Amiodarone: Biochemical Evidence for Binding to a Receptor for Class I Drugs Associated With the Rat Cardiac Sodium Channel

Robert S. Sheldon, Roger J. Hill, Nancy J. Cannon, and Henry J. Duff

Amiodarone has multiple pharmacological effects in heart. Electrophysiological data suggest that among its other effects, amiodarone is a sodium channel blocker. Using a radioligand assay, we determined whether amiodarone interacted with a previously described receptor for type I agents associated with the cardiac sodium channel. The radioligand was \[^{3}\text{H} \text{BTXB}]\) (\[^{3}\text{H} \text{BTXB}\]) a toxin that binds to the activated state of the sodium channel. We have previously shown that class I antiarrhythmic drugs inhibit \[^{3}\text{H} \text{BTXB}\] binding. The purpose of this study was to assess whether amiodarone and other class HI agents interact with this receptor. Amiodarone inhibited \[^{3}\text{H} \text{BTXB}\] binding in a dose-dependent fashion, with an estimated IC\(_{50}\) value of 3.6 \mu M. This IC\(_{50}\) value is similar to reported clinically effective serum concentrations of amiodarone. In contrast to amiodarone, the IC\(_{50}\) values for other class III drugs (bretylium, sotalol, betahaneiline, N-acetylprocainamide) were much higher than their therapeutic concentrations and bore no relation to them. Scatchard analysis of \[^{3}\text{H} \text{BTXB}\] binding showed that amiodarone reduced the maximal binding for \[^{3}\text{H} \text{BTXB}\]; this finding indicates irreversible inhibition or (more likely) allosteric inhibition by amiodarone. The latter agrees with electrophysiological data suggesting that amiodarone binds to inactivated sodium channels. Sodium channel blockade by amiodarone may contribute to its overall electrophysiological effect. (Circulation Research 1989;65:477-482)

Amiodarone is perhaps the most effective agent currently available for the treatment of ventricular tachyarrhythmias.\(^1\) Its mechanism of action is as yet unclear although it has been classified as a class III antiarrhythmic agent because it prolongs action potential duration and refractoriness.\(^1\) However, several recent reports have suggested that amiodarone may also block the cardiac sodium channel. Heger et al\(^2\) originally showed that amiodarone prolongs His-to-ventricular conduction time. Mason et al\(^5\) then demonstrated that amiodarone reduced the maximum rate of rise of the action potential upstroke (\(V_{\text{max}}\)) in guinea pig papillary muscle during voltage-clamp experiments. Yabek et al\(^5\) obtained similar results with canine Purkinje fibers and ventricular muscle. The latter three studies showed that amiodarone exhibited both frequency-dependent and voltage-dependent block and suggested that amiodarone, like class I agents, blocks inactivated sodium channels.

We have recently developed a radioligand assay for a receptor for the class I agents that is associated with the cardiac sodium channel.\(^5-8\) Binding of class I agents to this receptor is saturable, reversible, stereospecific, and occurs at pharmacologically relevant concentrations with a similar rank order of potency in vitro and in vivo. We hypothesized that amiodarone, because of its class I-like effect on conduction time and \(V_{\text{max}}\), would also bind to the class I drug receptor at pharmacologically relevant concentrations. Other class III agents have little effect as sodium channel blockers except at suprapharmacological levels.\(^9,10\) These data suggested that they should have little affinity for the class I receptor. Therefore, we compared the abil-

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Radioisotopes of amiodarone and other class III agents to bind to the class I receptor associated with the cardiac sodium channel.

