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Mitochondrial benzodiazepine receptor linked to inner membrane ion channels by nanomolar actions of ligands

(patch clamp/transport/protoporphyrin/permeability transition/heart)

KATHLEEN W. KINNALLY*†, DMITRY B. ZOROV*‡, YURI N. ANTONENKO*‡, SOLOMON H. SNYDER§, MAUREEN W. MCENERY§, AND HENRY TEDESCHI*

*Department of Biological Sciences, State University of New York at Albany, NY 12222; ‡A. N. Belozersky Laboratory of Molecular Biology and Bioorganic Chemistry, Moscow State University, Moscow, Russia; and §Departments of Neuroscience, Pharmacology and Molecular Sciences, and Psychiatry, The Johns Hopkins University School of Medicine, Baltimore, MD 21205

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ABSTRACT **The mitochondrial benzodiazepine receptor (mBzR) binds a subset of benzodiazepines and isoquinoline carboxamides with nanomolar affinity and consists of the voltage-dependent anion channel, the adenine nucleotide translocator, and an 18-kDa protein. The effect of ligands of the mBzR on two inner mitochondrial membrane channel activities was determined with patch-clamp techniques. The relative inhibitory potencies of the drugs resemble their binding affinities for the mBzR. Ro5-4864 and protoporphyrin IX inhibit activity of the multiple conductance channel (MCC) and the mitochondrial centum-picosiemen (mCtS) channel activities at nanomolar concentrations. PK11195 inhibits mCtS activity at similar levels. Higher concentrations of protoporphyrin IX induce MCC but possibly not mCtS activity. Clonazepam, which has low affinity for mBzR, is at least 500 times less potent at both channel activities. Ro15-1788, which also has a low mBzR affinity, inhibits MCC at very high concentrations (16 μM). The findings indicate an association of these two channel activities with the proteins forming the mBzR complex and are consistent with an interaction of inner and outer membrane channels.**

Benzodiazepines exert their well-known antianxiety, anticonvulsant, and sedative effects through a receptor in nervous tissue that is associated with a chloride channel regulated by the inhibitory neurotransmitter γ-aminobutyric acid (1). Benzodiazepines also bind with nanomolar affinity to a distinct site in many tissues that is selectively associated with mitochondria and designated the mitochondrial benzodiazepine receptor (mBzR). Drugs with high affinity for mBzR influence diverse biological processes, including mitochondrial respiration (2–4), neural excitability (5), leukocyte respiration (6), and adrenal steroid synthesis (7–9). These pleiotropic actions are consistent with influences on some fundamental aspect of mitochondrial function.

Purification of mBzR in a form retaining reversible ligand binding yielded a complex containing the voltage-dependent anion channel (VDAC, or mitochondrial porin), the adenine nucleotide translocator (ANT), and an 18-kDa protein (10). Since VDAC and the 18-kDa protein are localized to the outer membrane and ANT is an inner membrane protein, the functional mBzR is most likely associated with "contact sites" where the inner and outer membranes adhere (11) and manifest a number of ionic conductance levels (12). While the multiple conductance channel activity (MCC, defined with patch-clamp studies as ranging in conductance between 40 and >1000 pS) is thought to be associated with contact sites (for a review see ref. 13), this has not been postulated for mCtS activity [mitochondrial centum-picosiemen, the ≈100-pS voltage-dependent activity originally described by Sorgato *et al.* (14)].

We now demonstrate that nanomolar levels of mBzR ligands influence both MCC and mCtS activities with drug specificities appropriate for mBzR (15, 16) in patch-clamp studies of mitoplasts (mitochondria whose outer membrane is removed). Some of these results were previously presented in abstract form (17). Our findings indicate that MCC and mCtS conductances are associated with the mBzR protein complex and that these channel activities account for at least some of the biological manifestations of mBzR.

