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Benzoquinoid Tyrosine Kinase Inhibitors Are Potent Blockers of Cardiac Muscarinic Receptor Function

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SUMMARY

The whole-cell mode of the patch-clamp technique was used to study the effect of methyl-2,5-dihydroxycinnamate (MDC), a specific protein tyrosine kinase inhibitor, on the K^+ currents induced by muscarinic cholinergic agonists in atrial myocytes. Extracellular MDC abolished muscarinic K^+ currents irreversibly, with an apparent inactivation constant K_{inact} of 1.3 μM. Binding studies using purified cardiac sarcolemma indicated that MDC disrupts functional interactions between muscarinic receptors and G proteins with an IC_{50} of 0.7 μM but does not change significantly the distribution of muscarinic binding sites between forms with low and high affinity for agonists. The effects of MDC on muscarinic receptors appear to be unrelated to changes in tyrosine phosphorylation, because (i) the binding experiments were performed in the total absence of phosphorylating nucleotides; (ii) lavendustin-A, a tyrosine kinase inhibitor that is active *in vitro* but not *in vivo*, presumably because it does not cross plasma membranes, inhibited the muscarinic K^+ currents of atrial cells similarly to MDC; and (iii) vanadate, a well known inhibitor of phosphotyrosine phosphatases that potentiates the effects of tyrosine phosphorylation, did not affect K^+ currents when applied extracellularly or into the cytosol of atrial myocytes. The effects of MDC and lavendustin-A were abolished by reducing agents and were mimicked by hydroquinone (or *p*-benzoquinone), indicating that the common quinol moiety is involved in the antimuscarinic activity of the tyrosine kinase inhibitors. It is suggested that these compounds inhibit muscarinic receptor function through oxidation to the quinone form, followed by covalent reaction with a nucleophilic group in the receptor molecule.

In atrial cells, binding of agonists to muscarinic receptors promotes the opening of a specific class of K^+ channels via the G protein G_k (1). The basic kinetic characteristics of this system can be interpreted on the basis of a model that requires interactions between only three elements, receptor, G protein, and channel (2, 3). However, some salient features of $I_{K(ACh)}$ have yet to be rationalized in molecular terms. For instance, after exposure to agonist, $I_{K(ACh)}$ rises quickly to a peak and then decays to a steady state level within 1–2 sec; the molecular mechanism of this rapid desensitization is unknown. A regulatory mechanism based on phosphorylation was proposed to account for this process; it appears to involve an unknown protein kinase that is distinct from cyclic nucleotide-dependent protein kinases or protein kinase C (4). Recent observations suggesting that functional interactions between G protein-coupled pathways and tyrosine kinases may be of physiological significance (5) led us to investigate the possibility that tyrosine phosphorylation might underlie rapid desensitization of $I_{K(ACh)}$. To this end we used an erbstatin analog, MDC, which was previously shown to inhibit specifically a number of tyrosine kinases *in vitro* as well as in intact cells (6–8). Our results indicate that exposure of atrial myocytes to low doses of MDC abolishes whole-cell $I_{K(ACh)}$ rapidly and irreversibly. Unexpectedly, further experimentation has shown that these effects take place at the level of the muscarinic receptor and do not appear to involve tyrosine kinase inhibition. Rather, our results are consistent with a mechanism in which oxidation of the quinol moiety of MDC or lavendustin-A is followed by covalent reaction between an unsubstituted position on the quinone ring and a nucleophilic group in the receptor molecule. Although the present work casts doubts on the usefulness of compounds such as MDC and lavendustin-A for studies of tyrosine phosphorylation in intact cells, it suggests the possibility of utilizing benzoquinoid compounds to aid in the characterization of the structural domains involved in the cycling of muscarinic receptors between forms with high and low affinity for agonists.

Experimental Procedures

Materials. GTP, GMP-PNP, and GTPγS were from Boehringer Manheim. Tetrodotoxin and HEPES were from Calbiochem. MDC (GIBCO or Research Biochemicals Inc.) and lavendustin-A (GIBCO)

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ABBREVIATIONS: $I_{K(ACh)}$, muscarinic K^+ current; ACh, acetylcholine; MDC, methyl-2,5-dihydroxycinnamate; DMSO, dimethylsulfoxide; QNB, quinuclidinyl benzilate; I_{Ca}, calcium current; NEM, *N*-ethylmaleimide; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; GTPγS, guanosine-5′-*O*-(3-thio)triphosphate; GMP-PNP, 5′-guanylyl-imidodiphosphate.

were dissolved in DMSO, whereas hydroquinone (Baker), benzoquinone (Sigma), menaquinone (Aldrich), and duroquinone (Aldrich) were dissolved in ethanol. Stock solutions were stored at −70°; care was taken to protect stock and working solutions of MDC and other photosensitive compounds from light. [^{3}H]QNB was from Amersham Co. Collagenase type 1 was from Worthington and protease was from Sigma. All other chemicals were from J.T. Baker or Sigma Chemical Co.

Electrophysiology. Membrane currents were recorded at 22–24° in the whole-cell configuration of the gigaseal patch-clamp technique (9), using a custom-design current-to-voltage converter with a 1.2-GΩ feedback resistor and an ultra-low noise input field effect transistor. The signals were stored on video tape, using a PCM/videocassette recording system. For analysis and display of the recordings, the data were sampled at 100 Hz using a DAP 2400/5 signal processor board (Microstar Laboratories). The standard internal solution contained 80 mM potassium aspartate, 30 mM KCl, 1 mM EGTA, 2.5 mM Mg·ATP, 0.2 mM Li_4GTP, and 5 mM HEPES, adjusted to pH 7.4 with KOH. Patch pipettes were made from square bore glass, using a Flaming/Brown micropipette puller (Sutter Instruments); their resistance when filled with internal solution was 1.5–5 MΩ. The external Ringer's solution contained 90 mM NaCl, 5 mM $MgCl_2$, 2.5 mM $CaCl_2$, 2.5 mM KCl, 20 mM HEPES (pH 7.4 with NaOH), and 5 μM tetrodotoxin to block sodium currents. The membrane potential was held at −85 mV for 0.25 sec and alternatively stepped to −135 mV, −90 mV, and −5 mV for 0.25 sec. Using this protocol, the steady state currents measured during the last 10 msec of a voltage step are chiefly carried by K^+ ions. This sequence of voltages also provides the means to distinguish between currents through background potassium channels (I_{K1}) and $I_{K(ACh)}$, because the former do not contribute to the outward currents measured at −5 mV. When desired, Ca^{2+} currents were blocked by cadmium (0.5 mM); alternatively, I_{Ca} was minimized by stepping the membrane potential to −45 mV instead of −5 mV, because in these cells I_{Ca} is not activated at voltages negative to −30 mV. When GTP analogs were used, the extracellular solution also contained 3 μM glybenclamide to block ATP-sensitive channels. Superfusion of cells with various bath solutions was performed as described elsewhere (3). In preliminary experiments it was established that, at the concentrations used (maximum of 0.02%, v/v), the organic solvents introduced by test compounds in the external solutions had no effect on membrane currents.

Cell and membrane preparation. Atrial myocytes were isolated from bullfrog hearts by proteolytic digestion. Briefly, hearts were initially perfused with nominally Ca^{2+}-free buffer A (in mM: NaCl, 110; KCl, 5.4; $MgCl_2$, 1; HEPES, 10). When the heartbeat had stopped and most of the blood in the atrial lumen had been expelled, collagenase (0.9–1.4 mg/ml) and trypsin (0.3 mg/ml) were added to the perfusion solution. The heart was digested with enzyme solution for up to 30 min. The atria were removed, rinsed, and incubated with buffer B (buffer A with 10 mM sodium pyruvate and 0.2 mM $CaCl_2$ added) containing 4 μg/ml aprotinin. After 30 min the tissue was transferred to a vial containing buffer B, and isolated cells were obtained by gentle shaking. Viable cells were dissociated from digested atria for up to 36 hr, provided that the tissue was stored at 5° during this period.

