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Effects of mitoxantrone on action potential and membrane currents in isolated cardiac myocytes

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1 The effects of mitoxantrone (MTO), an anticancer drug, on the membrane electrical properties of cardiac myocytes were investigated using the whole-cell clamp technique.

2 In isolated guinea-pig ventricular myocytes, 30 μM MTO induced a time-dependent prolongation of action potential duration (APD) which was occasionally accompanied by early afterdepolarizations. APD prolongation was preserved in the presence of 10 μM tetrodotoxin and showed reverse rate-dependence.

3 Both the inward rectifier K^+ current (I_{K1}) and the delayed rectifier K^+ current (I_K) of guinea-pig ventricular myocytes were significantly depressed by 30 μM MTO. The rapidly activating component of I_K (I_{Kr}) seemed to be preferentially blocked by MTO. The transient outward current was not affected by MTO in rat ventricular myocytes.

4 Thirty μM MTO had no direct effect on the L-type Ca^{2+} current ($I_{Ca(L)}$), but reversed the inhibitory effect of 1 μM carbamylcholine but not the A_1-adenosine receptor agonist (−)-N^6-phenylisopropyladenosine (1 μM) on $I_{Ca(L)}$ enhanced by 50 nM isoprenaline in guinea-pig ventricular myocytes. In guinea-pig atrial mycotyes, 30 μM MTO inhibited by 93% the muscarinic receptor gated K^+ current ($I_{K,ACh}$) evoked by 1 μM carbamylcholine, whereas $I_{K,ACh}$ elicited by 100 μM GTPγS, a nonhydrolysable GTP analogue, was only decreased by 12%.

5 The specific binding of [^{3}H]QNB, a muscarinic receptor ligand, to human atrial membranes was concentration-dependently displaced by MTO (1–1000 μM).

6 In conclusion, MTO blocks cardiac muscarinic receptors and prolongs APD by inhibition of I_{K1} and I_{Kr}. The occasionally observed early afterdepolarizations may signify a potential cardiac hazard of the drug.

Keywords: Cardiac effects; ventricular myocytes; atrial myocytes; mitoxantrone; membrane currents; action potential; muscarinic receptors; early afterdepolarization

Abbreviations: APD, action potential duration; APD_{90}, action potential duration measured at 90% repolarization; CCh, carbamylcholine; C_m, membrane capacitance; GTPγS, guanosine-5′-O-(3-thiotriphosphate); $I_{Ca(L)}$, L-type Ca^{2+} current; I_K, delayed rectifier K^+ current; $I_{K,ACh}$, muscarinic receptor gated K^+ current; I_{Kr}, rapidly activating delayed rectifier K^+ current; I_{Ks}, slowly activating delayed rectifier K^+ current; ISO, isoprenaline; MTO, mitoxantrone; R-PIA, (−)-N^6-phenylisopropyladenosine; TTX, tetrodotoxin

Introduction

Mitoxantrone (MTO) is an anthracene-based antineoplastic agent that was developed as an alternative to cardiotoxic anthracyclines (Johnson *et al*., 1979; Murdock *et al*., 1979). Its therapeutic efficacy has been demonstrated in patients with advanced breast cancer, non-Hodgkin's lymphoma and several forms of leukaemia (Weiss, 1989; Faulds *et al*., 1991; Wiseman & Spencer, 1997). Although MTO displays a more favourable tolerability profile than anthracyclines, cardiotoxic effects have been clearly documented with this drug. Described cardiac effects include decreases in left ventricular ejection fraction, congestive heart failure and dysrhythmias (Shenkenberg & von Hoff, 1986; Gams & Wesler, 1984). The incidence of cardiotoxicity was increased in patients receiving cumulative MTO doses (Henderson *et al*., 1989) and in patients with a history of anthracycline therapy, mediastinal irradiation or cardiovascular diseases (for review see Wiseman & Spencer, 1997). Although at clinically equivalent doses, cardiotoxicity has been considered to be significantly less severe than that of doxorubicin (Posner *et al*., 1985; Dorr *et al*., 1991; Alderton *et al*., 1992), cardiac ultrastructural changes are similar to those of anthracyclines (Unverferth *et al*., 1983; Benjamin, 1995; Herman *et al*., 1997). Myofibrillar loss and dilatation of sarcotubular structures are regularly observed in cardiomyopathic animals chronically treated with either doxorubicin or MTO, while mitochondrial swelling appeared to be more specific for the anthracene derivative (Dodd *et al*., 1993; Herman *et al*., 1997).

In the present study we investigated, for the first time, the acute effects of MTO on isolated cardiac preparations. The whole-cell clamp technique was used to record action potentials and membrane currents in guinea-pig and rat cardiomyocytes. Furthermore, the effect of MTO on cardiac muscarinic receptors was assessed by radioligand binding experiments in human atrial membranes.

Methods

Action potentials in multicellular preparations

Guinea-pigs of either sex weighing 250–350 g were anaesthetized with ether and subsequently killed by cervical

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dislocation. Right ventricular papillary muscles (diameter, 0.5–0.8 mm) were rapidly excised from the isolated heart and mounted horizontally in a two-cambered organ bath with internal circulation of the bath solution (volume, 50 ml). The bath solution was constantly gassed and kept in circulation by 95% O_2/5% CO_2; the temperature was maintained at 35°C, pH 7.4. The bath solution was a modified Krebs-Henseleit solution of the following composition (mM): NaCl 115, KCl 4.7, $MgSO_4$ 1.2, $CaCl_2$ 2.0, $NaHCO_3$ 25, KH_2PO_4 1.2, and glucose 10. Transmembrane electrical activity was recorded with conventional glass microelectrodes, which were filled with 3 M KCl and had tip resistance of 10–20 MΩ. Transmembrane potentials were measured by means of an electrometer amplifier (model 773, World Precision Instruments), stored on a DAT-recorder (DTR-1202, Bio-Logic) and subsequently evaluated by a computer. Only experiments with microelectrode impalements lasting throughout the experimental period were accepted for evaluation.

Single-cell isolation

Isolated myocytes were prepared from ventricles or atria of adult guinea-pigs or rats by enzymatic dissociation according to Powell *et al.* (1980) with small modifications. Briefly, the heart was retrogradely perfused at 37°C and at a constant rate of 10 ml min^{-1} with the following solutions: 5 min with a nominally Ca^{2+}-free Joklik solution (Joklik-MEM, Biochrom) supplemented with $NaHCO_3$ and then, 5–15 min with the same solution to which had been added 50 μM $CaCl_2$, collagenase (Worthington type II, 25 mg 50 ml^{-1}, Biochrom), protease (type XIV, 10 mg 50 ml^{-1}, Sigma), and 0.1% bovine serum albumin (fraction V, Sigma). All solutions were gassed with 5% CO_2 in O_2; the pH was 7.4. After perfusion, the ventricles or the atria were separated, minced and incubated in fresh enzyme solution. The cells were then disaggregated by gentle mechanical agitation. After filtration through a nylon mesh, the cells were centrifuged at $37 \times g$ for 3 min and then resuspended in Joklik solution containing 300 μM $CaCl_2$ and 1% bovine serum albumin and kept for use at room temperature under a continuous stream of 5% CO_2 in O_2.

