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Anti-muscarinic actions of mitoxantrone in isolated heart muscles of guinea pigs

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Abstract

A hypotheses that mitoxantrone is a competitive antagonist at muscarinic cholinergic receptors was examined in guinea-pig hearts. In isolated left atrial muscle preparations, electrically paced at 2 Hz, the muscarinic agonist, carbachol, caused a concentration-dependent decrease in developed tension. Mitoxantrone caused a parallel right-ward shift of the concentration–response curve for carbachol. Schild plots for the effect of mitoxantrone on the carbachol concentration–response relationship were linear with a slope of 0.88 which was not significantly different from the unity. The right-ward shift of the carbachol concentration–response relationship by mitoxantrone significantly reversed after an additional incubation with a mitoxantrone-free solution, although the reversal was incomplete after a 2-h incubation in the mitoxantrone-free solution. Mitoxantrone caused a concentration-dependent displacement of specific [^{3}H]quinuclidinyl benzilate binding to membrane preparations obtained from ventricular muscles of guinea-pig hearts. These results indicate that mitoxantrone acts as a competitive antagonist for the muscarinic receptors.

Keywords: Heart; Mitoxantrone; Anti-muscarinic action; (Guinea pig)

1. Introduction

We observed previously that the highly effective anthracycline antibiotics doxorubicin and pirarubicin caused a parallel right-ward shift of the concentration–response curve for the negative inotropic effect of acetylcholine in left atrial muscle preparations isolated from guinea-pig hearts (Temma et al., 1992, 1993). These agents also caused a concentration-dependent displacement of specific [^{3}H]quinuclidinyl benzilate binding in membrane preparations obtained from ventricular muscles of guinea-pig hearts. Therefore, we concluded that these anthracyclines act on muscarinic receptors as competitive antagonists in heart cells. In addition, it has been reported that anthracyclines cause both acute and chronic cardiotoxicity (Henderson and Frei, 1979; Singal et al., 1987; Doroshow, 1991). The acute cardiotoxicity is observed during or shortly after the treatment, and is characterized by electrocardiogram abnormalities, primarily tachycardia (Bonadonna et al., 1969; Henderson and Frei, 1979; Singal et al., 1987; Doroshow, 1991). The tachycardia appears when the parasympathetic nervous system is depressed (Brown, 1990). Therefore, our results suggest that the anti-muscarinic action by these anthracyclines may be one of the possible causes of the acute cardiotoxicity (Temma et al., 1992, 1993).

Mitoxantrone, which is an anthracedione derivative, structurally similar to anthracycline, was synthesized in an attempt to develop an agent with reliable anticancer activity free from cardiotoxicity (Wallace et al., 1979; Murdock et al., 1979; Henderson et al., 1982; Sparano et al., 1982; Iatropoulos, 1984). Mitoxantrone was shown to have a significant anticancer activity, especially for human breast

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cancers (Jones et al., 1985; Henderson et al., 1989; Faulds et al., 1991), with relatively minor cardiotoxic effects compared to that of doxorubicin. Mitoxantrone, however, is not free from cardiotoxicity. Recently, in clinical studies (Schell et al., 1982; Unverferth et al., 1983; Benjamin, 1995) and in experimental animal models (Alderton et al., 1992; Herman et al., 1997), this agent was shown to possess significant cardiotoxic effects which have similar features as those observed with doxorubicin. Most recently, Wang et al. (1999) reported that mitoxantrone prolonged action potential duration, and inhibited muscarinic receptor-gated K^+ current ($I_{k,ACh}$) in whole cell voltage clamp studies using myocytes isolated from atrial muscles of guinea-pig heart. Therefore, these investigators concluded that mitoxantrone may block the cardiac muscarinic receptors.

In the present study, the action of mitoxantrone on muscarinic receptors was examined in isolated heart muscles obtained from guinea pigs.

2. Materials and methods

All protocols in this study were approved by the Institutional Animal Care and Use Committee at the Kitasato University for School of Veterinary Medicine and Animal Sciences and were in accordance with the Guide for the Use of Laboratory Animals issued by the US Department of Health and Human Services.