Cardiac sarcolemma was prepared from bullfrog hearts as described previously (10) and was stored in liquid nitrogen. For alkaline treatment (11), membranes (3–4 mg/ml) were diluted 10-fold with 50 mM sodium phosphate, pH 11.5, the pH was readjusted to 11.5 with NaOH, and the suspension was incubated for 1 hr on ice. After centrifugation in a Beckman Airfuge (10 min, 30 psi), the pellet was resuspended in sucrose buffer (80 mM sucrose, 50 mM KCl, 10 mM Tris, pH 7.4 with HCl) and used for assays.

Binding assays. Binding of [^{3}H]QNB to frog cardiac sarcolemma was measured at 22–24° as described (10), using 7.5–10 μg of protein/assay in a standard volume of 2.5 ml. For determination of the binding parameters for [^{3}H]QNB, the assay volume was increased to 5 ml and the amount of membranes was reduced to 5 μg/assay. Nonspecific binding was determined in the presence of 4 μM atropine. Vesicles (1.5–2.0 mg/ml) were permeabilized with 0.8 mg/ml saponin for 20 min at room temperature before assays. MDC or other compounds were added to membranes already diluted in assay medium, 10 min before the addition of carbamylcholine, GMP-PNP, and [^{3}H]QNB; an equal volume of DMSO (or ethanol, when appropriate) was added to controls, to a maximum of 0.2% (v/v). After a 90-min incubation in the dark, bound and free [^{3}H]QNB were separated using GF/F filters that had been pretreated with 0.3% polyethyleneimine. Experimental points were determined in duplicate and each experiment was repeated two or three times, using different batches of sarcolemmal membranes whenever possible. Unless otherwise stated, relatively high (0.8–1.2 nM) concentrations of [^{3}H]QNB were used in competition binding experiments to ensure a rapid approach to equilibrium.

Results were analyzed by nonlinear curve-fitting using the Marquardt-Levenberg algorithm. Saturation binding isotherms of [^{3}H]QNB were fitted by the equation

$$B = B_{max}[Q]/(K_Q + [Q]) \quad (1)$$

where B and B_{max} are the amounts of [^{3}H]QNB bound at a free ligand concentration [Q] and at saturation, respectively, and K_Q is the dissociation constant for [^{3}H]QNB. Competition binding results were fitted to the two-site equation

$$y = \{[F_H/(1 + (C/K'_H))] + [(1 - F_H)/(1 + C/K'_L))]\} \quad (2)$$

where y represents the fractional saturation of [^{3}H]QNB binding sites, C is the concentration of carbamylcholine, F_H is the fraction of high affinity sites that bind agonist with apparent dissociation constant K'_H, and K'_L is the apparent dissociation constant for low affinity sites. The values of the dissociation constants K_H and K_L were derived from K'_H, K'_L, and K_Q, the dissociation constant for [^{3}H]QNB, using the expression

$$K = K'K_Q/[Q] + K_Q \quad (3)$$

Results

Effect of MDC on agonist-induced $I_{K(ACh)}$. Fig. 1 shows the typical effect of muscarinic agonists on atrial myocytes; superfusion with 1 μM ACh elicited a large increase in the inward currents measured at −135 mV, as well as the appearance of outward current at −5 mV. Both outward and inward components of the response were characterized by an initial peak, followed by a phase in which the currents declined slowly. If the cell was allowed to recover from the first contact with agonist for a period of 5 min, a second exposure to ACh produced a response of comparable magnitude (Fig. 1). However, if the bath was then perfused with the tyrosine kinase inhibitor MDC at 10 μM for 4.5 min, a third application of ACh had no effect on K^+ currents (Fig. 1). At this concentration the effect of MDC on $I_{K(ACh)}$ was rapid, because a complete block of $I_{K(ACh)}$ was produced by a 1-min exposure to the inhibitor (data not shown). In addition, the block of $I_{K(ACh)}$ by MDC was essentially irreversible; there was no recovery of the muscarinic response after washout of the drug.

Throughout the exposure to MDC, the currents recorded at −135 mV remained constant, indicating that the background K^+ currents were not altered; thus, MDC blocked only the K^+ channels gated by ACh (Fig. 1). Frequently, the earliest consequence of superfusion of cells with MDC was a transient increase of the basal I_{Ca}.[1] The resulting Ca^{2+} influx does not appear to be causally related to the effects of MDC on $I_{K(ACh)}$, because the extent and rate of inhibition of $I_{K(ACh)}$ were undi-

[1] A. S. Otero. Effect of guinones on cardiac calcium currents. Manuscript in preparation.

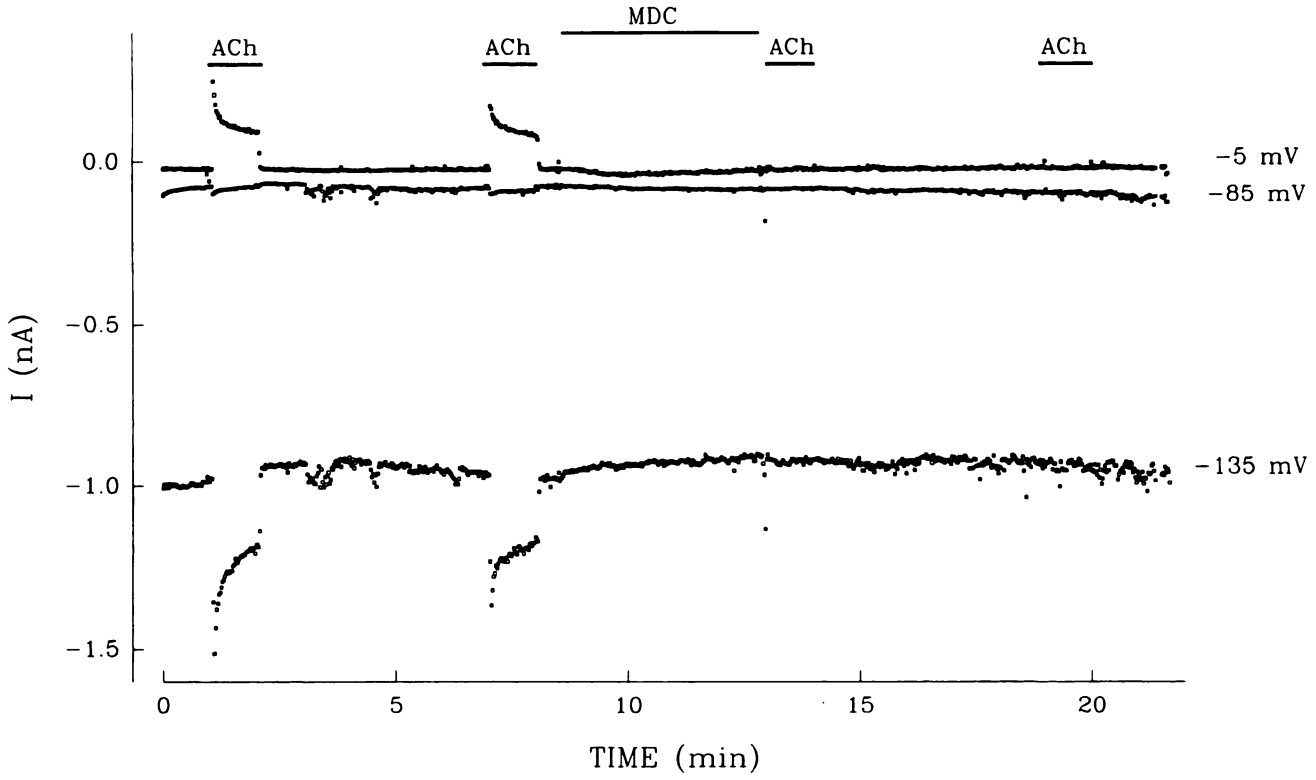

Fig. 1. Treatment of cells with MDC blocks $I_{K(ACh)}$ but has no effect on background K^+ currents. Membrane currents were elicited by stepping the voltage periodically to −5 mV (outward currents) and −135 mV (inward currents) from a holding potential of −85 mV; voltage steps lasted 0.25 sec. Each *symbol* represents the current recorded at the end of an individual step to the voltages indicated, measured every 1.5 sec and plotted as a function of time. The pipette contained standard internal solution. At time zero the whole-cell recording mode was established, and the cell was bathed in external solution supplemented with ACh (1 μM) or MDC (10 μM) during the periods indicated by the *bars*.