MATERIALS AND METHODS

Preparation of the Mitoplasts. Hearts from male rats of the Sprague–Dawley strain (100–250 g) were homogenized in 230 mM mannitol/70 mM sucrose/5 mM Hepes/1 mM EGTA, pH 7.4, with a glass and Teflon homogenizer of the Potter–Elvehjem type. Large mitochondria were isolated as previously described (e.g., ref. 18), and mitoplasts were prepared from these mitochondria by using the French-pressure-cell [2000 psi (1 psi = 6.89 kPa)] method of Decker and Greenawalt (19) to remove the outer membrane. After isolation the mitoplasts were resuspended in 150 mM KCl/5 mM Hepes/1 mM EGTA/0.75 mM $CaCl_2$, pH 7.4, which contains ≈0.1 μM free Ca^{2+} and is referred to as medium I. In general, MCC and/or mCtS activity was observed upon patching. Mitoplast patches without activity were not used.

Patch Clamping. The conditions and procedures were essentially the same as we previously reported (20). The bathing medium and the solution in the patching pipettes contained medium I. Experiments were carried out at room temperature (≈25°C). Typically, effectors were delivered in a solution of medium I (<0.5% ethyl alcohol), by perfusion of 3–6 ml through a 0.7-ml bath. Controls were carried out by the same procedure but without the drug, and little or no effect was observed with medium I containing up to 0.5% ethyl alcohol. The criterion for MCC activation was reversibility, to distinguish it from patch deterioration or seal loss. In this study only membrane patches excised from mitoplasts or phosphatidylcholine liposomes [prepared as previously described without added protein (21)] were used. Computer analysis of current signals stored on video tape at 10 kHz was bandwidth limited to 2 kHz with a low-pass filtering device (Frequency Devices, Haverhill, MA, model 902) and a sampling frequency of at least 5 kHz unless otherwise stated.

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Abbreviations: ANT, adenine nucleotide translocator; mBzR, mitochondrial benzodiazepine receptor; PPIX, protoporphyrin IX; MCC, multiple conductance channel; mCtS, mitochondrial centum-picosiemen channel; nP_o, open probability; V_o, voltage with nP_o of 0.5; VDAC, voltage-dependent anion channel.

†To whom reprint requests should be addressed.

Computer analysis of data was done with Strathclyde electrophysiological data analysis software (PAT, courtesy of J. Dempster, University of Strathclyde, U.K.) with a 2801A D/A board from Data Translation (Marlboro, MA). Open probability, nP_o, was calculated from amplitude histograms as the ratio of percent time at open current levels over the total time.

Chemicals. Stock solutions of 0.1–10 mM Ro5-4864 [7-chloro-1,3-dihydro-1-methyl-5-(*p*-chlorophenyl)-2*H*-1,4-benzodiazepine-2-one], PK11195 [1-(2-chlorophenyl)-*N*-methyl-*N*-(1-methylpropyl)isoquinoline-3-carboxamide], and clonazepam [5-(2-chlorophenyl)-1,3-dihydro-7-nitro-2*H*-1,4-benzodiazepine-2-one] were in ethyl alcohol. Protoporphyrin IX (PPIX) from Sigma was dissolved in ethyl alcohol with HCl to dissolve the crystals and diluted 1:100 with medium I for a 40 μM stock at pH 7.4. The drugs were obtained through the courtesy of P. F. Sorter (Hoffman–La Roche).

RESULTS

Studies of mCtS Channel Activity. In an effort to identify endogenous mBzR ligands, PPIX was found to be very potent in inhibiting mBzR ligand binding (16). The inhibitory effect of nanomolar levels of PPIX on mCtS activity is shown in Fig. 1, which includes typical current traces and total current amplitude diagrams. In this patch, PPIX (20 nM) decreased the mean open time from 4.17 to 0.95 ms, increased the closed time from 0.86 to 2.94 ms, and decreased the burst length from 352 to 17.1 ms. The changes in these characteristics are consistent with a shift in V_o (defined as the voltage giving an nP_o of 0.5) and stabilization of the closed state.

mBzR is classically defined by its binding affinities for Ro5-4864, PK11195, and clonazepam (10). Fig. 2 shows the effect of PK11195 on mCtS nP_o at various voltages, and Fig. 3 shows comparable experiments with Ro5-4864 and clonazepam. The high-affinity mBzR ligands PK11195 and Ro5-4864 reduced open probability with nanomolar concentrations most effectively at negative and low positive potentials, while the results with 5–20 μM clonazepam (a central benzodiazepine receptor ligand with low affinity for mBzR) cannot be distinguished from the control. The nP_o vs. voltage curves for the control and in the presence of PK11195 or Ro5-4864 are nearly parallel (Figs. 2 and 3) and show a V_o shift. Removal of Ro5-4864 or PK11195 by perfusion with drug-free medium at positive potentials relieved mCtS inhibition (n = 4 patches).