Whole-cell voltage clamp

A drop of cell suspension was added to a Tyrode solution in the recording chamber (volume, 0.5 ml) mounted on an inverted microscope (Zeiss Axiovert). The Tyrode solution contained (mM): NaCl 138, $MgSO_4$ 1.2, $CaCl_2$ 2, KCl 5, Glucose 10, HEPES 5; the pH was 7.4. After the cells had attached to the bottom, the bath was perfused at a flow rate of 4 ml min^{-1} with prewarmed Tyrode solution continuously gassed with O_2. The temperature in the bath (34–35°C) was continuously monitored.

Voltage-clamp experiments were performed in the whole-cell clamp configuration (Hamill *et al.*, 1981). Patch electrodes were fabricated from borosilicate glass capillaries (World Precision Instruments) and filled with prefiltered solutions of different composition (see below). The resistance of the electrodes ranged from 1.5–3 MΩ. The whole-cell voltage clamp was achieved by the use of a patch-clamp amplifier (EPC7, List Medical Electronics), connected *via* a 16 bit A/D interface to a pentium IBM clone computer. The data were sampled at 3 kHz, data acquisition and analysis was performed with an ISO-3 multitasking patch-clamp program (MFK, Neidernhausen). To determine current densities,

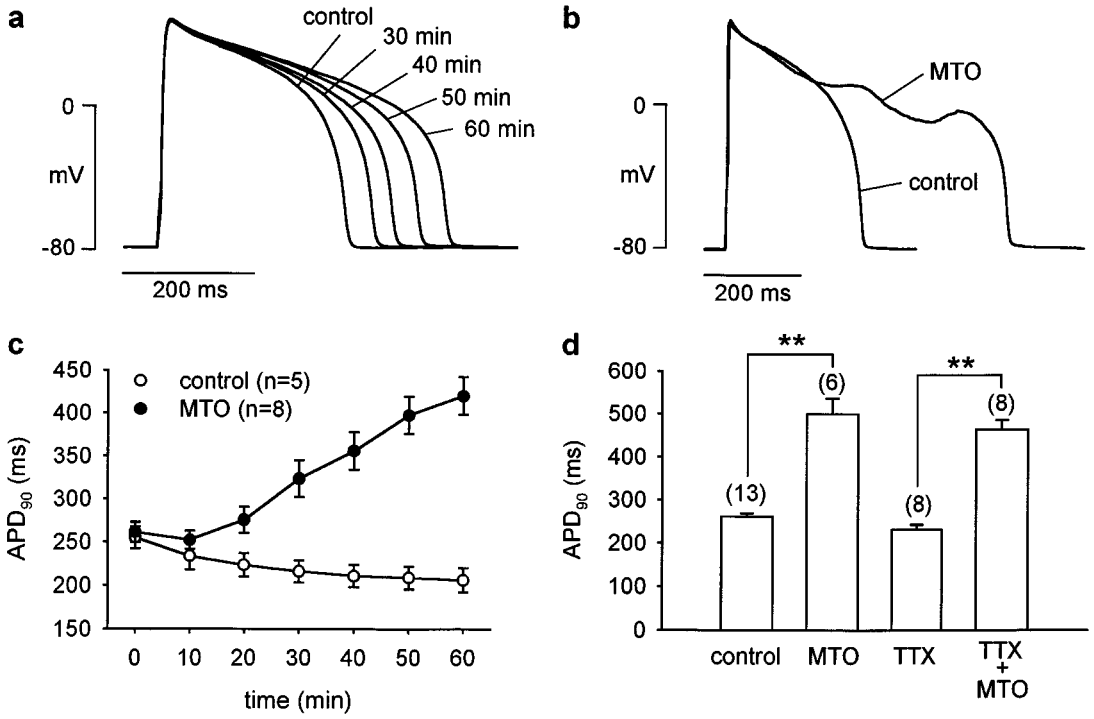

Figure 1 Effect of MTO on the action potential duration (APD) in guinea-pig ventricular myocytes. Action potentials were evoked with current-clamp at a frequency of 0.5 Hz. (a) Recording showing time-dependent prolongation of APD induced by 30 μM MTO. All action potentials were continuously recorded from one cell. (b) Superimposed recordings showing early afterdepolarization caused by superfusion of a myocyte with 30 μM MTO for 1 h. (c) Time-dependent change of APD_{90} in control cells and in cells superfused with 30 μM MTO. (d) Failure of tetrodotoxin (TTX) to prevent the prolongation of APD_{90} induced by superfusion of myocytes with 30 μM MTO for 60 min. APD_{90} was measured in the absence (control) and in the presence of either 30 μM MTO, 10 μM TTX, or 30 μM MTO plus 10 μM TTX. Data from four different cell groups are compared. Numbers of cells in each group are given in parentheses. $^{**}P<0.01$.

membrane capacitance (C_m) was calculated as the area under the uncompensated capacitative transient divided by the amplitude of a hyperpolarizing pulse of 5 mV. The L-type Ca^{2+} current ($I_{Ca(L)}$) was recorded by applying a test pulse of 300 ms every 5 s from a holding potential of −40 mV. The amplitude of $I_{Ca(L)}$ was measured as peak inward current with respect to the zero current level. To eliminate interfering K^+ current, KCl of the Tyrode solution was replaced by equimolar CsCl. Steady-state membrane K^+ currents were obtained by applying hyperpolarizing and depolarizing test pulses for 1 s from a holding potential of −40 mV at a rate of 0.1 Hz. The steady-state membrane K^+ current was measured as the net current at the end of the clamp set with respect to the zero current level. In some cases the inward rectifier K^+ current (I_{K1}) was blocked by 1 mM $BaCl_2$ in order to measure other background currents activated by 100-ms test pulses to potentials between −100 and 0 mV. Delayed rectifier K^+ (I_K) was determined by measuring the outward tail currents elicited on repolarization to −40 mV at the end of 1-s or 250-ms depolarizing clamp steps. The amplitude of the deactivating I_K tail was measured as the difference between the peak outward tail current and the steady-state current at −40 mV. In some experiments nominally Ca^{2+}-free Tyrode solution was used to allow measurement of the rapidly activating I_K (I_{Kr}) without contamination of the slowly activating I_K (I_{Ks}) (Sanguinetti & Jurkiewicz, 1990b; Jurkiewicz & Sanguinetti, 1993). In this case, cells were depolarized to −10 mV for 500 ms from a holding potential of −40 mV. Both the steady-state current at the end of depolarization and the tail current upon repolarization were evaluated. The transient outward current (I_{to}) was measured in single rat myocytes by applying test pulses of 500 ms every 5 s from a holding potential of −80 mV. To inactivate interfering Na^+ current, a prepulse of 100 ms to −60 mV preceded each test pulse. When measuring K^+ currents or background currents, the external bath solution contained 0.3 μM nisoldipine in order to block interfering $I_{Ca(L)}$. For the measurement of muscarinic receptor gated K^+ currents ($I_{K,ACh}$), rat atrial myocytes were clamped at −50 mV. Action potentials of single cells were measured in the current-clamp mode at a frequency of 0.5 Hz.