minished in the presence of the Ca^{2+} channel blocker Cd^{2+} in the external solution or after changes in the pulse protocol so that the voltage used to elicit outward currents was below the threshold potential for I_{Ca}. Moreover, MDC inhibited $I_{K(ACh)}$ even in those cells in which it did not affect I_{Ca}.

The block of $I_{K(ACh)}$ by MDC was found to depend on concentration and length of the application. At 10 nM MDC had no detectable effect on the currents even after prolonged exposures; at a 10-fold higher concentration the inhibition was already significant. When MDC was added in the continued presence of agonist, $I_{K(ACh)}$ was suppressed after a short lag, at a rate that depended on the concentration of the inhibitor. The MDC-induced decay of outward $I_{K(ACh)}$ could be fitted by a single-exponential function (Fig. 2A), indicating that the process is first order. The kinetics of $I_{K(ACh)}$ block in the presence of 1 μM ACh and different concentrations of MDC were used to analyze this process. A plot of the half-time (τ) of inactivation versus the reciprocal concentration of MDC was fitted by a straight line (Fig. 2B), with a y-intercept larger than zero. This suggests that the block of $I_{K(ACh)}$ by MDC may be described by a model in which the initial interaction between the MDC and its target (P) is followed by a slow modification step that produces a stably inactivated species, P-MDC:

$$P + MDC \underset{k_1}{\overset{k_{-1}}{\leftrightarrows}} P\cdot MDC \rightarrow P - MDC$$

This process can be described by the equation:

$$\tau = T\{1 + (K_{inact}/[MDC])\} \quad (4)$$

where T is the minimum half-time of inactivation at saturating concentrations of MDC, and the apparent inactivation constant K_{inact} is defined as $(k_{-1} + k_2)/k_1$. From the data plotted in Fig. 2B, T is estimated to be 0.19 ± 0.03 min, whereas K_{inact} is 1.3 ± 0.1 μM (0.25 μg/ml).

Effect of MDC on $I_{K(ACh)}$ induced by intracellular GTP analogs. The aforementioned results indicate that MDC disrupts one or more of the processes leading to the activation of $I_{K(ACh)}$. Because these involve at least three basic components, i.e., receptor, G_k, and channel, the next objective was to define the element(s) inactivated by MDC. To do so, we applied the inhibitor to cells dialyzed with poorly hydrolyzable GTP analogs. Inclusion of GMP-PNP or GTPγS in the intracellular solution elicits atrial $I_{K(ACh)}$ in an agonist-independent manner, because GTP analogs can bind directly to G_k, forming an active species that cannot be deactivated by hydrolysis. Because unliganded muscarinic receptors are known to accelerate the binding of GTP analogs to G_k (12), this set of experiments was performed in the presence of the antagonist atropine (10 μM), which promotes the dissociation of muscarinic receptor-G protein complexes (13). Under these conditions, the contribution of effects of MDC on the channel or G proteins should be magnified at the expense of any receptor-related actions. Fig. 3 illustrates the effects of MDC on outward $I_{K(ACh)}$ when the internal solution contains 1 mM GTPγS. Application of 10 μM MDC after full activation had been attained caused only a slight decay of $I_{K(ACh)}$; this effect was slow to develop and was totally reversible (Fig. 3). Similar observations were made in cells dialyzed with 2 mM GMP-PNP (data not shown). The