2.1. Left atrial muscle preparations

Male Hartley guinea pigs weighing 350–400 g were anesthetized with thiopental (50 mg/kg). The hearts were immediately removed and perfused via the aorta using a Langendorff apparatus with Krebs–Henseleit bicarbonate buffer solution with the following millimolar composition; 118 NaCl, 27.2 $NaHCO_3$, 4.8 KCl, 1.0 KH_2PO_4, 1.2 $MgSO_4$, 1.2 $CaCl_2$ and 11.1 glucose. The solution was saturated with a 95% O_2 and 5% CO_2 gas mixture, yielding a pH value of 7.4 at 30°C. After all visible blood was washed out, left atrial muscles were excised.

Left atrial muscle preparations were suspended vertically in a temperature-controlled bath containing 20 ml of the above Krebs–Henseleit bicarbonate buffer solution. The preparations were electrically paced at 2 Hz with square-wave pulses of 3-ms duration at a voltage approximately 15% above the threshold using a pair of platinum field-stimulation electrodes. The resting tension was adjusted to 1.0 g. Developed tension was continuously monitored using a force-displacement transducer and a polygraph recorder (model TB 651T and WI840G, respectively, Nihon Kohden Kogyo, Tokyo). Experiments were started after a 90–120 min equilibration period. Cumulative concentration–response curves for the negative inotropic effect of carbachol were generated by increasing the drug concentration at approximately 2-min intervals or when the effect reached a steady state at each concentration.

2.2. [^{3}H]quinuclidinyl benzilate binding

Partially purified membrane preparations obtained from ventricular muscle of guinea-pig hearts (Temma et al., 1992) were used for [^{3}H]quinuclidinyl benzilate binding study. Ventricular muscles were minced, and homogenized with a Dounce ball type homogenizer with 20 volumes of an ice cold buffer solution (250 mM, sucrose; 5 mM, $MgCl_2$; 20 mM, Tris–HCl buffer, pH 7.5). The homogenate was centrifuged at $900 \times g$ for 10 min, and resultant supernatant was centrifuged at $43{,}000 \times g$ for 20 min. The sediment was resuspended in a buffer solution containing 600 mM KCl, and was incubated for 10 min at 4°C. The suspension was centrifuged again at $43{,}000 \times g$ for 20 min. The sediment was washed twice with resuspending in the above mentioned buffer solution without KCl and centrifugation, and was used for [^{3}H]quinuclidinyl benzilate binding study.

The binding reaction was started by the addition of a 25-μl aliquot of membrane preparations to 225 μl of an incubation solution (final protein concentration, 0.05 mg/ml) containing 1 nM [^{3}H]quinuclidinyl benzilate, 75 mM $MgCl_2$, 25 mM Tris–HCl buffer (pH 7.5 at 26°C) and mitoxantrone (0, 0.3, 1, 3, 10 or 30 μM) with or without 2 μM atropine. After the mixture was incubated for 2 h at 26°C, the reaction was terminated by the addition of 3 ml of an ice-cold "stopping" solution containing 75 mM $MgCl_2$ and 25 mM Tris–HCl buffer (pH 7.5 at 4°C). Bound [^{3}H] quinuclidinyl benzilate was separated from unbound [^{3}H]quinuclidinyl benzilate by a nitrocellulose filter (pore size, 0.8 μm, Advantic Toyo, Japan) under vacuum. The filters were washed three times with 3 ml each of the above stopping solution. Radioactivity trapped by the filters (bound [^{3}H]quinuclidinyl benzilate) was estimated using a liquid scintillation spectrometer. Specific [^{3}H]quinuclidinyl benzilate binding is calculated as the difference between values observed in the absence (total binding) and presence (non-specific binding) of 2 μM atropine (Temma et al., 1992).