Electrode filling solutions

The electrode filling solutions contained (a) for single cell action potential recordings (mM): KCl 125, NaCl 5, $MgSO_4$ 2, K_2ATP 5, HEPES 5; pH 7.3 adjusted by adding KOH; (b) for $I_{Ca(L)}$: CsCl 125, TEA-Cl 20, MgATP 5, EGTA 10, HEPES 5; pH 7.3 adjusted with CsOH; (c) for K^+ currents or background currents: KCl 125, $MgSO_4$ 2, K_2ATP 5, EGTA 5, HEPES 5; pH 7.3 adjusted with KOH; for the measurement of $I_{K,ACh}$ either 100 μM GTP or 100 μM guanosine-5′-O-(3-thiotriphosphate((GTPγS) was added to the pipette solution.

Membrane preparation

Washed 100,000 × *g* membranes were prepared from frozen left atrial tissue from a patient with dilated cardiomyopathy who underwent cardiac transplantation. Tissue homogenization and membrane preparation was as described previously (Abi-

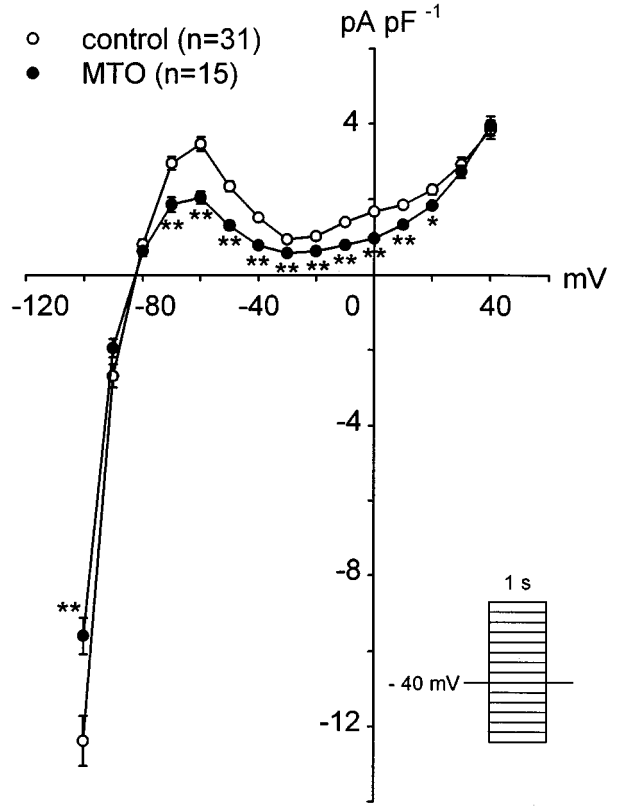

Figure 2 Current-voltage relation of the steady-state K^+ currents of guinea-pig ventricular myocytes in the absence (control) and in the presence of 30 μM MTO. The drug effect was measured 1 h after its addition to the cells. Currents were evoked by applying 1-s depolarizing or hyperpolarizing pulses in 10 mV steps from a holding potential of −40 mV every 10 s. The mean current densities are plotted against the respective test potentials. Data from two different cell groups are compared. $*P<0.05$ and $**P<0.01$ vs control.

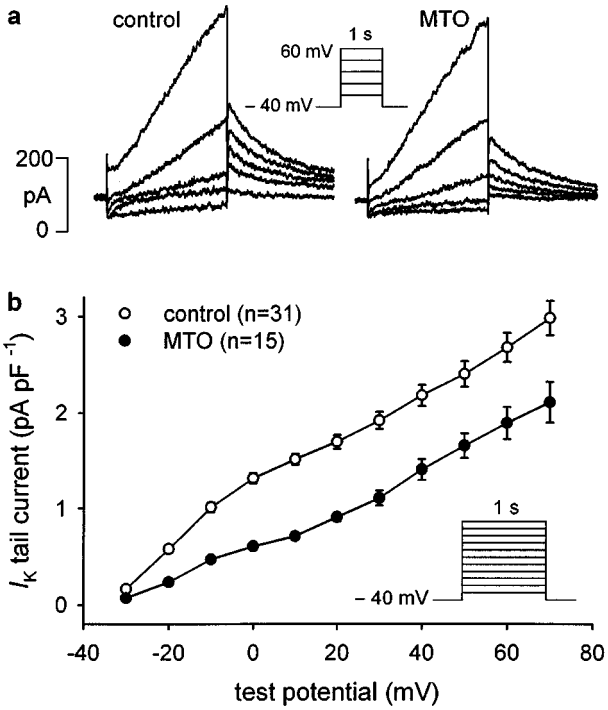

Figure 3 Inhibition of I_k by MTO in guinea-pig ventricular myocytes. I_k was elicited every 10 s by voltage protocols indicated in the inset of panel (a) and (b). The drug effect was determined 60 min after its addition to the myocytes. (a) Superimposed traces showing I_k steady-state currents and tail currents of a control cell (C_m = 80 pF) and of a cell superfused with 30 μM MTO (C_m = 83 pF). (b) I_k tail current-voltage relation in the absence (control) and in the presence of 30 μM MTO. The mean I_k tail current densities from two different cell groups at the respective test potentials are compared.

Gerges *et al*., 1997). Membranes were stored in aliquots at −80°C. Protein yield was 9.92 mg g^{-1} wet weight.