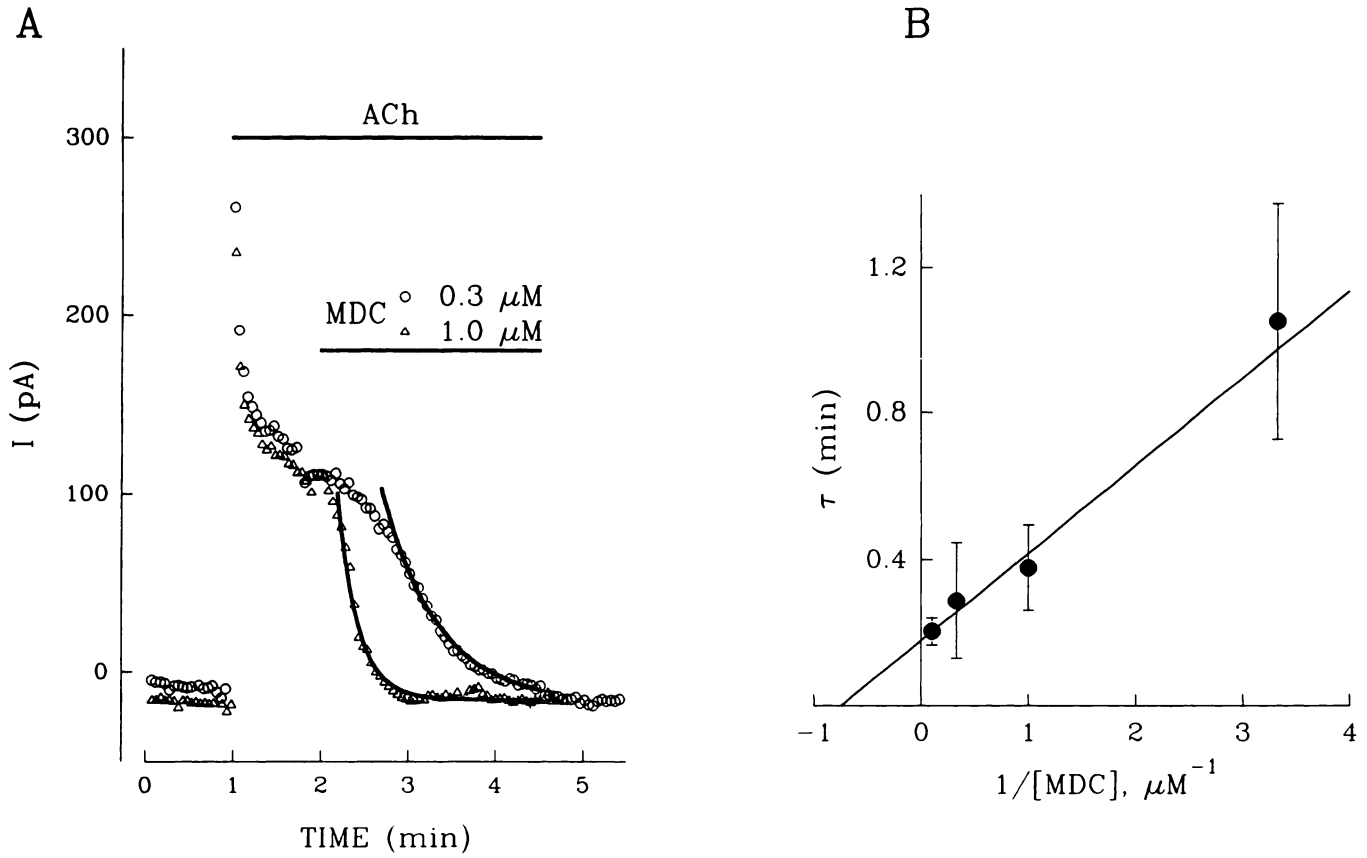

Fig. 2. Kinetics of $I_{K(ACh)}$ inhibition by MDC. Conditions were as in Fig. 1. The cell was exposed first to 1 μM ACh for approximately 1 min and then to ACh plus 0.3–10 μM MDC for a period of 2.5 min. The rate of $I_{K(ACh)}$ decay in the presence of MDC was calculated by fitting the data to the function $f = a \cdot \exp(-t/\tau)$, where a is the amplitude of the exponential function and τ is the half-time of inactivation of $I_{K(ACh)}$. A, Shown are whole-cell outward currents measured at the end of 0.25-sec pulses from −85 mV to −5 mV. Exposure to ACh or ACh and MDC took place as marked by *bars*. ○, 0.3 μM MDC; △, 1.0 μM MDC. The exponential fits obtained for these two experiments are shown as *solid lines*. B, The half-time for inactivation of $I_{K(ACh)}$ (τ) was determined at 0.3, 1, 3, and 10 μM MDC (three experiments for each concentration of inhibitor), using the protocol shown in A; values (means ± standard errors) were then plotted against the inverse of the concentration of MDC to determine K_{inact}, the apparent inactivation constant, and T, the minimal half-time of inactivation.

slow and reversible inhibition of a small fraction of the receptor-independent $I_{K(ACh)}$ by 10 μM MDC is in marked contrast to the prompt disappearance of $I_{K(ACh)}$ caused by a 10-fold lower concentration of inhibitor in the presence of agonist and GTP (Fig. 2). Note that addition of atropine to MDC solutions did not protect $I_{K(ACh)}$ from inhibition in experiments similar to the one in Fig. 1; after washout of atropine and MDC there was no response to ACh (data not shown). One interpretation of the results observed in the presence of GTP analogs is that MDC might affect $I_{K(ACh)}$ at the level of the receptor, which is essential for activation of the system in the presence of GTP but plays a lesser role in the conditions of Fig. 3. On the other hand, the block observed in Figs. 1 and 2 could take place at the level of the G protein, and persistent activation of G_k by GTP analogs might stabilize a protein conformation that resists inhibition by MDC. The following experiments were designed to distinguish between these alternatives.

Binding characteristics of cardiac muscarinic receptors in the presence of MDC. To determine the effect of MDC on muscarinic receptors, we performed equilibrium binding studies using purified frog cardiac sarcolemma. Under our experimental conditions the antagonist QNB binds to a homogeneous population of sites (Table 1); the number of sites and the binding affinity for [^{3}H]QNB were not changed by the presence of 10 μM MDC in the assay medium (Table 1). As expected (14) (reviewed in Ref. 15), [^{3}H]QNB/carbamylcholine displacement curves were shallow (Fig. 4), indicating the presence of at least two distinct states of the receptor, based on their affinity for agonist. Analysis of the data using a two-site model showed that approximately 40% of the sites bound agonist with high affinity (K_H = 1.9 nM), whereas the remainder displayed low affinity (K_L = 1.4 μM) (Table 1). The appearance of the carbamylcholine displacement curve was altered only slightly by MDC, with the dissociation constants K_H and K_L remaining approximately the same. In contrast, MDC gave rise to marked changes in the behavior of the system towards guanine nucleotides. In control membranes the competition curves became steep (Fig. 4) in the presence of 10 μM GMP-PNP, and carbamylcholine bound to a single class of sites with low affinity (K_L = 4.2 μM). Incubation of membranes with 10 μM MDC all but eliminated the effect of GMP-PNP on carbamylcholine affinity (Fig. 4), restoring the agonist binding profile obtained in the absence of guanine nucleotides. This loss of guanine nucleotide sensitivity reveals that MDC disrupts interactions between cardiac muscarinic receptors and G proteins. In contrast to procedures known to result in the uncoupling of receptors from G proteins, such as alkaline treatment (11) and alkylation with NEM (16–19), treatment with MDC

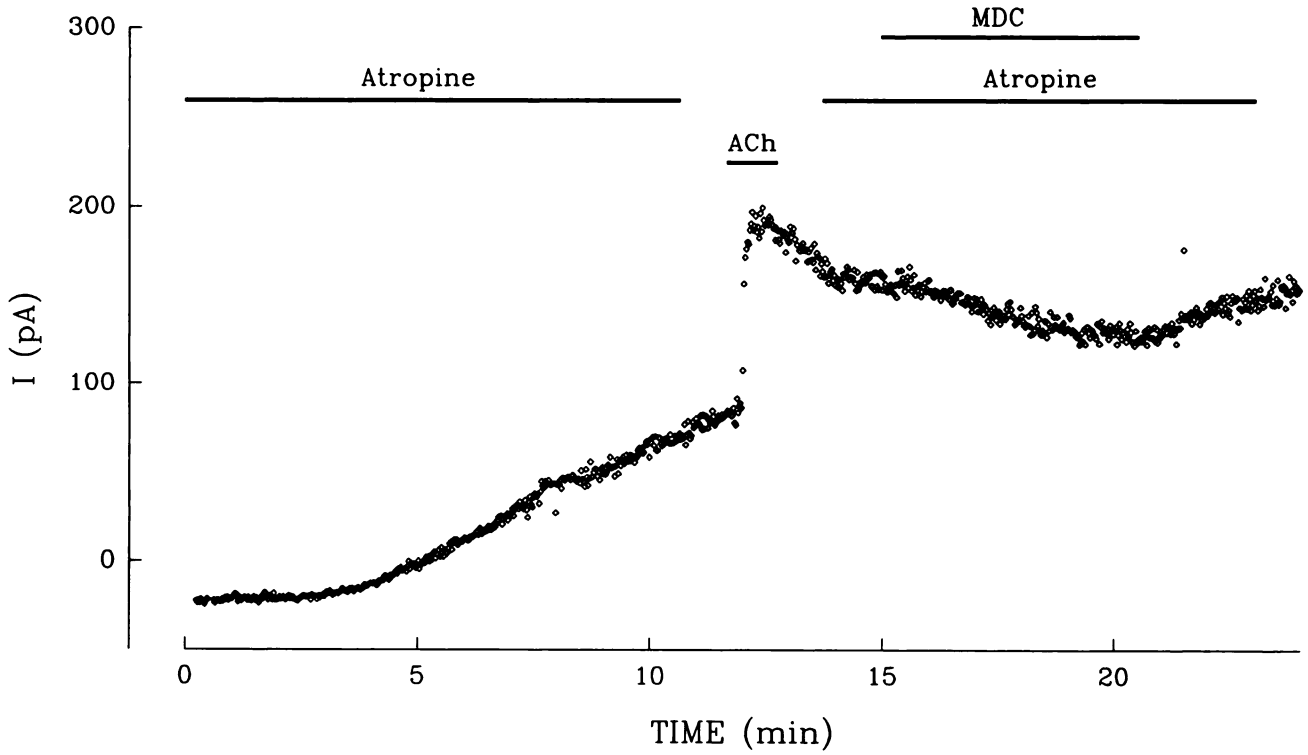

Fig. 3. GTPγS-supported $I_{K(ACh)}$ is resistant to MDC inhibition. Shown are outward currents elicited by stepping the membrane potential from −85 mV to −5 mV. The solution used to fill the patch pipette contained 1 mM GTPγS and 2.5 mM ATP·Mg, so GTPγS was dialyzed into the cell from the beginning of the recording. The bath contained MDC (1 μM), atropine (10 μM), and ACh (1 μM) for the periods indicated by *bars*.