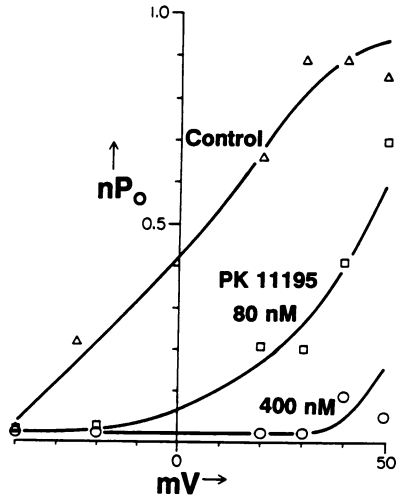

FIG. 2. mCtS nP_o dependence on voltage and PK11195. Amplitude diagrams (bin width, 0.1 pA) were used to calculate nP_o in the absence (△) and presence of 80 nM (□) and 400 nM (○) PK11195. Otherwise, details are as in Fig. 1.

The relative potencies of drugs on mCtS activity fit well with mBzR binding affinity (Table 1). The three mBzR ligands Ro5-4864, PPIX, and PK11195 affect mCtS activity at nanomolar concentrations whereas clonazepam is about 500 times less potent despite only minor differences in chemical structure from Ro5-4864. Overall, potencies of the ligands on channel activity parallel their binding affinities for mBzR (15, 16).

Studies of MCC Activity. The effect of various mBzR high- and low-affinity ligands on MCC activity is shown in Table 2. A study of PPIX effects on MCC activity is shown in Fig. 4. Fig. 4*A* corresponds to the control, showing a current trace and total current amplitude histogram with an nP_o of 0.99. Fig. 4*B* shows the dramatic inhibitory effect of 8 nM PPIX. The channel opened only for short periods (nP_o = 0.22). Strikingly, the inhibition (which was only slightly reversible

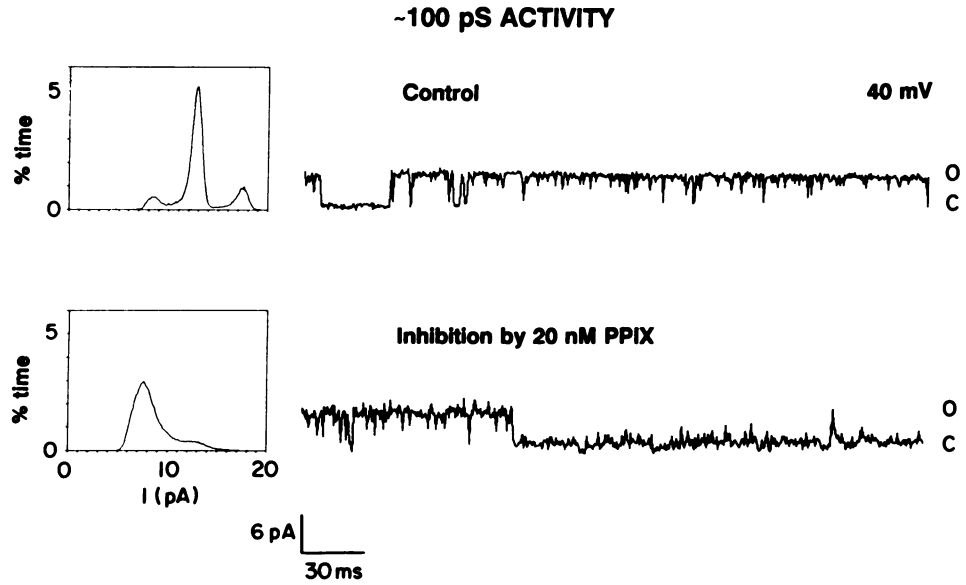

FIG. 1. Inhibition of mCtS activity by PPIX. Total amplitude diagram shows occupancy of current levels as percent time (bin width, 0.1 pA) relative to zero-voltage current (I) level. Current traces illustrate data used to generate amplitude diagrams in the absence (control) and presence of 20 nM PPIX at 40 mV for two channels in this patch. O, open; C, closed.