Materials and Methods

Myocyte Preparation

Cardiac myocytes were isolated from adult male Sprague-Dawley rats (200–250 g) by the method of Kryski et al. Rats were killed by cervical dislocation, and the heart was rapidly removed. The aorta was cannulated, and the heart was perfused in a retrograde fashion in a Langendorff perfusion apparatus. The heart was perfused and later incubated with a series of solutions that were equilibrated with 95% O₂–5% CO₂ at 37°C. The solutions were based on Joklik’s Minimal Essential Medium (MEM) supplemented with 1.2 mM MgSO₄ and 1 mM L-carnitine (MEM). They included a rinse solution (MEM), a digestion solution (MEM with 0.1% wt/vol fatty acid–free bovine serum albumin [BSA] and 0.1% collagenase), a calcium solution (MEM with 1 mM CaCl₂ and 1% fatty acid–free albumin), and an incubation solution (MEM with 50 μM CaCl₂ and 1% dialyzed BSA). The heart was perfused at 20°C for 5 minutes with rinse solution and then perfused at 37°C for 20 minutes with digestion solution. The ventricles were then removed, minced with scissors, and rinsed at 37°C for 15 minutes with calcium solution. Calcium solution was then removed by aspiration, and the tissue pieces were incubated at 37°C for 15 minutes with digestion solution in a shaking water bath. Dispersed cells were decanted into a plastic centrifuge tube, and the cell suspension was shaken again with digestion solution. This procedure resulted in almost total dispersion of the heart. The pooled myocytes were then filtered through a 185-μm silk screen mesh, collected by gentle centrifugation, and rinsed with incubation solution. The cells were then collected again by gentle centrifugation and resuspended in incubation solution.

This method routinely yielded about 100 mg (dry wt) of myocytes, which corresponds to 2×10⁶ cells. The cells were 82–92% viable rod-shaped cells that excluded trypan blue. The cells maintained a resting membrane potential of −75 mV to −80 mV and had been metabolically characterized by Kryski et al.

Radioligand Binding

Myocytes (6×10⁵ per assay) in 50 μl incubation buffer were incubated with 1.3 μM sea anemone toxin II (ATX), 13 nM [³H]batrachotoxinin A 20α-benzoate (BTXB) (50 Ci/mM), and 0.13 mM tetrodotoxin for 45–60 minutes at 37°C. Tetrodotoxin was added to prevent depolarization induced by sodium influx. Various concentrations of drugs and toxins were included in the incubations. Assays were done in triplicate with each containing 0.6 mM aconitine to define non-specific binding. Reactions were terminated by adding 10 ml Krebs-Henseleit-BSA buffer, which consisted of (mM) NaCl 127, KCl 2.33, KH₂PO₄ 1.30, MgSO₄ 1.23, NaHCO₃ 25, glucose 10, CaCl₂ 50 (μM), and BSA 1% equilibrated with 95% O₂–5% CO₂ and incubated at 37°C for 1 minute; then the reactions were filtered through a Whatman GF-C 24-mm filter and washed four times with 5 ml rinse buffer that contained (mM) Tris Cl 25 (pH 7.4), NaCl 130, KCl 5.5, MgSO₄ 0.8, glucose 5.5, CaCl₂ 50 (μM). The filters were then dried and counted in Econofluor scintillation fluid (New England Nuclear, Boston, Massachusetts). The retained radioactivity represents [³H]BTXB bound to myocytes.

The rationale for the incubation and filtration conditions was described by Sheldon et al. The conditions provide a maximal reduction in background and scatter with a minimal reduction in specific binding. The total wash time is 45 seconds. Initial control experiments showed that under these conditions, less than 10% of the specifically bound [³H]BTXB dissociated from the complex. Under these reaction conditions (13 nM [³H]BTXB, 1.3 μM ATX), about 60–75% of the total radioactivity retained on the filters was bound specifically to the receptor.

Amiodarone Preparation

Amiodarone was prepared daily as a 25 mM stock solution in warm 100% ethanol and then diluted with incubation solution. The amiodarone did not form visible precipitates at final concentrations less than 1 mM. Control experiments showed that ethanol up to 2% did not inhibit [³H]BTXB binding.

Aqueous Concentrations of Amiodarone

Amiodarone concentrations were measured by high-performance liquid chromatography, which has a coefficient of variation of less than 7%. The high-performance liquid chromatography system consisted of a Waters 510 pump (Waters Associates, Milford, Massachusetts), WISP sample injector (Waters), reverse-phase Brownlee RP18 (10 μm) column (220 mm×4.6 mm i.d.) (Brownlee Lales, Santa Clara, California), Shimadzu ultraviolet detector operated at 254 nm (Shimadzu Corporation, Kyoto, Japan), and a Hewlett-Packard 3390A integrator (Hewlett-Packard, Mississauga, Ontario, Canada) with a chart speed of 0.5 cm/min. The mobile phase was methanol-water-ammonium hydroxide (89.7:10:0.3 vol/vol) with a flow rate of 3 ml/min.