TABLE 1

Effect of MDC on binding parameters for muscarinic ligands

Membranes were treated with alkali as described in Experimental Procedures. When present, MDC and GMP-PNP were at 10 μM. Values are means ± standard errors of the fitted parameters from *n* separate experiments. B_{max} is the amount of [^{3}H]QNB bound at saturation, K_D is the dissociation constant for [^{3}H]QNB, F_H is the fraction of high affinity sites that bind agonist with dissociation constant K_H, and K_L is the dissociation constant for low affinity sites.

QNB (direct binding) (n = 3)

Additions to assay	K_D	B_{max}
	pM	*pmol/mg*
None	29.9 ± 0.3	6.1 ± 0.8
MDC	32.4 ± 2.8	6.2 ± 0.9

Carbamylcholine (competition binding)

Membrane treatment	Additions to assay	F_H	K_H	K_L
		%	*nM*	*μM*
None (n = 4)	None	39.9 ± 7.6	1.9 ± 1.1	1.4 ± 0.6
	GMP-PNP	0		4.2 ± 1.6
	MDC	42.1 ± 4.9	3.2 ± 2.3	1.7 ± 1.4
	GMP-PNP + MDC	22.6 ± 14.5	0.3 ± 0.1	0.7 ± 0.5
		0		2.8 ± 2.3
Alkaline extraction (n = 3)	None	35.2 ± 13.1	11.3 ± 1.4	0.9 ± 0.5
	MDC			

had little effect on the distribution of agonist binding sites between low and high affinity forms (Table 1).

Fig. 5 shows the concentration dependence of the MDC effect on the modulation of agonist binding by guanine nucleotides. Binding of [^{3}H]QNB or carbamylcholine alone was basically not changed by increases in the concentration of MDC. GMP-PNP was able to decrease the binding of carbamylcholine by approximately 35% in controls, but in the presence of increasing MDC this fraction gradually decreased until the nucleotide effect vanished at 10 μM MDC. From this type of experiment it was possible to estimate an IC_{50} for MDC; the value obtained, 0.7 ± 0.3 μM, is similar to the K_{inact} value determined for inhibition of $I_{K(ACh)}$ by MDC in intact cells (1.3 μM), suggesting that the same process underlies the actions of MDC on isolated membranes and atrial myocytes.

The marked reduction in receptor-G protein interactions brought about by MDC might be related to an action of the inhibitor at the level of either protein, or both; the next experiments were designed to distinguish between these possibilities.

Effect of MDC on muscarinic receptors in alkaline-extracted membranes. To delineate further the site of action of MDC, its effects were assessed with sarcolemmal preparations that had been incubated at pH 11.5 for 1 hr before the assay. This procedure strips membranes of peripheral proteins such as G protein α subunits (20) but does not affect the antagonist-binding properties of a number of receptors (β-, $α_1$-, and $α_2$-adrenergic) (11, 21).

In agreement with previous reports, alkaline-treated sarcolemma displays only low affinity, guanine nucleotide-insensitive, agonist binding, reflecting the loss of receptor-G protein interactions. Surprisingly, MDC was still able to promote high affinity binding of carbamylcholine to muscarinic receptors under these conditions (Fig. 6), restoring the shallow agonist binding profile characteristic of control membranes (Fig. 4). This result demonstrates that MDC affects agonist binding through the receptor itself, in a process that does not require the presence of functional G proteins.

Effect of vanadate and lavendustin-A on muscarinic receptor function. Because the binding assays described here

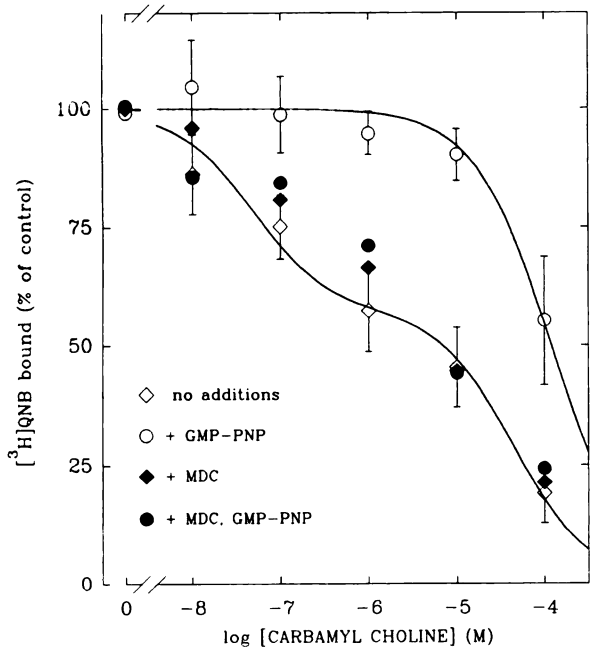

Fig. 4. Displacement by carbamylcholine of [^{3}H]QNB binding to muscarinic receptors in cardiac sarcolemma. Experiments were carried out as described in Experimental Procedures. Specific [^{3}H]QNB binding to untreated membranes in the absence of agonist or GMP-PNP (control) was taken as 100%. Data points are means ± standard errors from three determinations. *Open symbols*, untreated membranes; *closed symbols*, membranes incubated with 10 μM MDC before the addition of carbamylcholine at the concentrations indicated; assays were performed in the absence (*diamonds*) or presence (*circles*) of 10 μM GMP-PNP. *Solid lines* were obtained by fitting the data to eq. 1 or eq. 2. For the sake of clarity, error bars and theoretical curves were omitted in the case of MDC-treated membranes. The estimates for K_d values and fractions of high affinity sites are shown in Table 1.

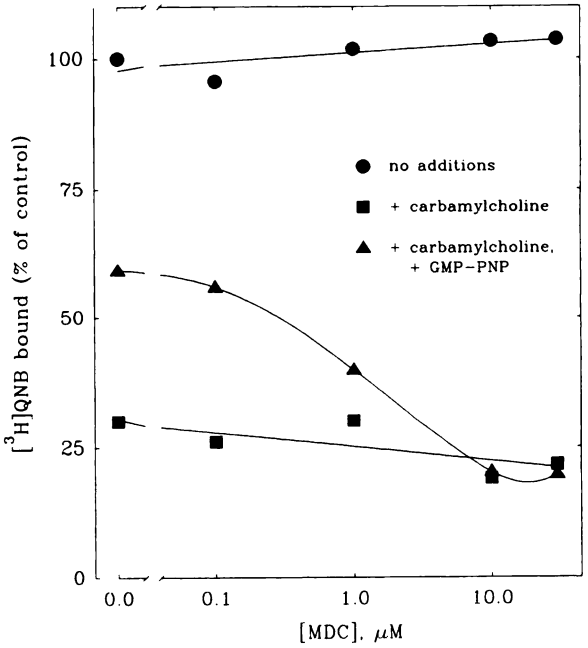

Fig. 5. Effect of increasing concentrations of MDC on guanine nucleotide sensitivity of carbamylcholine binding. Membranes were incubated with MDC at the concentrations shown. [^{3}H]QNB binding was measured in the absence of other ligands (●) or in the presence of 10 μM carbamylcholine with (▲) or without (■) 10 μM GMP-PNP. Results are expressed as in Fig. 4; shown are mean values from two experiments.

