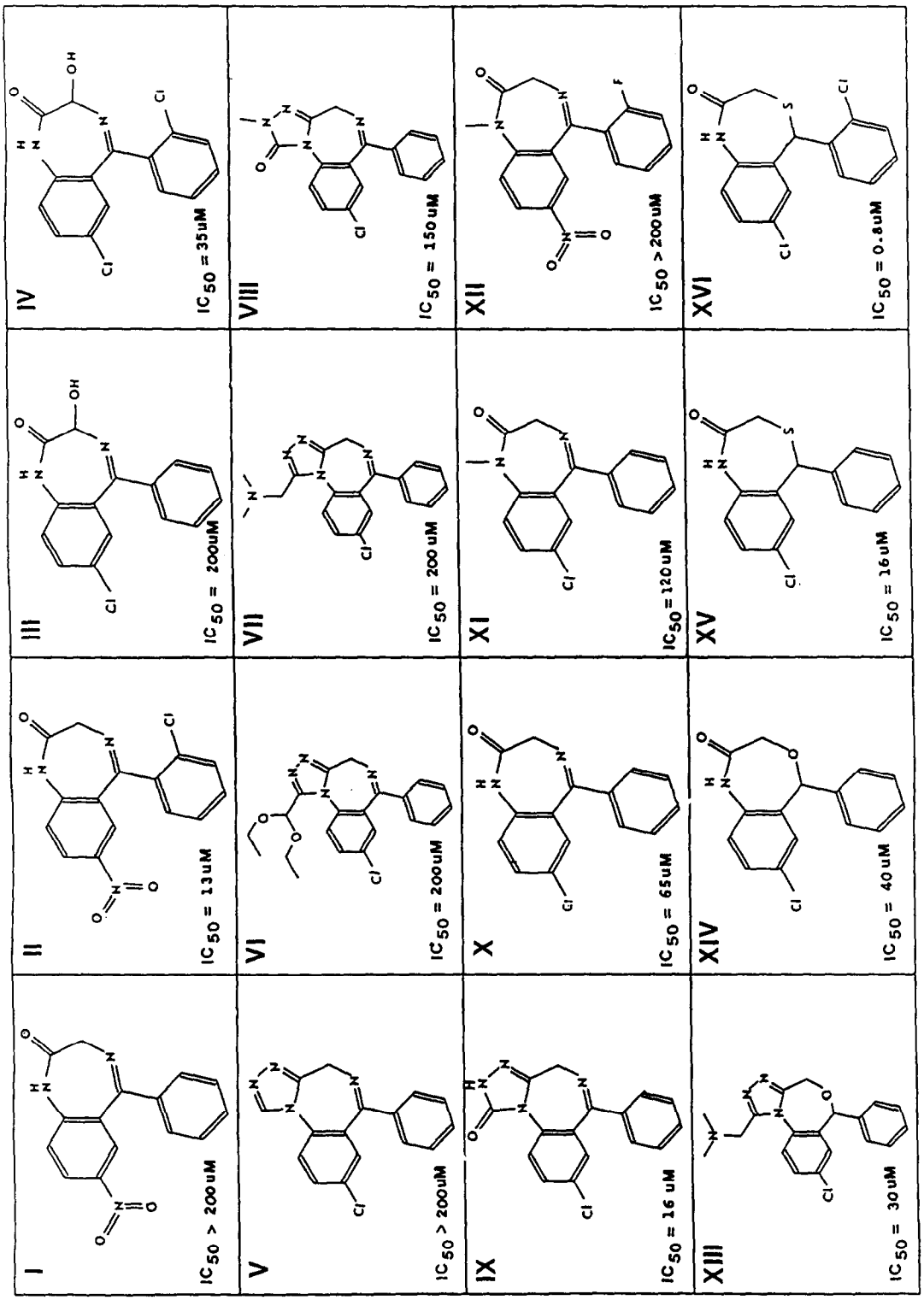

Fig. 2. Structure and inhibitory potency of various benzodiazepines and related compounds.