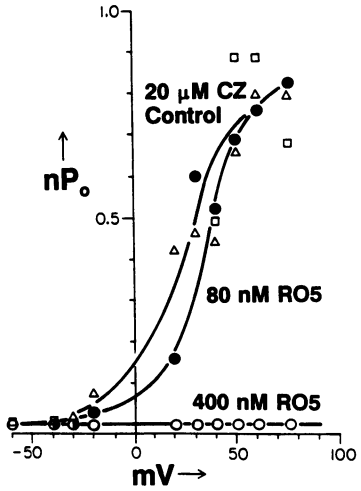

FIG. 3. Differential effect of benzodiazepines on mCtS activity voltage dependence. Amplitude diagrams (bin width, 0.1 pA) were used to calculate nP_o at various voltages. Control (□) and 20 μM clonazepam (CZ, △) were not distinguishable. Slight inhibition was seen in the presence of 80 nM Ro5-4864 (RO5, ●) and was complete at 400 nM (○). Otherwise, details are as in Fig. 1.

by perfusion with medium without PPIX) was overcome by subsequent perfusion with 400 nM PPIX, and, in turn, this activation was reversed by perfusion with medium lacking the drug (Fig. 4*D*, 70% showing this effect, $n = 7$ patches). A subsequent reactivation (Fig. 4*E*) was blocked by Ro5-4864 ($n = 2$ patches).

The PPIX dose dependence of nP_o at 20 mV showed inhibition at 8–40 nM and activation at 200–400 nM (Fig. 5*A*). Fig. 5*B* shows the dependence of nP_o on voltage and summarizes in detail the interplay between voltage and PPIX at an inhibiting (8 nM) and an activating (400 nM) concentration. The control shows the typical voltage sensitivity of the channel, which closes to lower conductance levels with positive voltage. Perfusion with 8 nM PPIX reduced nP_o at all voltages. The increase in nP_o induced by 400 nM PPIX was less prominent at high positive voltages, which suggests a V_o shift of at least 25 mV.

As with mCtS activity, the relative potencies of the four drugs tested on MCC activity correspond at least approximately with the mBzR pharmacology (4) and binding affinity (15, 16) (Table 2). Of the three benzodiazepines evaluated, Ro5-4864, which is mBzR-selective, had a nanomolar IC_{50}, whereas clonazepam and Ro15-1788 {ethyl 8-fluoro-5,6-dihydro-5-methyl-6-oxo-4*H*-imidazo[1,5*a*][1,4]benzodiazepine-3-carboxylate}, which are potent at central benzodiazepine receptors but inactive at mBzR, were about 500 times less effective. Clonazepam was not effective whereas 16 μM Ro15-1788 inhibited about 60% of the patches. PPIX, like Ro5-4864, had a nanomolar IC_{50}.

In contrast to our results with MCC activity, we did not usually observe activation of the mCtS activity by high

Table 1. Inhibition of mCtS activity by benzodiazepine-related ligands

Ligand	*n**	%†	IC_{50},‡ nM
Ro5-4864	10	100	26 ± 12
Clonazepam	8	25	>15,000
PPIX	4	100	35 ± 6
PK11195	6	100	<80

*No. of individual membrane patches used to determine effect.
†Percent patches showing effect.
‡Voltage-dependent inhibitory concentration giving an ≈50% decrease in nP_o observed at, generally, 20 mV. Values are means ± SE of experiments where drug concentration was varied with a minimum n of 4 patches.