2.3. Chemicals and statistical analyses

[^{3}H]quinuclidinyl benzilate (specific activity 1.81 TBq/mmol) was purchased from Amersham Japan (Tokyo, Japan). Mitoxantrone hydrochloride, carbachol (carbamylcholine chloride) and atropine sulfate were purchased from Sigma (St. Louis, MO, USA). Other Chemicals used were of reagent grade. The IC_{50} values (the concentration that decreases the developed tension or [^{3}H]quinuclidinyl benzilate binding to 50% of the control values) were calculated graphically. Non-linear regression analyses were carried out to obtain the concentration–response curves for

negative inotropic effects of carbachol in the presence or absence of mitoxantrone and a curve for the concentration-dependent reversal of the [^{3}H]quinuclidinyl benzilate binding by mitoxantrone using a data analysis soft (SimaPlot 2000: sigmoid, 3 parameter) from the SPSS Science software products, Chicago, IL, USA. K_i values for the [^{3}H]quinuclidinyl benzilate binding study were obtained from the pseudo Hill plot analyses. Analyses of statistical significance were performed with Student's *t*-test. Criterion for statistical significance is a P value of less than 0.05. Protein concentration was assayed using the Bio-Rad protein assay reagent (Bio-Rad Laboratory, Hercules, CA, USA).

3. Results

3.1. Force of contraction study

3.1.1. Effect of mitoxantrone on developed tension

In the present study, carbachol was employed as a specific muscarinic agonist because it is stable in an oxygen-saturated incubation solution (Temma et al., 1992). The concentration–response curve for carbachol was generated after a 2-h incubation in the presence of mitoxantrone. Negative inotropic effects were expressed as percentage of developed tension observed immediately before the addition of the lowest concentration of carbachol.

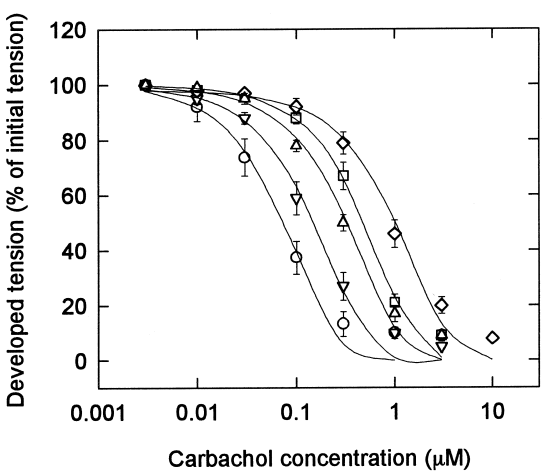

Fig. 1. Effect of mitoxantrone on the negative inotropic effect of carbachol. Electrically paced guinea-pig (2 Hz) left atrial preparations were incubated for 90–100 min at 30°C and then incubated for a further 120 min, either in the absence (○), or presence of mitoxantrone [10 (□), 30 (△) or 100 (▽) μM]. Carbachol concentration–response curves were then established. In one series of experiments, after incubation with 100 μM mitoxantrone for 120 min, atrial preparations were incubated for a further 120 min in drug-free solution, before the carbachol concentration–response curve was established (◇). The curves were fit to individual point using a logarithmic function curve-fit program (sigmoid, 3 parameter, SigmaPlot 2000, SPSS) for dose–response curves. Each point represents the mean of four to six experiments and the vertical lines indicate the standard errors.

Table 1
Effects of mitoxantrone on the IC_{50} values for the negative inotropic effect of carbachol

	IC_{50} value for carbachol (μM)[a]
Mitoxantrone (μM)	
0 (Control)	0.057 ± 0.014 (4)
10	0.180 ± 0.022 (7)[b]
30	0.421 ± 0.035 (6)[b]
100	0.770 ± 0.138 (6)[b]
Mitoxantrone, 100 μM, followed by a 2-h washout	0.353 ± 0.062 (4)[c]

The number in parentheses represents the number of experiments.
[a] The concentration of carbachol that decreases developed tension to 50% of the initial value (mean ± SE).
[b] Significantly different from the value observed in control experiments ($P < 0.05$).
[c] Significantly different from the values observed in the presence of 100 μM mitoxantrone ($P < 0.05$).

Increasing concentrations of carbachol were added after a steady state response to the previous concentration was observed or after 2 min in the absence of a response.

At the end of 90–120-min equilibration period, developed tension was 15.0 ± 0.7 mN ($n = 23$), and was gradually decreased with time in the absence of mitoxantrone or carbachol, reaching 80.0 ± 3.5% ($n = 4$) of the initial developed tension at 2 h later. The addition of mitoxantrone caused an increase in developed tension which reached a steady state within 2 h. Developed tension observed at 2 h after the addition of 10, 30 or 100 μM mitoxantrone was 117.6 ± 1.2% ($n = 7$), 127.2 ± 3.6% ($n = 6$) or 132.25.8% ($n = 6$), respectively, of the initial developed tension. These results show mitoxantrone has a positive inotropic effect on the atrial muscle preparations isolated from guinea-pig heart.