Radioligand binding

The density of M-cholinoceptors was determined by saturation binding experiments with the nonselective M-cholinoceptor antagonist [^{3}H]-QNB (42 Ci $mmol^{-1}$; DuPont-New England Nuclear; Boston, MA, U.S.A.) at room temperature (22–24°C), using 40–80 μg membrane protein in an assay buffer of Tris 20 mM, NaCl 100 mM, EDTA 0.5 mM, pH 7.4, as described previously (Abi-Gerges *et al*., 1997). To test whether MTO competes with [^{3}H]-QNB for binding to human atrial membranes, two sets of experiments were performed. First, saturation experiments with [^{3}H]-QNB were performed in the absence and presence of 30 μM MTO. Second, binding of several fixed concentrations of [^{3}H]-QNB (0.70–2.91 nM) was displaced by MTO (0.1–1000 μM). All reactions were performed at least twice in triplicate. The software GraphPads® was used to fit displacement curves and to calculate dissociation constants.

Drugs

Mitoxantrone hydrochloride (batch 331110) was kindly provided by Lederle GmbH (Münster, Germany) and was dissolved in distilled water to give a 30 mM stock solution. Appropriate portions of this stock solution were added to the bath solution just before use to achieve final concentrations. Tetrodotoxin, (−)-N^6-phenylisopropyladenosine (R-PIA), GTPγS, carbamylcholine and isoprenaline were obtained from Sigma (Deisenhofen, Germany). d-sotalol and nisoldipine were kindly provided by Bristol-Myers Co. (Wallingford, CT, U.S.A.) and Bayer AG (Wuppertal, Germany), respectively.

Statistics

Where appropriate, results are presented as means ± s.e.mean. Significance tests were performed using Student's *t*-test for paired or unpaired observations. Differences between means were regarded statistically significant at $P<0.05$.

Results

Action potential duration

The effect of MTO on action potential duration (APD) was investigated in isolated ventricular myocytes that had been current-clamped in the whole-cell clamp configuration (Figure 1a). In the absence of MTO, cells stimulated at 0.5 Hz had

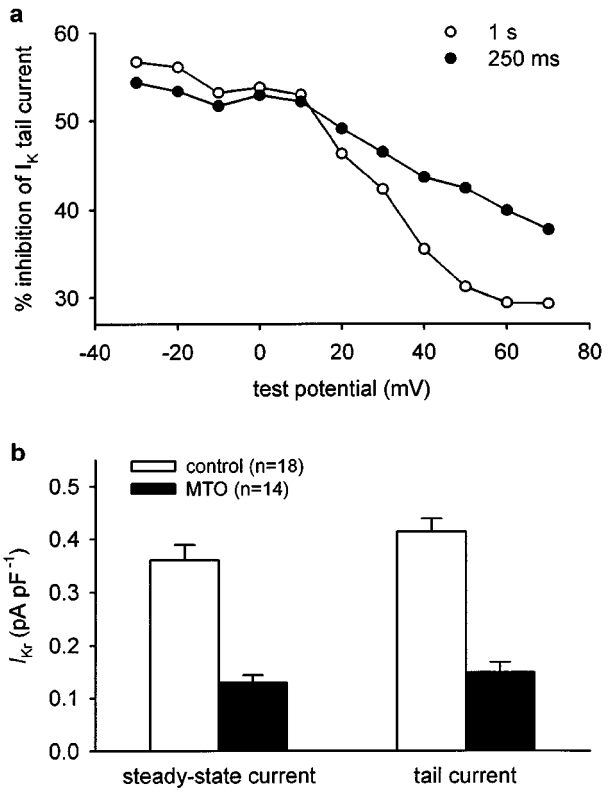

Figure 4 (a) Effect of test pulse duration on the voltage-dependent inhibition of I_k tail currents by 30 μM MTO. Guinea-pig ventricular myocytes were clamped every 10 s from a holding potential of −40 mV to test potentials up to +70 mV in 10 mV increments. Test pulses of 1-s and 250-ms were applied to every cell. Per cent inhibition of the I_k tail current was calculated by dividing the mean current densities of the cells pretreated with 30 μM MTO for 1 h ($n=15$) by that of control cells ($n=31$) at the respective test potential and test pulse duration. (b) Inhibition of I_{kr} by 30 μM MTO. Myocytes were superfused with nominally Ca^{2+}-free bath solution. I_{kr} steady-state current was evoked by a 500-ms depolarization pulse to −10 mV at 0.5 Hz, and I_{kr} tail current was elicited upon repolarization to a holding potential of −40 mV. The effect of MTO was measured after cells had been treated with the drug for 1 h. The mean current densities of two different cell groups are compared.

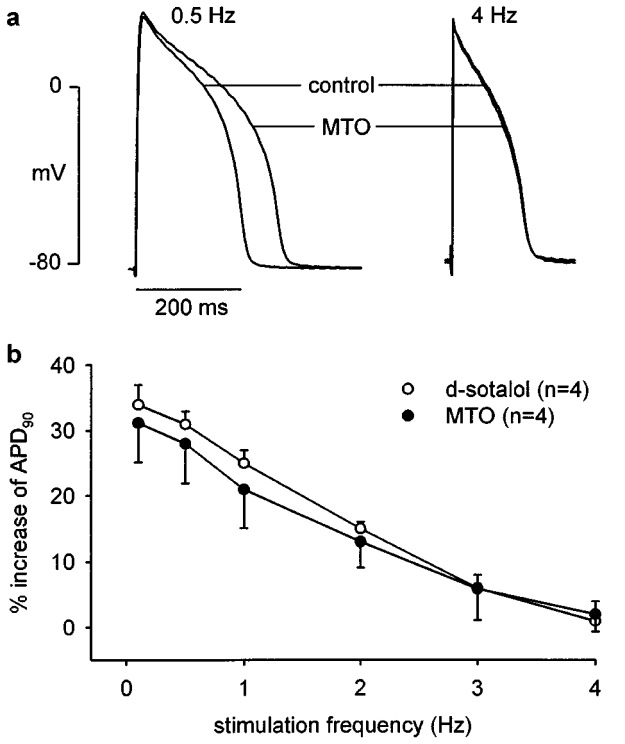

Figure 5 Reverse rate-dependence of the prolongation of action potential duration (APD) by MTO in guinea-pig ventricular papillary muscles. The effect of MTO was measured 2 h after its addition to the bath. (a) Superimposed recordings showing the effect of 30 μM MTO on APD in the same muscle at two different stimulation frequencies. (b) Comparison of the reverse rate-dependence of the prolongation of APD_{90} by 30 μM MTO and 30 μM d-sotalol. The effect of d-sotalol was evaluated 30 min after its addition to the bath. Two different groups are compared.

