Identification of a Novel, Inhibitory Action of Amiodarone on Vesicular Monoamine Transport

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ABSTRACT
The benzofuran antiarrhythmic drug, amiodarone, exhibits a wide range of pharmacological properties. Recent in vivo biochemical studies suggest that amiodarone may exert an antiadrenergic action in the heart, which resembles the effects of reserpine. To investigate the cellular basis for this apparent presynaptic, sympatholytic action we used Chinese hamster ovary (CHO) cells expressing type 2 vesicular monoamine transporter (VMAT2) as a synaptic vesicular model. Amiodarone inhibited the uptake of [3H]norepinephrine in VMAT2-transfected CHO cells in a concentration–dependent manner, with a −log EC50 of 6.44 ± 0.32. To further identify the site at which amiodarone suppressed vesicular monoamine transport, we examined the ability of amiodarone to displace [3H]reserpine from its binding site in membrane fractions prepared from CHO cells expressing VMAT2. [3H]Reserpine binding was inhibited in a concentration–dependent manner by amiodarone, with an −log EC50 of 6.76 ± 0.03, reaching 84 ± 5% inhibition of reserpine binding at 10 μM. A pH-dependent mechanism for this action of amiodarone was excluded in studies using the pH-sensitive fluorescent indicator 2′,7′-bis(carboxyethyl)-5,6-carboxyfluorescein (BCECF). These data indicate that amiodarone inhibits the uptake of monoamine into the axoplasmic storage vesicle by inhibiting VMAT. Furthermore, amiodarone competes specifically with reserpine for binding to VMAT. These findings suggest a novel presynaptic site of action for amiodarone.

Amiodarone, a benzofuran derivative, is a commonly used antiarrhythmic agent that was initially developed as an antianginal agent. This complex drug has been demonstrated to block K+, Na+, and Ca2+ channels, in addition to having an incompletely characterized antisympathetic action. The antiadrenergic actions of amiodarone are complex. In isolated tissues, amiodarone has been characterized as a noncompetitive β-adrenoceptor antagonist (Polster and Broekhuysen, 1976), whereas in intact animals, amiodarone has been shown to cause a bradycardia that is apparently independent of actions on β-adrenoceptors (Charlier, 1970). More recently, Du et al. (1995), demonstrated a “reserpine-like” effect of amiodarone characterized by an increase in the overflow of dihydroxyphenylglycol (DHPG), the major intraneuronal metabolite of norepinephrine (NE), from the rat heart. This action was accompanied by a reduction in the cardiac NE tissue content, and in conjunction, an attenuation of the release of NE in response to electrical stimulation of the cardiac sympathetic nerves.

Reserpine, a Rauwolfia alkaloid, prevents the transport of amines into chromaffin granules and synaptic storage vesicles (Darchen et al., 1989; Schuldiner et al., 1995) by binding with high affinity to the vesicular monoamine transporter (VMAT), most probably at the amine recognition site. The actions of reserpine on sympathetic neuronal biochemical integrity have been well documented, and in the rat heart include an increase the overflow of DHPG while decreasing the tissue content of NE (Du et al., 1995). The resultant increase in DHPG overflow is readily explained by the known mechanism of action for reserpine, and results from increased metabolism of intraneuronal NE by monoamine oxidase, yielding DHPG.

Thus, given physiological data suggesting that amiodarone exerts antiadrenergic actions, and neurochemical data indicating a possible reserpine-like action, the aim of the current study was to investigate the effect of amiodarone on vesicular monoamine transport. To examine this question as directly as possible, we studied the action of amiodarone on monoamine transport in Chinese hamster ovary (CHO) cells transfected with cDNA encoding type 2 VMAT (VMAT2). Subsequently, binding experiments were performed to ascertain whether amiodarone binds to the amine recognition site.

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ABBREVIATIONS: CHO, Chinese hamster ovary; VMAT2, type 2 vesicular monoamine transporter; BCECF, 2′,7′-bis (carboxyethyl)-5,6-carboxyfluorescein; DHPG, dihydroxyphenylglycol; NE, norepinephrine; pH, intracellular pH.
Materials and Methods

Cell Culture and Transfection. CHO cells grown in 10% fetal calf serum to 60% to 70% confluency in 24-well plates (Falcon Labware, Oxnard, CA) were transiently transfected (Lipofectamine, Gibco-Life Technologies, Gaithersburg, MD) with an expression vector (pcDNA3) containing full-length cDNA for the bovine VMAT2 (kindly provided by Dr. B. Gasnier, Institute de Biologie Physico-Chimique, Paris, France).