3.1.2. Effects of mitoxantrone on the concentration–response relationship for carbachol

Under the present experimental condition, carbachol caused a concentration-dependent decrease in developed tension (Fig. 1). The IC_{50} value for carbachol observed in the absence of mitoxantrone was 0.057 ± 0.014 μM (Table 1). The concentration–response curve for carbachol obtained from a curve fit program, observed in the presence of mitoxantrone, was shifted to the right in a parallel manner (Fig. 1). The magnitude of the right-ward shift was dependent on the concentration of mitoxantrone (Fig. 1, Table 1); slopes of the line observed in the presence of 10 (−1.11 ± 0.06), 30 (−1.17 ± 0.05) or 100 (−1.04 ± 0.11) μM mitoxantrone were not significantly different from the control value (−1.04 ± 0.07) observed in the absence of mitoxantrone. When the preparation were exposed to mitoxantrone for 2 h and then incubated for an additional 2 h period in a mitoxantrone-free solution, the magnitude of the right-ward shift of the carbachol concentration–re-

sponse curve was less than that observed immediately after 2 h exposure to mitoxantrone (Fig. 1). Fig. 2 shows Schild plots for mitoxantrone showing the 95% confidence limits. A straight regression line represented by the equation of ($Y = 0.88X + 4.57$) may be fitted to 17 points representing each experiment. The slope of the Schild regression was 0.88 ± 0.10, and was not significantly different from the unity. The correlation coefficient of the line was 0.833. The pA_2 value was 5.20 (correspond to the K_B values of $10^{-5.2}$; 6.31 μM) with the 95% confidence limit of 4.60–6.00 ($n = 17$).

3.1.3. Reversal of the negative inotropic effect of carbachol by mitoxantrone

Fig. 3 shows the reversal of the negative inotropic effects of carbachol by mitoxantrone. Carbachol at 0.1 μM caused a quick and marked decrease in developed tension, reaching the maximum negative inotropic effect within 2–3 min. Developed tension observed after a 15-min incubation (immediately before the addition of mitoxantrone) was $32.9 \pm 4.5\%$ ($n = 4$) of the initial value, and was relatively stable during the next 20-min period (Fig. 3). The addition of 10 or 30 μM mitoxantrone caused a gradual increase in developed tension. The developed tension observed at the end of a 20-min incubation with 10 and 30 μM mitoxantrone was $56.3 \pm 10.2\%$ ($n = 4$) and $87.0 \pm 1.6\%$ ($n = 4$), respectively, of the initial value observed before the addition of carbachol. A higher concentration of mitoxantrone (100 μM) caused a stronger and faster increase in the developed tension observed in the presence of 0.1 μM carbachol, reaching a peak 10 min after mitoxantrone addition. At this point, the developed tension was $36.3 \pm 10.1\%$ above the initial value observed before the addition of carbachol.

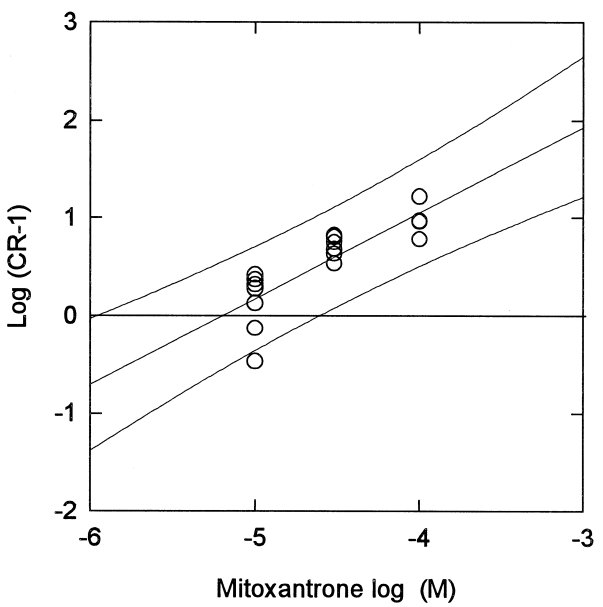

Fig. 2. Schild plots for the effect of mitoxantrone on the negative inotropic effect of carbachol in electrically paced guinea-pig left atrial preparations. Values of log (CR-1) are plotted against log concentration (M). Concentration ratio (CR) are the ratios of the IC_{50} values for carbachol, observed in the presence of given concentrations of mitoxantrone to the IC_{50} value of carbachol observed in the absence of mitoxantrone.