resting potentials in the range of −76 to −85 mV (mean of thirteen cells −79.3±0.7 mV) and displayed action potentials with a mean duration of 260.7±7.7 ms ($n=13$, control in Figure 1d) when measured at 90% repolarization (APD_{90}). As shown in Figure 1c, without drug-intervention APD_{90} shortened under continuous stimulation within 1 h by 19.3% ($n=5$), whereas MTO prolonged APD_{90} after 1 h by 60.6% ($n=8$). In three other cell groups, APD_{90} was determined after 1 h of incubation with either 30 μM MTO, 10 μM tetrodotoxin, or 10 μM tetrodotoxin plus 30 μM MTO. As shown in Figure 1d, APD_{90} of six cells treated with 30 μM MTO was 500.5±36.5 ms and thus significantly longer than APD in untreated cells ($P<0.01$). The ability of MTO to prolong APD was preserved in the presence of tetrodotoxin. Although tetrodotoxin significantly shortened APD_{90} as has previously been observed (Kiyosue & Arita, 1989), MTO prolonged APD_{90} in the presence of tetrodotoxin to 463.2±22.9 ms ($n=8$; Figure 1d). There was no significant difference in the prolongation of APD by MTO when tetrodotoxin was present; MTO increased APD_{90} by 91.8% in the absence and by 100.4% in the presence of 10 μM tetrodotoxin. The results clearly exclude the possibility of a tetrodotoxin-sensitive Na^+ window current contributing to the APD prolongation produced by MTO. Other parameters of the action potential, such as resting potential and action potential amplitude, were not significantly affected by MTO. Not surprisingly for cells with such a pronounced prolongation of APD, some cells exposed for more than 1 h to 30 μM MTO displayed early after depolarization (Figure 1b).

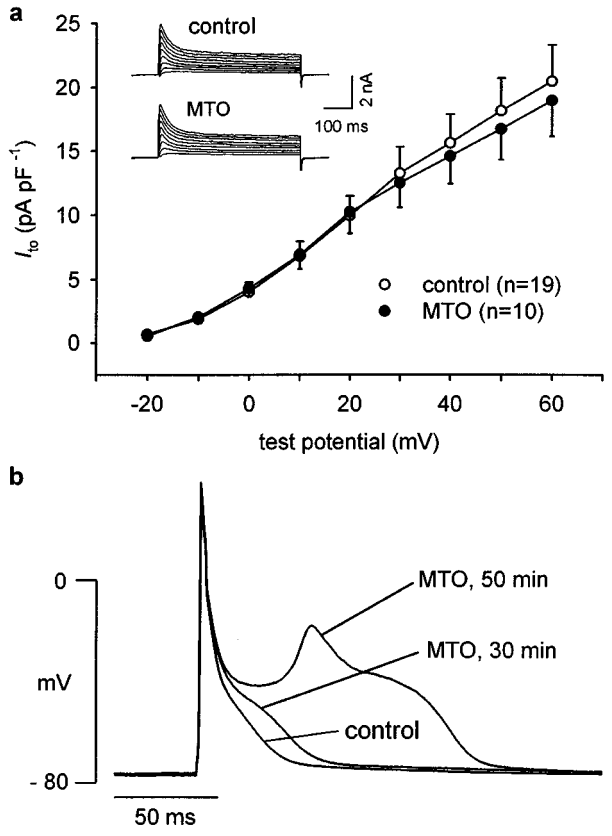

Figure 6 (a) Failure of 30 μM MTO to influence I_{to} of rat ventricular myocytes. I_{to} was elicited by depolarization to various test potentials for 500 ms after a 100 ms prepulse to −60 mV from a holding potential of −80 mV every 10 s. Current-voltage relation of I_{to} in control cells and in cells pretreated with 30 μM MTO for 1 h are compared. The inset shows representative I_{to} recordings of a control cell ($C_m=135$ pF) and of a MTO-treated cell ($C_m=129$ pF). (b) Superimposed traces showing the time-dependent effect of 30 μM MTO on the action potential of a rat ventricular myocyte, current-clamped at 0.5 Hz.

Voltage-dependent K^+ currents

To test for a possible interaction of MTO with K^+ currents, myocytes were clamped from a holding potential of −40 mV to voltages between −100 and +40 mV in 10-mV steps for 1 s. As shown by the current-voltage relations in Figure 2, pretreatment of cells with 30 μM MTO for 1 h produced a significant decrease of I_{K1}. When I_{K1} was blocked by 1 mM $BaCl_2$, the residual background currents activated between −100 to 0 mV were not significantly influenced by MTO (data not shown). K^+ currents activated at potentials positive to −30 mV, i.e., potentials at which I_K activates, were likewise suppressed by 30 μM MTO. Currents elicited by clamp steps to voltages positive to +20 mV, however, were not inhibited by MTO. The effect of MTO on I_K was therefore investigated in more detail by measuring the outward tail currents elicited on repolarization to −40 mV after depolarizing step potentials from a holding potential of −40 mV to +70 mV in 10 mV increments for 1 s. Typical recordings obtained from two different cells clamped from −40 to +60 mV in several steps are shown in Figure 3a; peak tail currents are clearly suppressed at all potentials in the MTO-pretreated cells whereas time-dependent I_K was not affected at potentials positive to +20 mV. In Figure 3b, tail currents were plotted as a function of membrane potential, and it can be seen that currents in the presence of MTO (closed circles) were suppressed at all voltages. Inspection of Figure 3b reveals that the current-voltage relations in the absence and in the presence of MTO are characterized by an outward hump which indicates that I_K is composed of a low (−30 to +10 mV) and a high voltage (> +20 mV) component. Figure 4a (open circles) demonstrates that inhibition of I_K tail currents by MTO is largest at lower voltages (by 50–60% at −30 to +10 mV) and decreases at more positive potentials. When I_K tail currents were elicited by short voltage pulses of 250 ms, inhibition by MTO did not differ from long pulses in the lower voltage range but was more sustained at more positive potentials (Figure 4a, closed circles). These results suggested that MTO preferentially inhibited the rapid activating component of I_K, i.e., I_{Kr}. When I_{Kr} was measured directly in cells bathed in nominally Ca^{2+}-free solution, both the steady-state current evoked by a 500-ms depolarizing step to −10 mV and the tail current elicited upon repolarization to a holding potential of −40 mV were significantly inhibited by 64% after 1 h treatment with 30 μM MTO (Figure 4b). The voltage dependence of I_{Kr} activation was not significantly influenced by MTO (data not shown). Class III antiarrhythmic drugs, specifically those which inhibit predominantly I_{Kr}, are known to exhibit reverse rate-dependence (Hondeghem & Snyders, 1990). Figure 5 shows that 30 μM MTO resembled d-sotalol in that APD prolongation was successively reduced by increasing the stimulation frequency. As shown by the original action potentials in Figure 5a, MTO completely lost its APD-prolonging effect at a stimulation frequency of 4 Hz. Note that the experiments shown in Figure 5 were carried out in guinea-pig papillary muscles.