Table 2. Effect of benzodiazepine-related ligands on MCC activity

Ligand	Effect	*n**	%†	IC_{50},‡ nM
Ro5-4864	Inhibit	19	89	71 ± 37
Clonazepam	None	5	100	>20,000
PPIX	Inhibit	8	88	24 ± 10
	Activate	7	100	244 ± 38
Ro15-1788	Inhibit	8	63	16,000

*No. of individual membrane patches used to determine effect.
†Percent patches showing effect.
‡Voltage-dependent inhibitory (or activating) concentration giving an ≈50% change in nP_o or mean current level observed at, generally, 20 mV. Values are means ± SE of experiments where drug concentration was varied with a minimum n of 4 patches.

concentrations of the drugs. Since activation of MCC could mask the activity of the smaller channel, it is difficult to interpret this finding. Perfusion of patches excised from phosphatidylcholine liposomes (no added protein) with ethanol (up to 0.5%) or PPIX (8 nM–2 μM) had no effect on current traces ($n = 4$) at ±50 mV.

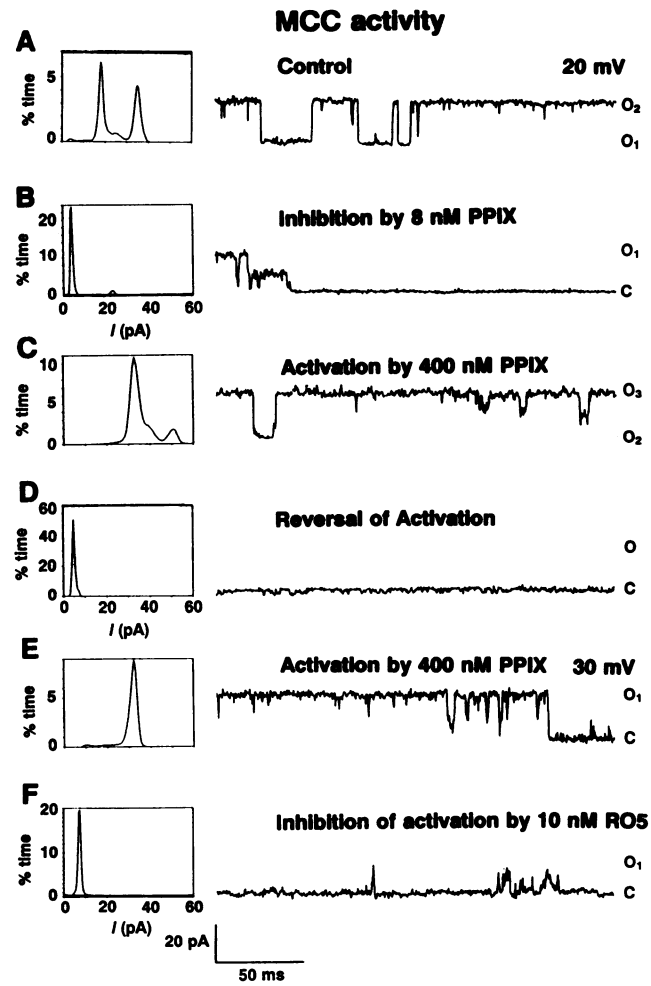

FIG. 4. Effect of PPIX on MCC activity. Total amplitude diagrams show occupation of current levels as percent time (bin width, 0.4 pA) relative to zero-voltage current (I) level. Current traces illustrate data used to generate amplitude diagrams. (*A*) Control MCC activity at 20 mV, medium I. (*B*) Inhibition after perfusion with 8 nM PPIX in medium I. (*C*) Induction of MCC activity with 400 nM PPIX in medium I. (*D*) Reversal of activation by washing with medium I. (*E*) Reactivation of MCC activity with 400 nM PPIX in medium I at 30 mV. (*F*) Perfusion with 10 nM Ro5-4864 (RO5) plus 400 nM PPIX in medium I reverses PPIX-induced MCC activity. O_1, O_2, and O_3 correspond to the open states of one, two and three channels. C is the closed state.