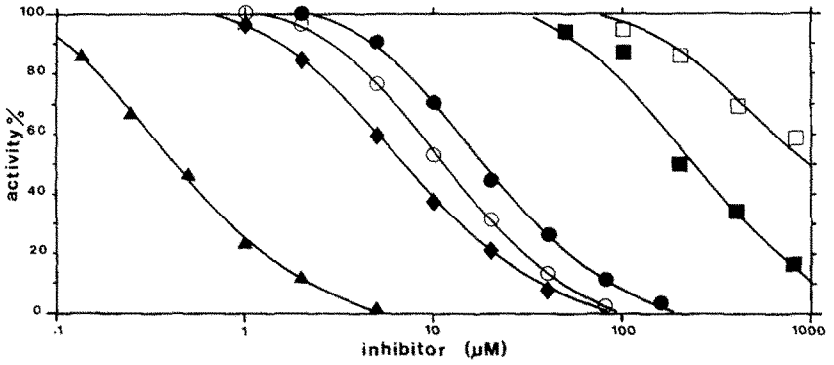

Fig. 3. A comparison of the potency of compound **XVI** with that of known inhibitors of the mitochondrial Na^+–Ca^{2+} exchanger. The experiments were carried out as described in Fig. 1 in the presence of various concentrations of inhibitors. The rate of Ca^{2+} efflux induced by the addition of 20 mM NaCl in the absence of inhibitors was taken as 100% activity. (▲) compound **XVI**, (◆) bepridil, (○) clonazepam, (●) (+)*cis* diltiazem, (■) benzamil, (□) amiloride.

independent Ca^{2+}-release from loaded mitochondria was not affected by compound **XVI**. At higher concentrations (above 20 μM), however, the compound started to inhibit the mechanism of Ca^{2+} accumulation in mitochondria.

DISCUSSION

Benzodiazepines interact with a variety of binding sites. In addition to the benzodiazepine receptors of the central type, which were initially identified in nerve tissue [12] where they modulate the GABA-regulated anion channels [13], another principal type of binding site has been described which is not linked to the GABA receptors. These latter binding sites, referred to as the peripheral-type sites, have been found in association with tissues characterized by a highly developed oxidative phosphorylating activity [14]. Interestingly enough, the peripheral-type receptors were recently shown to be localized mainly on the outer mitochondrial membrane [15]. The inner mitochondrial membrane contains a Na^+–Ca^{2+} exchange system which also seems to interact with benzodiazepines. Clonazepam, in fact, proved to be one of the most potent inhibitors of the exchange activity [5]. A direct comparison of the structural requirements that benzodiazepines must meet to inhibit the mitochondrial exchanger clearly shows that the binding site differs from both the peripheral-type and the central-type receptors. Methylation of the secondary amido group in the diazepine ring drastically reduces the capacity of compounds to inhibit the Na^+–Ca^{2+} exchanger. On the other hand, *N*-methylation has no appreciable effect on the binding to the central-type receptors and actually leads to a striking increase in affinity for the peripheral type receptors localized on the outer mitochondrial membrane [16]. The interaction of benzodiazepines with both the peripheral-type and the central-type receptors is optimalized by the introduction of a halogen in the 2′-position [16]. This modification considerably increases the inhibitory effect of benzodiazepines on the exchanger activity also. It seems that both the 2′-chloro substitution and the distal secondary amido group (which might form hydrogen bonds with the exchanger molecule) are necessary for optimal interaction of benzodiazepines, benzoxazepines, or benzothiazepines with the mitochondrial Na^+–Ca^{2+} exchanger. By compounding the various structural features which seemed to be important for an effective inhibitory activity, we succeeded in obtaining a potent inhibitor of the mitochondrial exchanger active in the submicromolar range. The improved selectivity and potency render compound **XVI** a valuable tool for future investigations of the physiological role of the Na^+-induced rapid release of Ca^{2+} ions from mitochondria in excitable tissues and also for biochemical studies of the molecular nature of the exchange mechanism.