In some tissues such as rat and human heart, I_{to} plays an important role in determining APD. Because I_{to} is functionally absent in guinea-pig ventricular myocytes (Josephson *et al.*, 1984), experiments were also carried out on isolated rat cardiomyocytes. Cells were depolarized from a holding

potential of −80 mV by a prepulse to −60 mV and then clamped to test potentials ranging from −30 to +60 mV for 500 ms. As shown by the original recordings in Figure 6a (inset), and verified in additional 19 control and ten cells pretreated with 30 μM MTO for 1 h, no significant effect on I_{to} at any of the test potentials could be detected by the drug. The lack of MTO to affect I_{to} is also demonstrated by continuous action potential measurements in current-clamped rat ventricular myocytes as shown in Figure 6b. In the voltage range where I_{to} is dominant, no influence on action potential repolarization can be detected. However, when I_{K1} becomes activated at around −30 mV, MTO produced a time-dependent slowing of repolarization which eventually led to early afterdepolarizations. As shown before in guinea-pig cells, MTO also inhibited I_{K1} of rat cardiomyocytes (not shown) and this effect can explain the pronounced prolongation of the late phase of the action potential in the rat.

L-type Ca^{2+} current

When $I_{Ca(L)}$ was measured at various potentials in guinea-pig ventricular myocytes either in the absence or in the presence of 30 μM MTO, no significant difference in the peak or the inactivation kinetics of the current was observed (data not shown). In the experiment shown in Figure 7a and b, $I_{Ca(L)}$ was enhanced from 0.7−2.0 nA by superfusing the myocyte with 50 nM isoprenaline. The isoprenaline-stimulated $I_{Ca(L)}$ was then inhibited by 1 μM carbamylcholine to 1.3 nA. Adding 30 μM MTO to the isoprenaline- and carbamylcholine containing bath solution partially reversed $I_{Ca(L)}$ to 1.9 nA. After washing out all three drugs, $I_{Ca(L)}$ returned to the predrug control level.

In Figure 7a, the original current traces of the experiment depicted in Figure 7b are superimposed at times when the respective effects were maximal. Results similar to those shown in Figure 7a and b were obtained in eleven other cells (Figure 7c). In order to investigate whether MTO antagonized the effect of carbamylcholine by blocking muscarinic receptors or by interfering with mechanisms downstream to receptor activation, experiments with the A_1-adenosine receptor agonist R-PIA were carried out. As shown in Figure 7d, addition of 1 μM R-PIA to myocytes stimulated by 50 nM isoprenaline induced a signfiicant decrease of $I_{Ca(L)}$ from 2.2±0.2 (ISO; $n=7$) to 1.4±0.2 nA (ISO+R-PIA). Contrary to carbamylcholine, the enhancing effect of additional MTO on $I_{Ca(L)}$ was not observed in the presence of isoprenaline and R-PIA (Figure 7d ISO+R-PIA+MTO). These findings indicate that MTO enhanced $I_{Ca(L)}$ most likely by blocking muscarinic receptors.

Muscarinic receptor gated K^+ current

Cardiac muscarinic receptor gated K^+ channels (K_{ACh} channels) are present in high density in the atria, sinoatrial- and atrioventricular node, and are a major target of the vagal autonomic system in the heart. The effect of MTO on $I_{K,ACh}$ induced by carbamylcholine in GTP (100 μM)-loaded guinea-pig atrial cells was therefore investigated. When 1 μM carbamylcholine was added to the superfusion medium, an outward K^+ current was rapidly activated at a holding potential of −50 mV (Figure 8a). After activation, the current slowly declined, possibly due to desensitization (Kurachi *et al*., 1987). Addition of 30 μM MTO after 1 min superfusion of

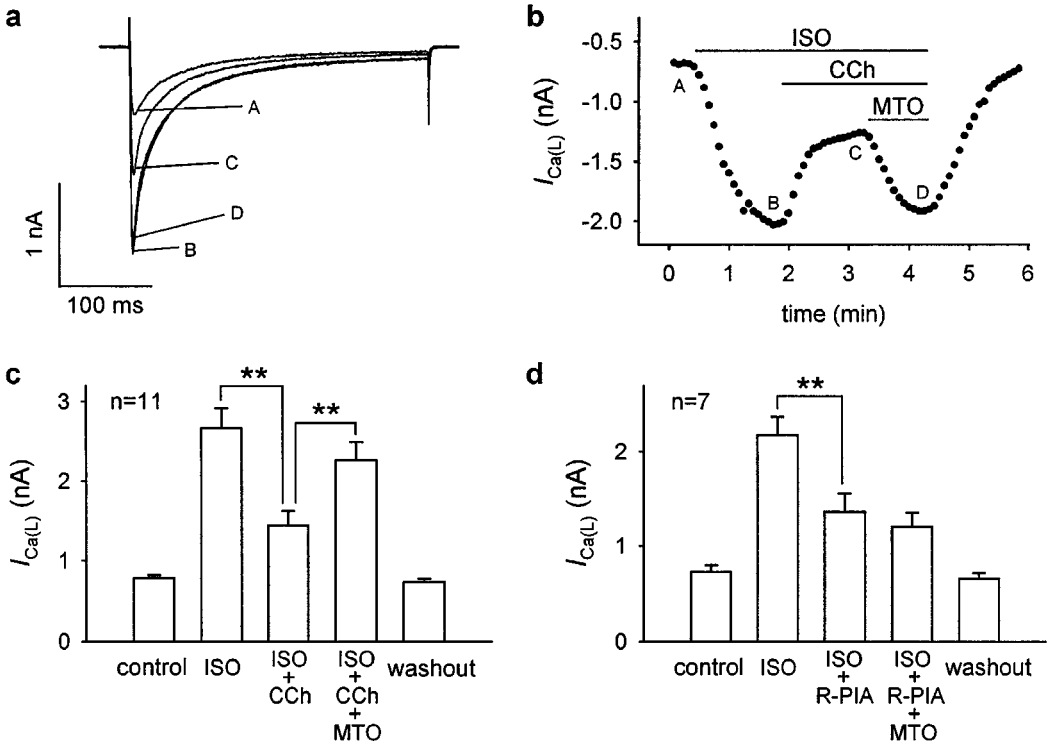

Figure 7 Reversal by MTO of the inhibitory effect of carbamylcholine on $I_{Ca(L)}$ activated by isoprenaline in guinea-pig ventricular myocytes. $I_{Ca(L)}$ was elicited by depolarizing steps from a holding potential of −40 mV to 0 mV for 300 ms at a frequency of 0.2 Hz. MTO: 30 μM mitoxantrone; ISO: 50 nM isoprenaline; CCh: 1 μM carbamylcholine; R-PIA: 1 μM (−)-N^6-(2-phenylisopropyl)-adenosine. (a) Superimposed current recordings showing the reversing effect of MTO on peak $I_{Ca(L)}$ inhibited by CCh. The current traces were recorded at the times indicated by the corresponding letters in panel (b). (b) Time course of the change of peak I_{Ca} in the same cell as in (a). (c) and (d) Summary of the effect of MTO on peak $I_{Ca(L)}$ inhibitited by CCh and R-PIA, respectively. $^{**}P<0.01$.