were carried out in the absence of ATP or other high energy phosphate donors, alteration of agonist binding by MDC is clearly unrelated to inhibition of any phosphorylation processes. Instead, MDC acts directly on muscarinic receptors and eliminates their functional coupling to G proteins, preventing the interconversion of high into low affinity agonist binding sites by guanine nucleotides. Given that MDC affects agonist binding to muscarinic receptors in membranes and $I_{K(ACh)}$ with similar affinities and considering that receptors unable to cycle between high and low affinity forms are likely to be inactive, the effects of MDC on muscarinic receptors in purified membranes account fully for the block of $I_{K(ACh)}$ observed in intact cells. Nevertheless, these arguments are not sufficient to eliminate the possibility that *in vivo* the mechanism of inhibition of $I_{K(ACh)}$ by MDC does involve tyrosine phosphorylation. To clarify the issue of tyrosine kinase involvement in the actions of MDC we used two independent approaches. The question of tyrosine kinase participation in $I_{K(ACh)}$ was first addressed with vanadate, a well known inhibitor of phosphotyrosine phosphatases. Because cellular levels of phosphotyrosine are controlled by the ratio of phosphorylation and dephosphorylation, vanadate can be used either to increase tyrosine phosphorylation in the absence of stimulation or to amplify signals mediated by activated tyrosine kinases. However, the presence of 0.5–1 mM vanadate in the bath solution for 5–10 min had no significant effect on atrial K^+ currents (data not shown). Similarly, addition of 0.2 mM vanadate to the internal solution did not change

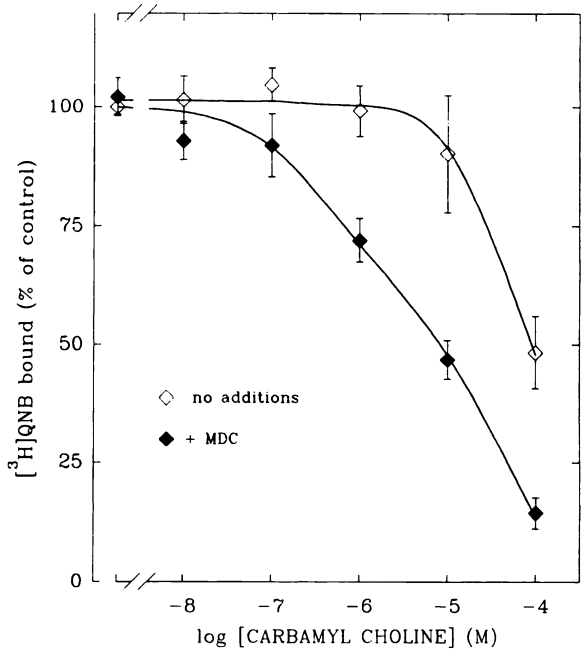

Fig. 6. Effect of MDC on carbamylcholine binding to alkaline-treated membranes. Alkaline treatment was as described in Experimental Procedures. Membranes were preincubated with DMSO (0.2%, v/v) (◇) or 10 μM MDC (◆); binding of [^{3}H]QNB was then determined in the presence of the concentrations of carbamylcholine indicated. Data were analyzed as in Fig. 4. Values plotted are means ± standard errors of four determinations. The corresponding estimates for binding parameters are displayed in Table 1.

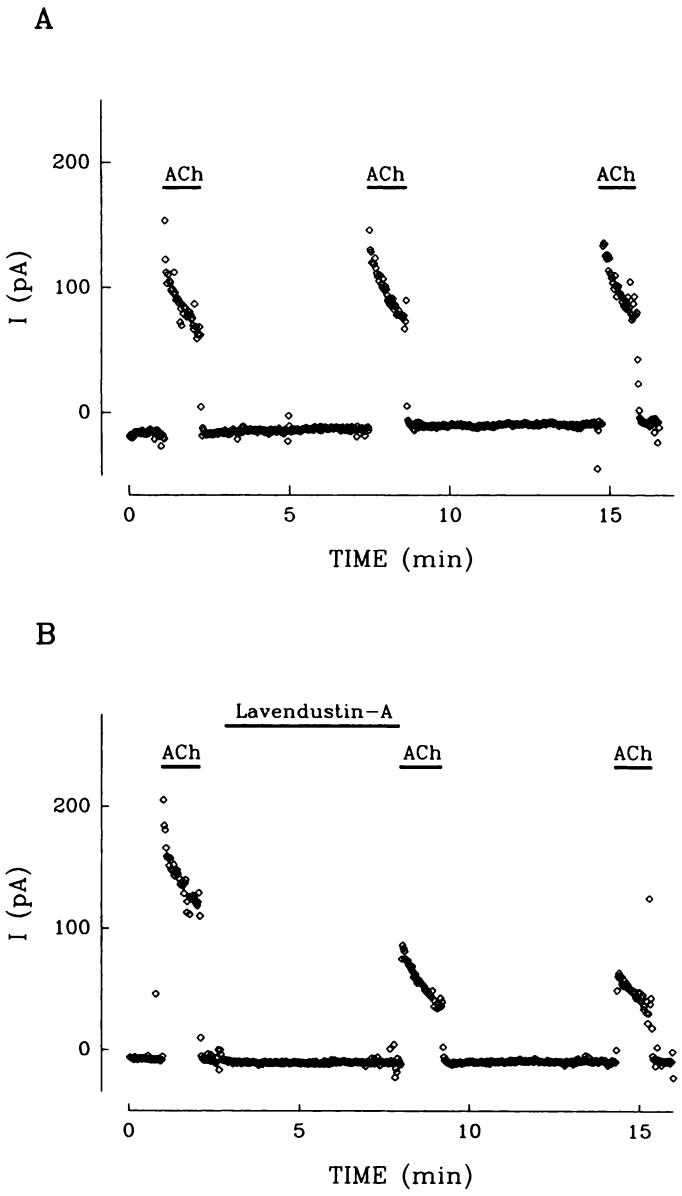

Fig. 7. Effect of vanadate and lavendustin-A on $I_{K(ACh)}$. Experimental protocol was as in Fig. 1; as in Figs. 2 and 3, only outward currents are shown. A, The patch pipette was filled with standard internal solution containing 0.2 mM sodium vanadate. During the periods indicated, ACh (1 μM) was present in the bath. B, During the periods indicated the external solution contained lavendustin-A (10 μM) or ACh (1 μM).

$I_{K(ACh)}$ in experiments lasting 16–20 min (Fig. 7A), an interval that is more than adequate for diffusion of small molecules such as GTPγS from the patch pipette into the cell interior (Fig. 3). A second approach made use of lavendustin-A, a tyrosine kinase inhibitor that is very potent *in vitro*, inhibiting the activity of the epidermal growth factor receptor kinase with an IC_{50} of 10 nM (22). Nevertheless, lavendustin-A was found to be inactive in intact cells even when used at very high (>250 μM) concentrations, presumably because it does not permeate plasma membranes and therefore cannot reach intracellular targets (22). Thus, lavendustin-A should not reproduce the effects of MDC if those result from inhibition of intracellular tyrosine kinases. However, as seen with MDC (Fig. 1), lavendustin-A blocked $I_{K(ACh)}$ persistently, albeit less markedly than MDC (Fig. 7B). Taken together, the lack of effect of vanadate and the inhibition by lavendustin-A suggest that the ability to inhibit tyrosine kinases is of little relevance to the effects of MDC (and lavendustin-A) on $I_{K(ACh)}$.

Similarities between the behavior of lavendustin-A and MDC imply that their effects are produced by a common structural element. Because the most salient feature common to both compounds is the quinol moiety, the following group of experiments was designed to determine whether this portion of the molecule is important for the antimuscarinic effects of MDC and lavendustin-A.

Role of the quinone moiety of MDC in its interaction with muscarinic receptors. Quinols are highly reactive in the oxidized (quinone) form; to test the hypothesis that the benzoquinone form of MDC inhibits muscarinic receptor function, cells and membranes were exposed to MDC in the presence of reducing agents. MDC did not affect $I_{K(ACh)}$ in atrial myocytes in the presence of 0.1 mM ascorbic acid (Fig. 8); likewise, when added to assay mixtures containing an excess (0.1 mM) of sodium metabisulfite or ascorbic acid, MDC was unable to stabilize high affinity binding of agonist (data not shown). Note that the reducing agents protected muscarinic receptors when present in binding media or bath solutions simultaneously with MDC but were unable to reverse the effects of exposure of membranes or cells to MDC. Similar results were obtained with lavendustin-A (data not shown).