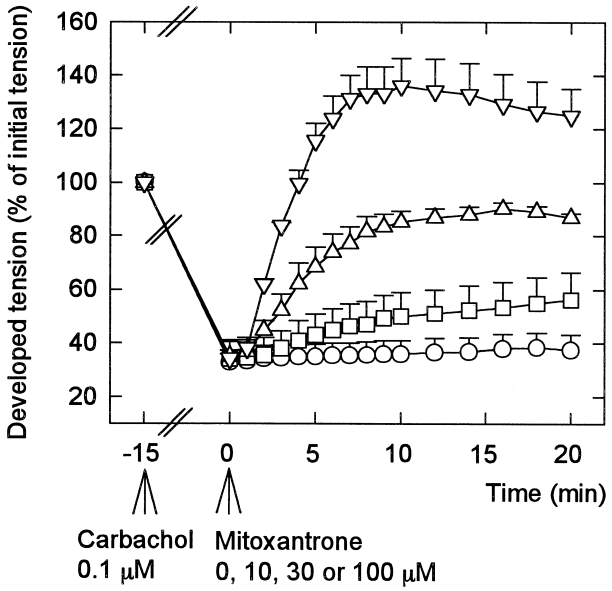

Fig. 3. Reversal of the negative inotropic effects of carbachol by mitoxantrone. After 90–120 min equilibration, 0.1 μM carbachol was added as indicated by the arrow. Mitoxantrone 10 (□), 30 (△) or 100 (▽) μM was added 15 min after the addition of carbachol. Control experiments (no mitoxantrone) are also shown (○). Each point represents the mean of four experiments and the vertical lines indicate the standard errors.

3.2. [^{3}H] quinuclidinyl benzilate binding study

Whether the right-ward shift of the concentration–response curve for carbachol and restoration of the negative inotropic effect of carbachol by mitoxantrone are due to a direct interaction with muscarinic receptors, were examined from the displacement of the specific [^{3}H]quinuclidinyl benzilate binding. [^{3}H]quinuclidinyl benzilate binding to partially purified membrane preparations isolated from ventricular muscle of guinea-pig hearts was observed in the presence of 1 nM [^{3}H]quinuclidinyl benzilate and the indicated concentration of mitoxantrone (Fig. 4). The [^{3}H]quinuclidinyl benzilate binding observed in the absence of mitoxantrone increased with time, and reached an apparent plateau at 2 h (data no shown). At this point, specific [^{3}H]quinuclidinyl benzilate binding was 393 ± 58 fmol/mg protein ($n = 3$), and was $67.5 \pm 8.6\%$ of the total binding (data not shown).

Scatchard plot analyses of the specific binding using 0.03, 0.1, 0.3, 1, 3 and 10 nM [^{3}H]quinuclidinyl benzilate revealed a single class of the binding site with apparent dissociation constant of 0.278 nM and a binding site concentration of 838 fmol/mg protein. Mitoxantrone reduced the [^{3}H]quinuclidinyl benzilate binding in a concen-