Monoamine Transport Assays. Measurement of transport of monoamines was performed as previously described (Steiner-Murdoch et al., 1996). In brief, 36 h after transfection, CHO cells were washed with uptake buffer containing 110 mM potassium tartrate, 5 mM glucose, 0.2% bovine serum albumin, 5 mM MgCl2, 1 mM ascorbic acid, 10 mM MgCl2, and 20 mM K-HEPES, pH 7.8. The cells were permeabilized for 10 min at 37°C in uptake buffer containing 10 mM digitonin. The medium was then replaced with fresh buffer in the absence of digitonin containing 5 mM Mg-ATP and the corresponding radiotracer ([3H]NE or [3H]dopamine) and drugs for the specific experiments and incubated for 45 min at 37°C. At the end of the experiment the uptake buffer was aspirated and cells washed with 1 ml of ice-cold uptake buffer containing 5 mM MgSO4 with no tracer or drug added. Cells were lysed with 0.1% Triton, and radioactivity subsequently assessed by liquid scintillation counting. All experiments were repeated at least three times.

[3H]Reserpine Binding. [3H]reserpine binding was performed in lysates prepared from transfected and untransfected CHO cells. Cells were collected by a rubber policeman and suspended in a lysis buffer containing 0.15 M NaCl, 10 mM K-HEPES, pH 8.5, 5 mM Na EGTA, and 1 μg/ml leupeptin, as previously described (Steiner-Murdoch et al., 1996). The cell lysate suspension was then sonicated for 10 s using a Sonicator B-30 Cell Disruptor (Branson Ultrasonics Corp., Danbury, CT). After centrifugation (3500g, 5 min), cell debris was discarded. The lysate then underwent ultracentrifugation (50,000g, 60 min) in a Beckman TLX ultracentrifuge (Beckman Instruments, Fuller, CA) and the supernatant discarded. The remaining pellet was resuspended at a final concentration of 0.25 mg protein/ml in binding buffer containing 320 mM sucrose, 10 mM K-HEPES, pH 8.5, 4 mM KCl, 5 mM ATP, and 5 mM MgSO4 and stored at −80°C until use.

Binding studies were performed by incubating 100 μg protein with [3H]reserpine (3 nM, specific activity 20 Ci/mmol) in a water bath at 32°C. The reaction was terminated by placing the reaction on ice and then rapidly diluting it with 1 ml of ice-cold binding buffer and subsequently filtering it on a glass fiber filter (Whatman GFC, Whatman Inc., Clifton, NJ). Filters were washed three times with ice-cold phosphate-buffered saline. Radioactivity was subsequently determined by liquid scintillation spectroscopy. Nonspecific binding was determined in membrane fractions obtained from untransfected CHO cells and in membrane fractions from VMAT2-transfected CHO cells in which 2 μM unlabeled reserpine was added to the binding reaction.

Intracellular pH (pHi) Recording. To establish the effect of amiodarone on pHi, CHO cells were also plated on glass coverslips for fluorescence spectroscopy. Following transfection with an expression vector containing VMAT2 cDNA (as described above), studies of pHi were performed with the pH-sensitive fluorescent indicator 2',7'-bis(carboxyethyl)-5,6-carboxyfluorescein (BCECF; Molecular Probes, Eugene, OR) (Weissberg et al., 1987). Cells were loaded in buffer containing: 135 mM NaCl, 5 mM KCl, 1.8 mM CaCl2, 0.8 mM MgSO4, 10 mM HEPES, 5.5 mM glucose, and 5 μM BCECF/AM, pH 7.4, for 30 min at 37°C, followed by washing in buffer solution for a further 10 min at room temperature. Coverslips containing CHO cells loaded with BCECF were subsequently loaded into a cuvette containing the above buffer at pH 7.8 and 37°C, and positioned in a SPEX CM2 dual excitation spectrofluorometer (SPEX Industries, Edison, NJ). Cells were alternately excited at 440 nm and 495 nm, and the emitted fluorescence was monitored at 530 nm.

Effect of Amiodarone and DEA on Monoamine Transport. CHO cells transfected with an expression vector containing full-length cDNA for VMAT2 robustly accumulated both [3H]NE and [3H]dopamine in comparison with untransfected cells (data not shown). Amiodarone inhibited VMAT2-mediated NE transport in a concentration-dependent manner, with an EC50 of 346 nM (Fig. 1A). At a concentration of 10 μM, amiodarone and its metabolite DEA potently inhibited VMAT2-mediated transport of dopamine and NE, reaching a similar degree of transport inhibition as that elicited by reserpine (Fig. 1B). Furthermore, amiodarone at a concentration of 10 μM also significantly inhibited the VAMT2-mediated transport of [3H]dopamine, with similar efficacy to that exhibited by 10 μM reserpine (Fig. 1C).