The lack of antimuscarinic activity of MDC (and lavendustin-A) in a reducing environment supports the hypothesis that it is the quinone moiety of these drugs that alters muscarinic receptor function. However, this finding leaves unanswered the question of the basic mechanism by which muscarinic receptor function is modified. Quinones affect cellular processes notably by participating in redox cycling, causing oxygen activation and oxidative stress, or by forming adducts with nucleophiles such as thiol, amino, and phenolic hydroxyl groups; in particular, reactions of quinones with proteins are often ascribed to the formation of sulfhydryl addition products. To assess the relative importance of these two mechanisms in the actions of MDC, a series of quinones were tested for their ability to

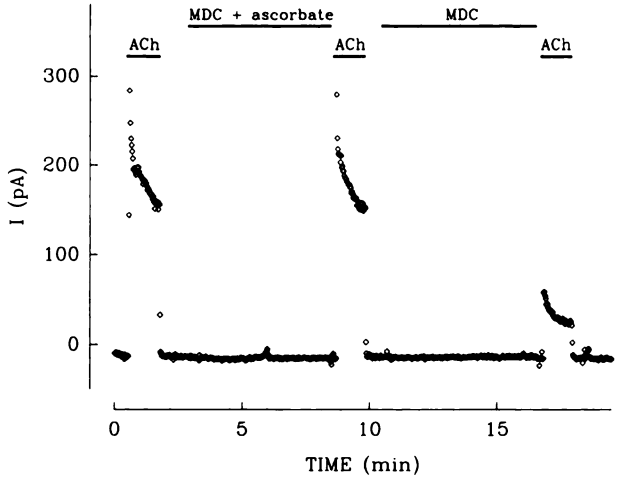

Fig. 8. The effect of MDC is abolished by reducing agents. Experimental conditions were as in Fig. 1; the *plot* shows outward currents measured at −5 mV as a function of time. *Bars*, intervals during which the external solution contained ACh (1 μM) or MDC (10 μM), in the absence or presence of 0.1 mM sodium ascorbate, as indicated.

abolish the guanine nucleotide sensitivity of agonist binding to muscarinic receptors in purified cardiac sarcolemma. The compounds selected were 1,4-hydroquinone and its oxidation product 1,4-benzoquinone, the fully substituted tetramethyl-1,4-benzoquinone (duroquinone), and 2-methyl-1,4-naftoquinone (menaquinone) (see Fig. 9 for chemical structures). Benzoquinone was chosen because it arylates nucleophiles but is unable to undergo redox cycling, whereas duroquinone is effective at inducing oxidative stress but does not react with nucleophiles. Menaquinone can do both and is better at redox cycling than duroquinone; in addition, although capable of forming adducts with nucleophilic groups, menaquinone reacts with glutathione with a rate constant that is 10^6-fold smaller than the value measured with benzoquinone (23, 24). Finally, hydroquinone was included to verify our hypothesis that spontaneous oxidation to the quinone form is an intermediate step required for antimuscarinic activity; if so, hydroquinone should be less effective than benzoquinone.

A summary of the results obtained is presented in Fig. 10A. Clearly, some of these compounds act as does MDC, eliminating guanine nucleotide-sensitive binding of carbamylcholine to high affinity sites. At 10 μM, the order of ability to reduce guanine nucleotide sensitivity was benzoquinone > hydroquinone ≈ MDC > lavendustin-A; duroquinone and menaquinone were essentially inactive, suggesting that oxidative stress does not contribute significantly to the effects of quinones on muscarinic receptors. In keeping with these findings, $I_{K(ACh)}$ was inhibited by 10 μM hydroquinone but not by duroquinone (Fig. 10B). The analogy between the patterns of effectiveness observed *in vivo* and *in vitro* for these compounds implies that both the block of $I_{K(ACh)}$ and the elimination of guanine nucleotide-sensitive agonist binding are achieved through the same mechanism. Based on these results, we conclude that oxidation to the quinone form, followed by arylation of a nucleophile, possibly a thiol, is the mechanism of action of benzoquinoid compounds on muscarinic receptors.

Discussion

The original objective of this study was to investigate the possible involvement of tyrosine phosphorylation in the activation of atrial K^+ currents by muscarinic receptors. The hypothesis was initially tested with the aid of a tyrosine kinase inhibitor, the erbstatin analog methyl-2,5-dihydroxycinnamate, which was selected on the basis of its relative stability in solution, its low IC_{50} for inhibition of the tyrosine kinase activity of the epidermal growth factor receptor, and the observation that it is a noncompetitive inhibitor with respect to ATP (6–8). The latter property is of considerable importance, because drugs that compete with ATP might conceivably interact also with the G protein nucleotide binding site. However, the main finding reported here is that tyrosine kinase inhibitors that carry a hydroquinone moiety block $I_{K(ACh)}$ irreversibly, by an unanticipated mechanism that involves alterations in muscarinic receptor function.

The rapid onset of the inhibition of $I_{K(ACh)}$ by MDC, as well as the relatively low doses of tyrosine kinase inhibitor required for elimination of the muscarinic cholinergic response, were the first signs of a possible direct action of MDC on some element of this signal transduction pathway. Thus, as a rule *in situ* inhibition of tyrosine kinases by erbstatin or MDC requires long incubation times (in the hour range), relatively high concentrations of the drugs (30–500 μM), or both (6–8, 25). The pronounced effects of MDC (and lavendustin-A) on muscarinic receptor binding in the absence of phosphorylating nucleotides, the inhibition of $I_{K(ACh)}$ by lavendustin-A in spite of the inability of the latter to cross cell membranes, and the insensitivity of $I_{K(ACh)}$ to vanadate all suggest that tyrosine kinase inhibition is not the primary mechanism responsible for the effects of these inhibitors on cardiac myocytes.

The precise mechanism by which MDC and other quinols modify muscarinic receptor function may involve their reaction with protein thiol groups. Arylation of an essential sulfhydryl group in $pp60^{v\text{-}src}$ was the mechanism proposed for the reversal by herbymicin, also a benzoquinoid tyrosine kinase inhibitor, of transformation by Rous sarcoma virus (26). Reactive cysteine residues play an essential role in muscarinic receptor function (15, 27, 28) and therefore are likely targets for arylation. For instance, similarly to application of MDC, treatment of atrial cells with the alkylating agent NEM blocks $I_{K(ACh)}$ (29). Also, NEM is known to have concentration-dependent effects on muscarinic agonist binding to cardiac sarcolemma. At low concentrations (≤0.1 mM) NEM eliminates high affin-

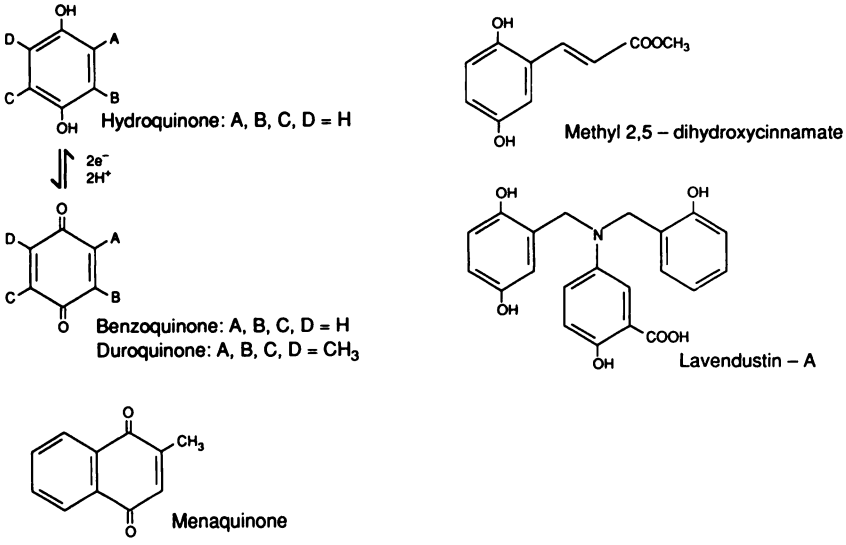

Fig. 9. Chemical structures of compounds used in this study.