To determine the fraction of amiodarone in the free aqueous phase, a known amount of amiodarone was added to incubation mixtures. The tubes were then centrifuged in an HS-N centrifuge at 2,500 rpm for 1 minute; then the reactions were filtered through a Whatman GF-C 24-mm filter and washed four times with 5 ml rinse buffer that contained (mM) Tris Cl 25 (pH 7.4), NaCl 130, KCl 5.5, MgSO₄ 0.8, glucose 5.5, CaCl₂ 50 (μM). The filters were then dried and counted in Econofluor scintillation fluid (New England Nuclear, Boston, Massachusetts). The retained radioactivity represents [³H]BTXB bound to myocytes.

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To determine the fraction of amiodarone in the free aqueous phase, a known amount of amiodarone was added to incubation mixtures. The tubes were then centrifuged in an HS-N centrifuge at 2,500 rpm for 30 seconds, and the amiodarone was determined in the supernatant fluid and cell pellet. The results showed that 13±3% of the specifically bound [³H]BTXB dissociated from the complex. Under these reaction conditions (13 nM [³H]BTXB, 1.3 μM ATX), about 60–75% of the total radioactivity retained on the filters was bound specifically to the receptor.

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Results

Amiodarone Inhibits $[^{3}H]$BTXB Binding

The affinity of drugs for the class I receptor can be estimated by their ability to inhibit the binding of $[^{3}H]$BTXB. The effect of two class III drugs (amiodarone and N-acetylprocainamide) on $[^{3}H]$BTXB binding is shown in Figure 1. The inhibition by the drugs is dose-dependent and follows a sigmoid curve characteristic of ligand binding to a single class of saturable sites. In this experiment, the estimated IC$_{50}$ of amiodarone is 3.6 $\mu M$ and that of N-acetylprocainamide is 2,100 $\mu M$. These are illustrative results of single experiments representative of those reported in Table 1.

The mean IC$_{50}$ values for inhibition of $[^{3}H]$BTXB binding by six class III drugs are listed in Table 1. The mean IC$_{50}$ values for amiodarone and desethylamiodarone are 3.6 $\mu M$ and 3.1 $\mu M$, respectively, whereas those for sotalol, bretylium, N-acetylprocainamide, and bethanidine are about 1,000-3,000 $\mu M$. Table 1 also contains the clinically effective serum concentrations and estimates of the concentrations at which these drugs block the sodium channel. The latter two sets of data are taken from the previously published works of others, which are listed in the table legend. In Figure 2, we have compared the IC$_{50}$ values from radioligand experiments with the clinically effective serum concentrations of the drugs. The values for type III drugs are represented in Figure 2 as open symbols in a plot of log (IC$_{50}$) versus log (serum concentration). For comparison, we have included similar data for eight type I antiarrhythmic drugs, which are thought to be effective clinically by causing sodium channel blockade. These are plotted as closed symbols. In contrast to the class I drugs, which have the same rank order of potency in vivo and in vitro and have IC$_{50}$ values at pharmacologically relevant concentrations, the type III drugs have neither the same rank order of potency nor are their IC$_{50}$ values at pharmacologically relevant concentrations. The relations between IC$_{50}$, serum concentration, and sodium channel blocking ability will be discussed below.

![Graph of effect of amiodarone and N-acetylprocainamide on $[^{3}H]$BTXB binding](image)

**TABLE 1. Comparison of IC$_{50}$ Values for Inhibition of $[^{3}H]$BTXB BINDING AND THERAPEUTIC SERUM CONCENTRATIONS FOR SIX CLASS III ANTIARRHYTHMIC DRUGS**

<table>
<thead>
<tr>
<th>Drug</th>
<th>Radioligand (IC$_{50}$, $\mu M$)</th>
<th>Therapeutic serum concentration ( SMA)</th>
<th>Electrophysiological effect on sodium channel</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Parameter</td>
<td>(ECC$_{50}$, $\mu M$)</td>
</tr>
<tr>
<td>Amiodarone</td>
<td>3.6±1.5</td>
<td>2.5</td>
<td>Voltage clamp</td>
</tr>
<tr>
<td>Desethylamiodarone</td>
<td>3.1±2.3</td>
<td>2.5</td>
<td>NA*</td>
</tr>
<tr>
<td>Bretylium</td>
<td>1,020±230</td>
<td>4</td>
<td>$V_{max}$</td>
</tr>
<tr>
<td>N-Acetylprocainamide</td>
<td>2,800±1,300</td>
<td>50</td>
<td>Conduction time</td>
</tr>
<tr>
<td>Sotalol</td>
<td>2,950±350</td>
<td>6</td>
<td>Voltage clamp</td>
</tr>
<tr>
<td>Bethanidine</td>
<td>104†</td>
<td>6</td>
<td>NA</td>
</tr>
</tbody>
</table>