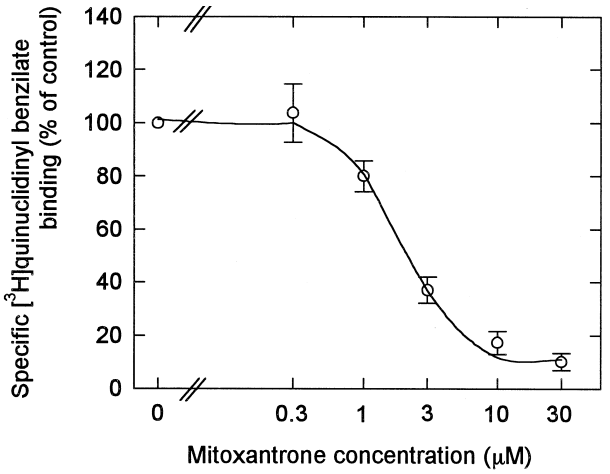

Fig. 4. Displacement of specific [^{3}H]quinuclidinyl benzilate binding by mitoxantrone. Partially purified membrane preparations obtained from ventricular muscles of guinea-pig hearts were incubated for 2 h at 26°C in an incubation solution containing 75 mM $MgCl_2$, 1 nM [^{3}H]quinuclidinyl benzilate, 25 mM Tris–HCl buffer and the indicated concentration of non-labeled mitoxantrone. Bound and unbound [^{3}H]quinuclidinyl benzilate was separated by nitrocellulose filters under a vacuum. Protein concentration in the incubation solution was approximately 0.05 mg/ml. A non-linear regression analyses were carried out to obtain a curve for the concentration-dependent reversal of the [^{3}H]quinuclidinyl benzilate binding by mitoxantrone. Each point represents the mean of three experiments. Vertical lines indicate the standard error.

tration-dependent manner (Fig. 4). The concentration of mitoxantrone to cause a 50% reduction in the [^{3}H]quinuclidinyl benzilate binding (the IC_{50} value) was 2.63 ± 0.63 μM ($n = 3$). Therefore, K_i values obtained from the pseudo Hill plot analyses were 0.573 ± 0.137 μM and slope factor was close to the unity (0.98 ± 0.08).

4. Discussion

Mitoxantrone caused a right-ward shift of the concentration–response curves for the negative inotropic effects of carbachol in isolated left atrial muscle preparations of guinea-pig hearts. The right-ward shift was concentration-dependent and parallel. These results are consistent with our earlier observations with doxorubicin and pirarubicin which are structurally similar to mitoxantrone (Temma et al., 1992, 1993). Furthermore, mitoxantrone caused a rapid reversal of the negative inotropic effects of carbachol, as also observed with doxorubicin (Temma et al., 1992). The question remains, however, as to whether the effect of mitoxantrone, in reversing the negative inotropic effect of carbachol, results from the antagonistic action of mitoxantrone at the cholinergic muscarinic receptor or from the intrinsic positive inotropic effect of mitoxantrone.

Mitoxantrone produced a positive inotropic effect in the absence of carbachol. This effect would not appear to be due to antagonism of the action of intrinsic acetylcholine since the developed tension was unaltered in the presence of atropine, a competitive antagonist of muscarinic receptors (data not shown). Apparently, the positive inotropic effect of mitoxantrone involves another mechanism, such as an alteration of free intracellular Ca^{2+}. Mitoxantrone has been shown to enhance Ca^{2+} release from the membrane preparations isolated from sarcoplasmic reticulum (Holmberg and Williams, 1990; Kim et al., 1994).

The parallel right-ward shift of the concentration–response curves for carbachol caused by mitoxantrone suggests that the effect of mitoxantrone is not caused by the simple addition of the two opposing effects; i.e., the negative inotropic effect of carbachol and the positive inotropic effect of mitoxantrone. Moreover, the magnitude of the positive inotropic effect of mitoxantrone would appear insufficient to reverse the negative inotropic effect of carbachol. Mitoxantrone at the concentration of 100 μM caused a 295 ± 22% increase in developed tension in the presence of 0.1 μM carbachol, whereas only a 19.0 ± 3.2% increase in developed tension was observed in the absence of carbachol at the comparable time point. These results indicate that the reversal of the negative inotropic effect of carbachol caused by mitoxantrone is at least mostly related to the antagonistic actions of mitoxantrone at the muscarinic receptors. Additionally, the slope for the Schild plot for the effect of mitoxantrone on the concentration-dependent negative inotropic response to carbachol was not divergent from the unity. Therefore, it is suggested that the antagonistic action would be competitive. These findings are consistent with observations in electrophysiological studies in isolated ventricular myocytes of guinea-pig or rat hearts that mitoxantrone inhibits muscarinic receptor-gated K^+ current which is evoked by 1 μM carbachol (Wang et al., 1999). This concept is further supported by the present finding that mitoxantrone inhibited the [^{3}H]quinuclidinyl benzilate binding.