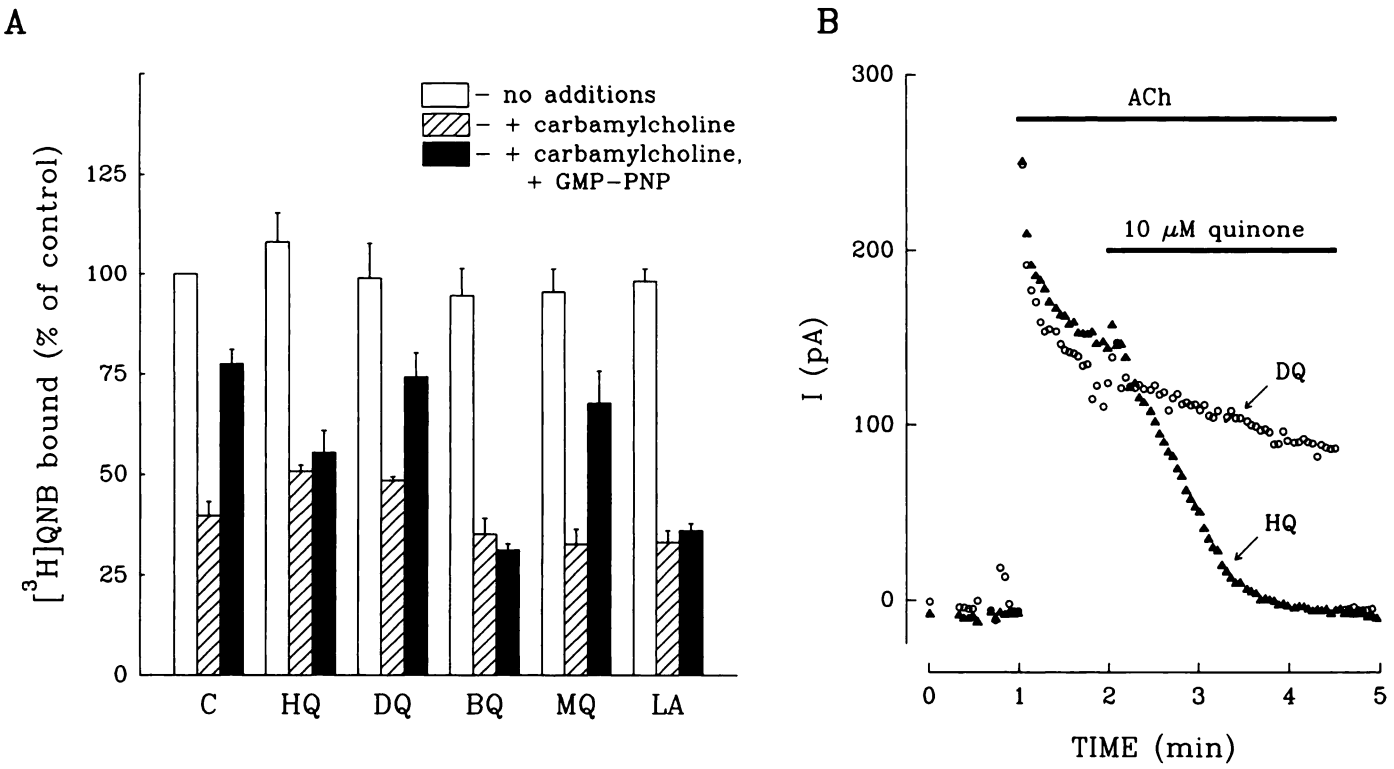

Fig. 10. Effect of quinones on cardiac muscarinic receptor function. A, Effect of quinones on the guanine nucleotide sensitivity of carbamylcholine binding. Binding results are expressed as percentages of the value measured in the presence of vehicle only, with no additions; values are means ± standard errors from three experiments. Membranes were incubated with ethanol (*C*) or with hydroquinone (*HQ*), duroquinone (*DQ*), benzoquinone (*BQ*), menaquinone (*MQ*) (all at 10 μM), or 40 μM lavendustin-A (*LA*). When indicated, assays contained carbamylcholine and/or GMP-PNP (both at 10 μM). B, Effect of quinones on $I_{K(ACh)}$. Experimental protocol was as described in the legend to Fig. 2A. Hydroquinone (▲) or duroquinone (○) (both at 10 μM) was present for the period shown.

ity, guanine nucleotide-sensitive, agonist binding (16, 17), whereas 1–2 mM NEM increases the agonist affinity (18, 19). It has been proposed that the dual effect of NEM is related to the sequential alkylation of specific sets of sulfhydryl groups in G proteins and receptors; namely, thiol groups in the G proteins react with NEM under mild conditions (30), preventing interaction with receptors, whereas the increase in the overall affinity for agonists at higher concentrations of reagent is due to alkylation of receptor sulfhydryl groups (19). Although the effects of MDC and other hydroquinone derivatives on cardiac muscarinic receptors resemble those of high concentrations of NEM, the quinones seem to be more potent, being active in the low micromolar range. This might simply indicate that the thiol groups involved are in an apolar environment, where the lipophylic quinones would be more effective than NEM. However, considering the relatively polar nature and consequent poor permeability of lavendustin-A, the site of action of the quinones tested is not likely to be very hydrophobic; instead, it would be expected to be located on the hydrophilic extracellular domains of the receptor. Therefore, the prominent effects of relatively low concentrations of benzoquinones may instead be the result of specific interactions with a particular site on the receptor molecule. The difference between the two types of alkylating agent might also arise from the fact that, whereas each molecule of NEM modifies only one group, the thioethers produced by the reaction of protein sulfhydryl groups with some quinones may undergo a second nucleophilic addition, leading to protein cross-linking.

In conclusion, the present results demonstrate that MDC and lavendustin-A modify muscarinic receptors directly and eliminate their functional linkage to G proteins. Exposure of membranes to either compound reduces markedly the sensitivity of agonist binding to guanine nucleotides; furthermore, in membranes exposed to MDC a significant fraction of the sites remain in the high affinity form. More importantly, the end result of incubation with MDC in alkaline-treated membranes is the same as observed in control membrane preparations, indicating that MDC restores the specific distribution of low and high affinity forms observed in the presence of functional G proteins. In fact, barring the loss of sensitivity to guanine nucleotides, addition of MDC to alkaline-extracted membranes is equivalent to reconstitution of the latter with exogenous G proteins (11). It seems that modification of the receptor by MDC simultaneously prevents and mimics interaction with a G protein, so that the proportion of high affinity sites detected in the presence of MDC does not differ significantly from that in the native system. Conceivably, the groups targeted by MDC are located in a region of the muscarinic receptors that translates coupling to G proteins into changes in the affinity for agonists; if so, benzoquinoid compounds might provide new insight into the mechanism by which interactions with G proteins provide the driving force for interconversion between muscarinic receptor states.

Finally, our observations show that derivatives of erbstatin or lavendustin-A that carry a reactive quinone moiety lack the selectivity required for utilization as tyrosine kinase inhibitors

or antitumor agents *in vivo*. Aside from their reactivity with proteins other than tyrosine kinases, during long term exposure these compounds may engage in redox cycling and form metabolites, such as oxidized glutathione and hydrogen peroxide, that are toxic to most cells and in particular to cardiac myocytes, which are known to be very vulnerable to oxidative damage (24).

Acknowledgments

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