IC$_{50}$ values are the means of 3-6 experiments. Serum concentrations are those thought to be effective in suppressing ventricular ectopy although there is no direct proof for the effectiveness of desethylamiodarone. Sources for serum levels are Mason (amiodarone), Heissenbuttel and Bigger (bretylium), Wyman et al. (N-acetylprocainamide); Steinbeck et al. and Somberg et al. (sotalol), and Somberg et al. (bethanidine). Electro-physiological data are from Mason et al. (amiodarone), Bigger and Jaffe (bretylium), Jaillon and Winkle (N-acetylprocainamide), and Carmeliet (sotalol). NA, not available.

*Desethylamiodarone and amiloride have similar effects on conduction time.

†One determination.
Amiodarone Allosterically Inhibits \(^{3}H\)BTXB Binding

Previous electrophysiological work\(^{5,4}\) led to the hypothesis that amiodarone binds preferentially to inactivated sodium channels. This hypothesis can be tested in the radioligand model.\(^{7}\) It is known from extensive work on the nerve sodium channel that alkaloid toxins (e.g., \(^{3}H\)BTXB) bind with high affinity to the activated state of the channel. If amiodarone binds to and stabilizes an inactivated state of the channel, then the channel will be unavailable for binding by \(^{3}H\)BTXB. Therefore, amiodarone should reduce the \(B_{\text{max}}\) for \(^{3}H\)BTXB. The effect of amiodarone on \(^{3}H\)BTXB binding is shown in Figure 3. Scatchard analysis of \(^{3}H\)BTXB binding indicates a single class of \(^{3}H\)BTXB binding sites with a \(K_{d}\) of 27 nM, in accord with previous results.\(^{7}\) Amiodarone markedly reduced the \(B_{\text{max}}\) capacity for \(^{3}H\)BTXB (Figure 3).

Discussion

Amiodarone is an efficacious antiarrhythmic drug whose mode of action is unclear.\(^{1}\) Although it has been categorized as a class III agent on the basis of its effect on action-potential duration, there is electrophysiological evidence that it has properties of a sodium channel blocker.\(^{2,5}\) This study assessed the ability of amiodarone to bind to a receptor for the class I antiarrhythmic agents associated with the cardiac sodium channel.\(^{6-8}\) Amiodarone inhibits the binding of \(^{3}H\)BTXB to the myocyte sodium channel; this occurrence suggests that amiodarone binds to a receptor associated with the channel.

The marked lipophilicity of amiodarone raises several issues regarding the distribution of amiodarone and its mode of access to the receptor associated with the sodium channel. Chatelain and Laruel\(^ {23}\) have shown that the distribution coefficient of amiodarone at pH 7.4 between red blood cell membranes and an aqueous phase is about 15,000. The partition coefficient of neutral amiodarone was much higher (approximately 10\(^ {6}\)) Our finding that 13±3% of amiodarone remained in the aqueous cell-free phase is not corrected for the relative volumes of the aqueous phase and the cell membranes, which occupy only a small fraction of total cell volume. Thus, although the fraction of amiodarone in the supernatant aqueous phase seems high, it probably reflects the large difference in the volumes of the two phases.

The distribution of a high proportion of amiodarone into the lipid cell membrane may be important in the pathway by which amiodarone (and indeed other antiarrhythmic drugs\(^ {23}\)) reach the receptor. The present study does not address this point. However, it should be stressed that \(IC_{50}\) values for the interaction of ligands with their receptors are conventionally expressed in terms of their aqueous concentrations. We have adopted this convention here although it remains possible that amiodarone does reach the sodium channel receptor via the membrane lipid pool.

One possible mechanism of action of amiodarone on \(^{3}H\)BTXB binding is that it could act indirectly by altering the physicochemical characteristics of the...
membrane. However, the sigmoidal concentration-response curve of amiodarone (Hill number 1.1±0.1) speaks to a single class of saturable sites.