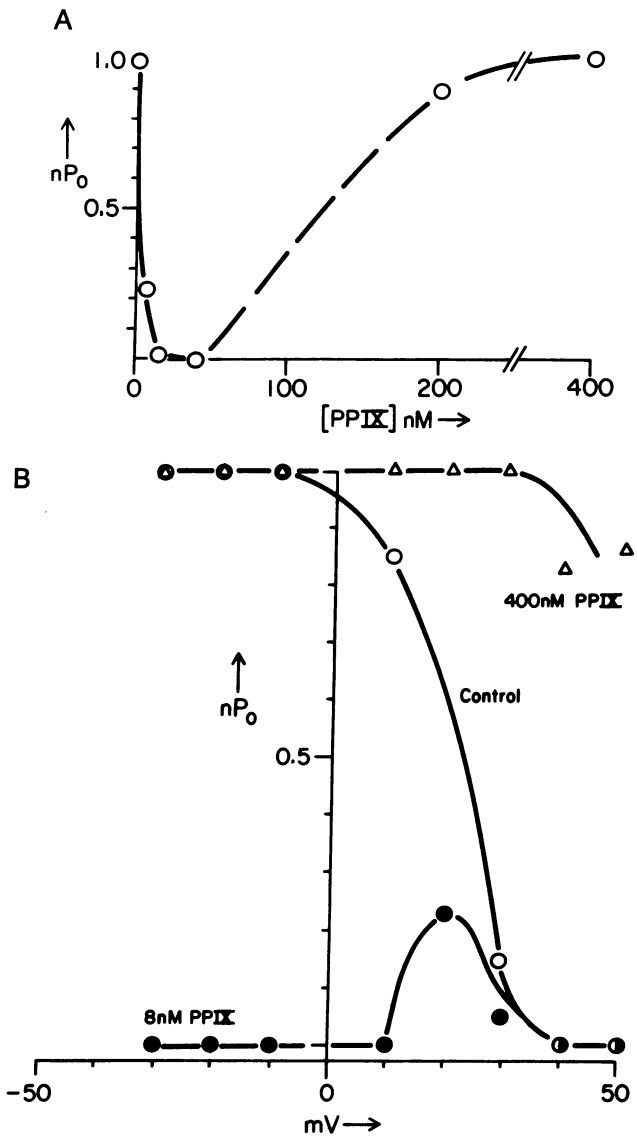

FIG. 5. MCC activity nP_o dependence on voltage and PPIX. (*A*) Amplitude diagrams were used to calculate nP_o of conductance levels above 300 pS at 20 mV. The plot of nP_o vs. PPIX concentration shows the inhibition at 8–40 nM and activation at 200–400 nM PPIX. (*B*) Voltage dependence of nP_o with 0 nM (○), 8 nM (●), and 400 nM (△) PPIX. Current records used for nP_o determinations were generally 30–90 s in duration. The control nP_o for the 250-pS level was 0 and 0.46 at 40 and 50 mV, respectively (data not shown).

For both mCtS and MCC activities, the inhibitory effects of nanomolar Ro5-4864, PPIX, and PK11195 were observed in 76% of the patches. Higher concentrations were necessary to inhibit 18% of the patches, and another 6% were not inhibited. This variability may be the result of a variety of factors: the geometry of the patch in the pipette (e.g., see ref. 22), which could prevent efficient mixing; differences in functional state (where presumably activating conditions could occur simultaneously); the presence of binding sites exogenous to the patch contributing to binding of the drug (e.g., the presence of other mitoplasts); variable partition of the hydrophobic drugs within the membrane patch; and the condition and availability of contact sites between the inner and outer membrane.

DISCUSSION

Our major finding is that ligands of mBzR influence MCC and mCtS channel activities at low nanomolar concentrations, consistent with their affinities in binding to mBzR (15, 16) and with relative effects corresponding to the pharmacology of mBzR (4). The only drug reported to affect mitochondrial channel activity as potently as mBzR ligands is cyclosporin A, which inhibits MCC activity at 10–100 nM (23). Both MCC and mCtS activities are affected by low micromolar levels of other drugs, including amiodarone (24) and antimycin A (18). At those concentrations, antimycin A and cyclosporin A also influence mBzR ligand binding (15), suggesting that the effects of these non-benzodiazepine drugs on MCC activity may involve mBzR.