Acknowledgements—We wish to express our thanks to Dr. Allgeier of Ciba-Geigy for providing us with most of the compounds used in this study and for his kind advice. We also thank Dr. V. Rasetti for helpful discussions.

REFERENCES

1. Carafoli E, Mitochondrial Ca-transport and Ca-binding proteins. In: *Mitochondria, Bioenergetics, Biogenesis and Membrane Structure* (Eds. Packer L and Gomez-Puyou A), pp. 47–60. Academic Press, New York, 1976.
2. Nicholls D and Akerman K, Mitochondrial calcium transport. *Biochim Biophys Acta* **683**: 57–88, 1982.
3. Crompton M, Capano M and Carafoli E, The sodium-induced efflux of calcium from heart mitochondria. *Eur J Biochem* **69**: 453–462, 1976.
4. Crompton M, Moser R, Luedi H and Carafoli E, The interrelations between the transport of sodium and calcium in mitochondria of various mammalian tissues. *Eur J Biochem* **82**: 25–31, 1978.
5. Matlib AM and Schwartz A, Selective effects of diltiazem, a benzothiazepine calcium channel blocker, and diazepam, and other benzodiazepines on the Na/Ca exchange carrier system of heart and brain mitochondria. *Life Sci* **32**: 2837–2842, 1983.
6. Scarpa A and Graziotti P, Mechanisms for intracellular Ca regulation in heart. *J Gen Physiol* **62**: 756–772, 1973.

7. Scarpa A, Brinley FJ and Dubyak G, Antipyrylazo III, a middle range Ca-metallochromic indicator. *Biochemistry* **17**: 1378–1386, 1978.
8. Vaghy PL, Johnson JD, Matlib MA, Wang T and Schwartz A, Selective inhibition of Na-induced Ca-release from heart mitochondria by diltiazem and certain other Ca-antagonist drugs. *J Biol Chem* **257**: 6000–60002, 1982.
9. Chiesi M, Rogg H, Eichenberger K, Gazzotti P and Carafoli E, Stereospecific action of diltiazem on the mitochondrial Na–Ca exchange system and on sarcolemmal Ca-channels. *Biochem Pharmacol* **36**: 2735–6002, 1982.
10. Jurkowitz MS, Altschuld RA, Brierley GP and Cragoe EJ, Inhibition of Na-dependent Ca-efflux from heart mitochondria by amiloride analogs. *FEBS Lett* **162**: 262–265, 1983.
11. Matlib MA, Action of bepridil, a new calcium channel blocker on oxidative phosphorylation, oligomycin-sensitive adenosine triphosphatase activity, swelling, Ca-uptake and Na-induced Ca-release processes of rabbit heart mitochondria *in vitro*. *J Pharmacol Exp Ther* **233**: 376–381, 1985.
12. Braestrup C and Squires RF, Specific benzodiazepine receptors in rat brain characterized by high affinity diazepam binding. *Nature (Lond)* **266**: 732–734, 1977.
13. Tallman JF, Paul SM, Skolnick P and Gallagher DW, Receptors for the age of anxiety: pharmacology of the benzodiazepines. *Nature (Lond)* **274**: 383–385, 1980.
14. Anholt RRH, DeSouza EB, Oster-Granite ML and Snyder SH, Depletion of peripheral-type benzodiazepine receptors after hypophysectomy in adrenal glands and testis. *J Pharmacol Exp Ther* **233**: 517–526, 1985.
15. Anholt RRH, Pedersen PL, DeSouza EB and Snyder SH, The peripheral type benzodiazepine receptor. *J Biol Chem* **261**: 576–583, 1986.
16. Wang JKT, Taniguchi T and Spector S, Structural requirements for the binding of benzodiazepines to their peripheral-type sites. *Mol Pharmacol* **25**: 349–351, 1984.