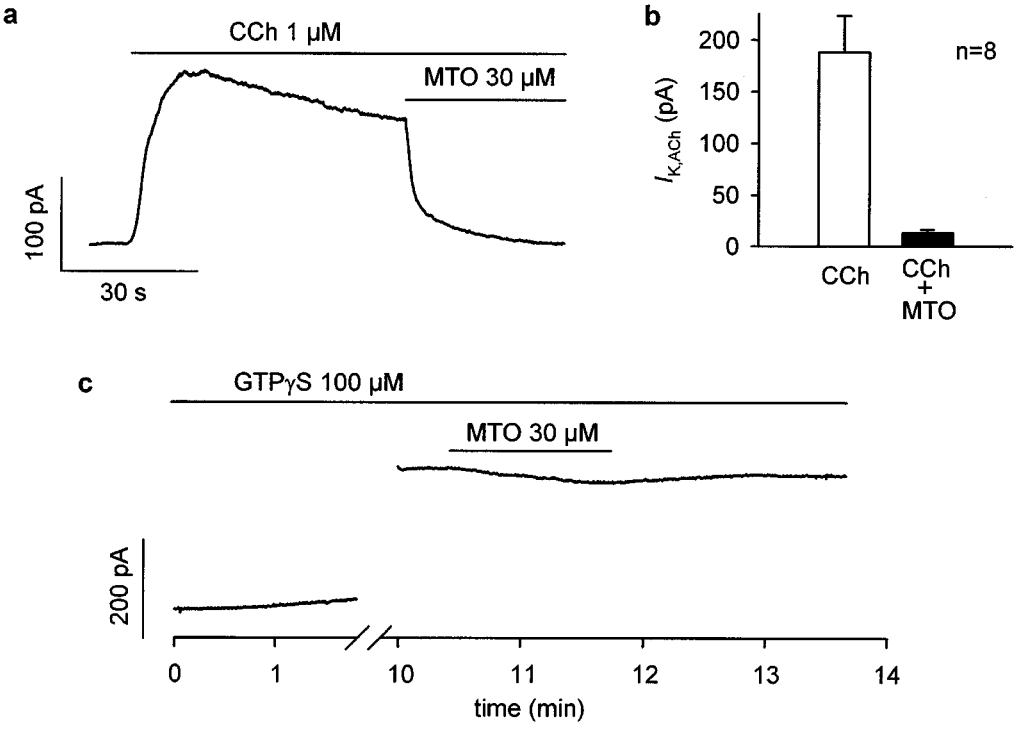

Figure 8 Effect of MTO on $I_{K,ACh}$ of guinea-pig atrial myocytes. The cells were continuously clamped at −50 mV. (a) Typical current recording showing the inhibitory effect of MTO on $I_{K,ACh}$ induced by carbamylcholine (CCh). (b) Summary of the inhibitory effect of 30 μM MTO on $I_{K,ACh}$ induced by 1 μM CCh. Data from the same cell group are compared. (c) Typical recording showing the effect of MTO on $I_{K,ACh}$ activated by loading the cell with GTPγS through a patch pipette.

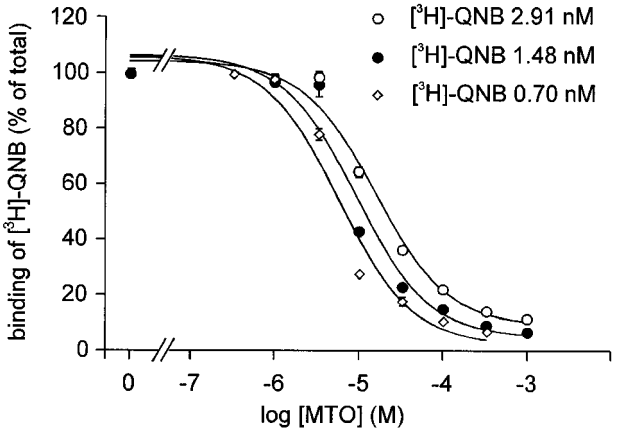

Figure 9 Displacement of specific [^{3}H]-QNB binding by MTO. MTO was added at concentrations ranging from 0.1–1000 μM in the presence of three different [^{3}H]-QNB concentrations. Fitted curves are representative of two independent experiments carried out in triplicate. Correlation coefficients of the fits are 0.99, 0.96 and 0.97 in the presence of 0.7, 1.48 and 2.91 nM [^{3}H]-QNB, respectively.

carbamylcholine induced within a few seconds a marked depression of $I_{K,ACh}$ (Figure 8a),. In eight cells, 1 μM carbamylcholine evoked an $I_{K,ACh}$ which amounted after 1 min to 188.3±35.0 pA and was markedly depressed to 13.3±3.5 pA, i.e. by 93%, when 30 μM MTO was added. Muscarinic receptors couple with K_{ACh} channels through a pertussis toxin-sensitive class of GTP-binding proteins in atrial cells (Pfaffinger *et al.*, 1985). When a nonhydrolysable GTP analogue such as GTPγS is applied intracellularly, activation of GTP-binding proteins occurs and induces a persistent activation of K_{ACh} channels which is resistant to inhibition by muscarinic receptor antagonists (Breitwieser & Szabo, 1985). With 100 μM GTPγS in the recording pipette, there was a continuous rise of a K^+ outward current in guinea-pig atrial cells clamped at −50 mV (Figure 8c). This current reached its maximum in 10 min and remained stable thereafter. MTO induced only a very weak (9%) but reversible inhibition of the current evoked by GTPγS. In five cells loaded with GTPγS, the K^+ current increased to 361.4±29.3 pA and 30 μM MTO reversed this current to 314.4±47.5 pA, i.e. by 12.6%. Although the experiment clearly shows that the predominant action of MTO on $I_{K,ACh}$ is *via* muscarinic receptors, a small direct inhibitory effect on K_{ACh} channels seems to contribute.