Finally, the physiochemical properties of amiodarone dictate its distribution between the aqueous phase and the lipid membrane. Therefore, the possibility that amiodarone partitions differently for live and dead cells, which both have lipid membranes, is unlikely. This is noteworthy because had amiodarone partitioned preferentially to dead cells, then the variable portion (8–18%) of dead cells might have spuriously influenced the apparent IC₅₀ of amiodarone.

The mean estimated IC₅₀ for amiodarone in this assay was 3.6 μM. In patients being treated with amiodarone for ventricular tachyarrhythmias, the serum concentration is similar, about 2.5 μM. This estimate is strikingly similar to the IC₅₀ of amiodarone for the receptor in our assay and suggests that one of the clinical effects of amiodarone is sodium channel blockade.

This work also sheds some insight into the nature of the interaction of amiodarone with the sodium channel. Previous work by Mason et al. suggested that amiodarone binds preferentially to inactivated channels with a Kᵢ of about 20 μM. The estimated affinities for the rested and activated states were much weaker. Given the extreme lipophilicity of amiodarone, it is likely that the estimate of 20 μM is misleadingly high and should be viewed as an upper limit, but the conclusion that the drug binds to inactivated channels remains valid. Similarly, Follmer et al. concluded from single cell voltage-clamp experiments that amiodarone bound with high affinity to both rested (estimated Kᵢ 3 μM) and inactivated channels (estimated Kᵢ 0.1 μM) but not to activated channels although their estimates are based on assumed rather than measured concentrations of amiodarone. The results in Figure 3 are consistent with an interpretation that amiodarone allosterically inhibits [3H]BTX binding and suggests that amiodarone binds to and stabilizes a state other than the toxin-activated state of the channel. Since the sodium channel may exist in several different activated and inactivated states, it is possible that amiodarone stabilizes the channel in one or more of these states, provided that [3H]BTX binding does not bind to that state.

The mechanism by which amiodarone might reversibly antagonize [3H]BTX binding is not yet resolved. We have shown that [3H]BTX binding to its receptor rapidly and reversibly (also, Hill, Duff, and Sheldon, unpublished results). In equilibrium competition experiments, the toxins and drugs are in a complex and dynamic interaction with sodium channels in various states. To account for the inhibition of [3H]BTX binding by amiodarone, it is not necessary to invoke amiodarone interaction with toxin-activated channels; in fact, amiodarone may be effective by stabilizing channels to which toxins are not bound. Thus, both electrophysiolog-

ical and biochemical data support the idea that amiodarone binds with high affinity to a site associated with either inactivated sodium channels or channels not activated by toxins. It is clear that amiodarone has properties of both class I and class III agents. It should be noted that the data in this report do not rule out an alternative but less likely explanation of the Scatchard data (that amiodarone irreversibly inhibits [3H]BTX binding).

We found that bretylium, sotalol, bethanidine, and N-acetylprocainamide, in contrast to amiodarone, had much less affinity for the class I receptor and only inhibited [3H]BTX binding at suprapharmacological concentrations. The concentrations at which these drugs block [3H]BTX binding and at which they appear to block the sodium channel (defined electrophysiologically) are compared in Table 1 and Figure 2. The electrophysiological data are derived from several techniques. These electrophysiological data are only estimates because concentrations producing maximum effects were not assessed. However, it has been shown (Table 1) that bretylium, sotalol, bethanidine, and N-acetylprocainamide function as very weak sodium channel blockers when studied with electrophysiological methods. The clinically effective concentrations for these drugs are also listed in Table 1. Bretylium, sotalol, and N-acetylprocainamide are clinically effective at much lower concentrations than the concentrations at which they block [3H]BTX binding. Thus, their antiarrhythmic effect is unlikely to be due to interaction with the class I receptor associated with cardiac sodium channels.

In conclusion, amiodarone binds to a receptor for class I antiarrhythmic agents associated with the cardiac sodium channel at a concentration similar to its myoccardial concentration. Other class III agents also bind to this site but do so at concentrations far higher than occur clinically.

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References


Key Words: amiodarone, sodium channel, antiarrhythmic drug receptor, cardiac myocytes, [H]batrachotoxinin benzoate
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