The voltage dependence curves of mCtS activity in the presence or absence of drugs (Figs. 2 and 3) are nearly parallel but show significant shifts in V_o. The changes in channel characteristics (decay constants, mean open and closed times, and burst lengths; data not shown) are consistent with a V_o shift. In line with the interpretation of others (25, 26), these findings suggest that the inhibition by Ro5-4864 and PK11195 does not alter the gating charge of the channel but rather involves a stabilization of the closed state. While the potencies are different, the similarities between these data and those of previous studies (18) suggest a similar mode of action for antimycin A, PK11195, and Ro5-4864 on mCtS activity.

Interestingly, the other class of mitoplast channel activity, MCC, exhibits a biphasic dose response to PPIX (Fig. 5*A*) with inhibition at low and a reversible activation at higher concentrations similar to that of antimycin A (18). The shift in V_o during activation may be the result of a stabilization of the open state with both drugs. Surprisingly, a biphasic dose response of the permeability transition pore [postulated equivalent of MCC activity (27)] to the MCC inhibitor amiodarone (24) was recently reported in mitochondrial suspensions (28). The biphasic dose response to different drugs suggests that binding at a second, lower affinity site may result in MCC activation by a variety of ligands. While the mechanism is unclear, it may involve alterations in contact-site integrity or coupling of proteins which make up or associate with mBzR. The reversal of 400 nM PPIX-induced activation by 10 nM Ro5-4864 (Fig. 4*D*) suggests an agonist–antagonist interaction. Additional binding sites are also supported by the micromolar inhibition of the inner membrane anion channel (IMAC) by Ro5-4864 in mitochondrial suspensions (29).

Inner and outer membranes make contact at specific sites (11) which may mediate unique mitochondrial functions. Patch-clamp studies of liposomes containing membrane preparations from brain mitochondria enriched in contact sites (30) have revealed channel activity that we believe corresponds to MCC activity (12). Because of these findings, it was suggested that outer and inner membrane channels may be in register at contact sites to provide a pathway between the matrix face and the patching pipette (13). Contact sites are organized in complexes probably involving VDAC, hexokinase, creatine kinase, and ANT (e.g., refs. 31–37) and possibly other proteins. The observation that mBzR-binding activity copurifies with both outer (18-kDa protein and VDAC) and inner (ANT) membrane components (10) supports this model.

Since drugs influence two distinct channel activities, MCC and mCtS, with potencies resembling their affinities for mBzR, it seems likely that the same proteins, or at least homologous binding sites on different proteins, are involved. MCC and mCtS activities might reflect different functional states of mBzR, perhaps related to variations in coupling of receptor subunits. As the mBzR ligand binding sites are on the outer membrane [PPIX and Ro5-4864 on VDAC (10, 38) and PK11195 on the 18-kDa protein (9)], it is also possible that mCtS and MCC may reflect differences in ANT, the inner membrane component of the mBzR receptor. If this were the case, the mBzR ligands would be indirectly influencing MCC

and mCtS activity by affecting either VDAC or the 18-kDa protein.

PK11195 inhibits mCtS activity (Fig. 2). Its putative target for inhibition is the nanomolar-affinity binding site on an 18-kDa protein whose primary structure does not indicate an associated membrane transport activity (39). Therefore, it is reasonable to postulate that the 18-kDa protein is a modulator of mitochondrial channel activity. This role may also explain the variable electrophysiological behavior of VDAC *in situ* and in reconstituted systems (40). As only a fraction of VDAC and ANT molecules are associated with the 18-kDa protein to form mBzR (38), some of the variability may be the result of an association of different proteins [e.g., ANT, 18-kDa protein, or modulator protein (41)] with VDAC.

The association of channels with mBzR can explain the principal observed actions of mBzR ligands. For example, the benzodiazepine-induced decrease in respiratory control ratios (4) may be anticipated from changes in the passage of crucial ions and other substances facilitated by MCC and mCtS activities. Similarly, the possible involvement of mBzR in diverse processes such as differentiation (42), immune cell proliferation (43), and steroidogenesis (7–9) could result from initial actions on MCC and/or mCtS activities.

Our findings may facilitate efforts to understand mitochondrial channels in terms of known protein constituents of mitochondrial membranes. Unlike VDAC, neither MCC nor mCtS has been ascribed to specific proteins. In investigating the relationship of mBzR to channel activity, mBzR ligands should be valuable pharmacological probes.

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