Mitoxantrone caused a concentration-dependent reduction of [^{3}H]quinuclidinyl benzilate binding in membrane preparations which are partially purified from ventricular muscles of guinea-pig hearts. Slope of the pseudo Hill plot calculated from this binding experiment was closed to the unity. In the present study, [^{3}H]quinuclidinyl benzilate binding was observed in ventricular muscle preparations whereas inotropic effects were examined in atrial muscle preparations. It has been reported that muscarinic receptors in ventricular muscle are coupled to different transduction pathways than those in atrial muscle. For example, muscarinic receptors are coupled to K^+ channels in the atrial muscle; however, in ventricular muscle, such pathways are not observed to cause the negative inotropic effects (Levy et al., 1981; Kurachi et al., 1986). Nevertheless, it is likely that the findings with respect to [^{3}H]quinuclidinyl benzilate binding to ventricular muscarinic cholinergic receptor sites are relevant to the interaction of mitoxantrone, with cholinergic muscarinic receptors in atrial muscle. The IC_{50} value for the displacement of 1 nM [^{3}H]quinuclidinyl benzilate

binding from ventricular muscle preparations by mitoxantrone was 2.63 ± 0.63 μM. The pA_2 value of 5.20 for antagonism of the negative inotropic effect of mitoxantrone in atrial preparations, corresponds to a concentration of 6.31 μM. These two values (2.63 and 6.31 μM) may be considered very close considering the difference in the assay systems.

It should be noted that the potency of mitoxantrone in displacing [^{3}H]quinuclidinyl benzilate binding was higher than that observed with doxorubicin or pirarubicin (Temma et al., 1993); the IC_{50} values for doxorubicin and pirarubicin are 54.0 ± 10.5 μM and 14.3 ± 1.3 μM, respectively. These results are consistent with our observations (Temma et al., 1999) that the right-ward shift of the negative inotropic effects of carbachol caused by 100 μM mitoxantrone (IC_{50} values: 0.770 ± 0.138 μM) was stronger than that caused by 100 μM doxorubicin (IC_{50} values: 0.465 ± 0.057 μM).

The anti-muscarinic actions of mitoxantrone are more potent than that of doxorubicin or pirarubicin; however, cardiotoxicity that appears after the repeated mitoxantrone treatment has been shown to be less severe than that observed with doxorubicin (Schell et al., 1982; Henderson et al., 1989; Alderton et al., 1992; Benjamin, 1995; Herman et al., 1997). Therefore, the anti-muscarinic action is unlikely to be involved in the chronic cardiotoxicity of these agents. Mitoxantrone has been shown to cause a transient increase in heart rate in experimental animals (Perkins et al., 1984) and human patients (Siegert et al., 1989). It would appear, therefore, the anti-muscarinic action may contribute to the development of the acute cardiotoxicity of mitoxantrone.

Recently, it has been shown that gallamine or W84 binds to the allsoteric site of acetylcholine muscarinic receptors, and modulate their functions (Holzgrabe and Mohr, 1998). Consequently, these compounds alter the action of carbachol in beating isolated heart muscles of guinea pigs, and also the equilibrium binding of *N*-[^{3}H]methyl-scopolamine observed with homogenates obtained from guinea-pig hearts. Thus, the possibility that mitoxantrone acts at the allosteric site instead of competitively binding at the muscarinic binding site should be considered. It should be noted that compounds which bind to the allosteric site contain one or more positively charged nitrogens often in conjugation with a ring system (Holzgrabe and Mohr, 1998). Mitoxantron, however, does not have such positively charged nitrogen(s). Therefore, it is unlikely that mitoxantrone acts as a modulator combining with the muscarinic receptors at the allosteric site. Consistent with this concept, doxorubicin or pirarubicin behaves as a competitive antagonist to the negative inotropic action of carbachol, although neither of these compounds contains positively charged nitrogen(s). Apparently, further studies are needed to examine the exact nature of the antagonism. It can be concluded, however, that mitoxantrone caused a concentration-dependent right-ward shift, and reversal of the negative inotropic effect of carbachol. These effects of mitoxantrone may be related to the acute cardiotoxic effect of this compound.

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