Effect of Amiodarone on [3H]Reserpine Binding. In an attempt to characterize the site at which amiodarone and DEA inhibit vesicular monoamine transport, we examined the influence of these compounds on the binding of [3H]reserpine to plasma membrane fractions purified from VMAT2-transfected CHO cells. Following incubation with [3H]reserpine, the radioactivity of membrane fractions obtained from control transfected CHO cells was 24084 ± 2510 dpm/μg. As depicted in Fig. 2, amiodarone competed for membrane binding with [3H]reserpine in a concentration-dependent fashion, with an EC50 of 173 nM, similar to that seen for the inhibitory action of amiodarone on VMAT2-mediated NE transport. In further experiments we also demonstrated that DEA also displaced reserpine from VMAT2-transfected plasma membranes, with a magnitude of effect similar to that of amiodarone (Fig. 2).

Influence of Amiodarone on pH. To investigate the potential role of a pH-mediated action of amiodarone on monoamine transport and reserpine binding, we investigated the effect of amiodarone on pHi in VMAT2-transfected CHO cells using a pH-sensitive fluorescent indicator, BCECF. Amiodarone was completely without action on pHi, whereas interventions that yield changes in pHi of approximately ±0.5 pH units could be readily detected (Borzak et al., 1990; Karwatowska-Prokopczuk et al., 1998).

Discussion

Amiodarone is a complex drug with many pharmacological properties. Aside from its actions on multiple ion channels, amiodarone also exhibits a sympatholytic effect, which has been described as resulting from both presynaptic and postsynaptic effects (Singh et al., 1989). Indeed, it has been
suggested that the most crucial pharmacological components of this clinically important antiarrhythmic agent are related specifically to actions on the sympathetic nervous system. Accordingly, the current study was designed to specifically examine the action of amiodarone and its direct metabolite DEA on the vesicular transport of monoamines.

To address this question as directly as possible, we examined the effect of amiodarone and DEA on monoamine uptake in CHO cells expressing VMAT2, and in conjunction, we examined the effect of these drugs on reserpine binding to VMAT2. The novel findings of this study are that both amiodarone and DEA appear to bind to VMAT2 at the amine recognition site and exhibit an inhibitory effect on the transport of monoamines. In complimentary experiments we showed that the inhibitory influence of amiodarone on VMAT2-mediated NE transport and [3H]reserpine binding were similar in magnitude to the actions of reserpine itself and occurred at an EC50 of 346 nM and 173 nM, respectively. Recently two distinct VMATs (VMAT1 and VMAT2) have been identified in the adrenal medulla and brain, respectively. In this study we examined the effect of amiodarone on VMAT2 activity because of indirect evidence suggesting an effect on the vesicular storage of NE in the rat heart. Although a number of compounds have been shown to inhibit the VMATs, reserpine and tetrabenazine continue to be the best characterized. Reserpine appears to bind directly to the amine recognition site (Darchen et al., 1989) and it has also been proposed that the binding site for reserpine is located on the external aspect of the vesicular membrane (Chaplin et al., 1985). In contrast, tetrabenazine does not appear to act at the amine recognition site, and its binding is not displaced by reserpine (Henry and Scherman, 1989). Together these data...
further support our hypothesis that amiodarone attenuates vesicular monoamine transport by binding to the reserpine binding site. The vesicular transport of monoamines can be also be modified by compounds that alter the vesicular transmembrane electrochemical gradient. Using fluorescence spectroscopy, we were unable to detect any influence of amiodarone on pH, in the same cellular model (transfected CHO cells) as that used for investigating the action of amiodarone on monoamine transport.

Given the wide range of substrates and inhibitors of the VMATs, it has been postulated that binding site plasticity results from the recognition of a small number of key elements within the substrate (Schuldiner et al., 1995). The key elements common to the inhibitors of VMAT are an aromatic ring and a positive charge, and furthermore hydroxyl, methoxy, or amino substituents in the ring improve affinity for the substrates. Consistent with this hypothesis, amiodarone has a diethylamino group located on an aromatic ring. Furthermore, a current model for monoamine transport by the VMATs, it has been postulated that binding site plasticity involves a conformational change of the protein with similar targets of action.

References


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