[^{3}H]-QNB binding study

Binding of [^{3}H]-QNB to two preparations of human atrial membranes was saturable between 500 and 1000 pM. Scatchard analysis indicated a homogenous population of binding sites, a B_{max} of 218 and 434 fmol mg^{-1} protein and a K_D of 104 and 234 pM, respectively. In the presence of 30 μM MTO, binding of [^{3}H]-QNB was almost completely abolished (not shown). MTO concentration-dependently displaced [^{3}H]-QNB from its binding sites with calculated equilibrium dissociation constants (K_i) of 1.29, 1.10 and 1.02 μM at 0.70, 1.48 and 2.91 nM [^{3}H]-QNB, respectively (Figure 9).

Discussion

The present study describes the acute effects of the anticancer drug MTO on the membrane electrical properties and the muscarinic receptor of isolated cardiac preparations.

The most prominent effect of MTO was a prolongation of APD which occurred in multicellular preparations as well as in

single myocytes. Prolongation of APD might have arisen from the elevation of depolarizing plateau currents, but it appears that enhancement by MTO either of fast Na^+ or of Ca^{2+} currents is not involved, since prolongation of APD persisted in the presence of tetrodotoxin and $I_{Ca(L)}$ remained unaffected in voltage-clamp experiments.

Delayed rectifier potassium channels play a key role in regulating cardiac APD. In guinea-pig ventricular myocytes, I_K is composed of two components, a rapidly activating, inwardly rectifying component, I_{Kr}, and a slowly activating component, I_{Ks} (Sanguinetti & Jurkiewicz, 1990a). At normal heart rates, both components of I_K seem to contribute to repolarization of the cardiac action potentials (Sanguinetti & Jurkiewicz, 1990a; Heath & Terrar, 1996). I_{Ks} has been shown to be more dominant than I_{Kr} during the plateau phase of action potential, whereas I_{Kr} is more important than I_{Ks} during phase 3 of the action potential (Sanguinetti & Jurkiewicz, 1990a; Zeng *et al*., 1995). Although I_{Kr} is the target of most clinically used class III antiarrhythmic drugs (Sanguinetti & Jurkiewicz, 1990a; Katritsis & Camm, 1993), selective blockers of the I_{Ks} channel complex have been developed recently and their APD prolonging effect has been clearly demonstrated (Busch *et al*., 1996; Salata *et al*., 1996; Schreieck *et al*., 1997). Although both components of I_K were present in most of our experiments, some of the findings are compatible with the view that MTO preferentially blocked I_{Kr}. Because I_{Kr}, by very fast inactivation of channels from open state (Shibasaki, 1987; Spector *et al*., 1996; Yang *et al*., 1997), exhibits inward rectification at positive potentials (0 to +40 mV), a specific I_{Kr} blocker should have no effect on time-dependent outward currents elicited at voltages above +40 mV when I_{Kr} is almost completely inactivated (Sanguinetti & Jurkiewicz, 1990a). This is exactly what was found with MTO, the time-dependent outward current measured at the end of 1 s pulses was only suppressed at voltages during which I_{Kr} was measurably activated (see Figure 2). Analysis of activation of I_K is difficult because of additional currents which may be simultaneously activated by depolarizing pulses. Upon repolarization from activating voltage steps, an outward tail current is observed which is thought to represent the slow deactivation of I_K and serves as a more reliable index of the I_K current (Heath & Terrar, 1996). With repolarizing steps, inactivated channels conducting I_{Kr} rapidly enter the open state, from which channels close slowly, thereby contributing to the peak and the decay of I_K tail currents also at very positive potentials (Shibasaki, 1987). Because I_{Ks} slowly activates with depolarization over a time course of many seconds, its contribution to I_K tail current increases over time and should therefore render peak I_K tail current more resistant to blockers of I_{Kr} at longer pulse durations. MTO suppressed I_K tail currents at all voltages (−30 to +70 mV) and, consistent with I_{Kr} block, suppression decreased at voltages above +20 mV and this decrease was more pronounced with long (1 s) as compared with short pulses (250 ms). Although the above results strongly suggested that MTO preferentially inhibited I_{Kr}, direct proof for this inhibition came from experiments carried out in nominally Ca^{2+}-free bath solution. It has been demonstrated that under this condition, I_{Kr} could be separated from I_{Ks} because the activating potentials of both currents were shifted in opposite direction (Sanguinetti & Jurkiewicz, 1990b; Jurkiewicz & Sanguinetti, 1993). When I_{Kr} was elicited by a 500-ms depolarizing pulse to −10 mV from a holding potential of −40 mV, MTO significantly inhibited both the steady-state- and the tail current. Specific blockers of I_{Kr} such as d-sotalol or dofetilide have been reported to display reverse rate-dependent effects, i.e., they prolong APD more at long than at short cycle lengths (Tande *et al*., 1990; Gwilt *et al*., 1991; Jurkiewicz & Sanguinetti, 1993; Gjini *et al*., 1996). In the present study we found that MTO also displayed reverse rate-dependence, its APD prolonging action in guinea-pig papillary muscle was almost completely abolished by increasing the stimulation frequency to 4 Hz. In this context, it is interesting to note that specific inhibitors of I_{Ks} and unspecific blockers of I_K such as ambasilide or amiodarone produced frquency-independent effects on APD (Bosch *et al*., 1997; Salata *et al*., 1996; Schreieck *et al*., 1997), indicating that reverse rate-dependence of APD is a characteristic feature of pure I_{Kr} blocking drugs. There are several explanations for reverse rate-dependence such as accumulation of I_{Ks} at high frequencies when cycle length becomes shorter than I_{Ks} deactivation time (Sanguinetti & Jurkiewicz, 1990a; 1993), $[Ca^{2+}]_i$-dependent increase in I_{Ks} at high stimulation rates (Hiraoka *et al*., 1995) and a decreased sensitivity of I_{Kr} at high $[K^+]_o$, due to accumulation of K^+ close to the outer cell membrane during rapid stimulation (Attwell *et al*., 1981; Yang & Roden, 1996). It should be noted, however, that none of these mechanisms has been firmly established as the cause of reverse rate-dependence of APD by I_{Kr} blockers.

The observation that MTO did not alter resting membrane potential does not by itself exclude an inhibitory effect on I_{K1} because only a fraction of the normal I_{K1} is required to maintain the membrane potential near E_K during diastole. Indeed, current-voltage relations disclosed a significant reduction by MTO of the outward current hump observed at potentials between −70 and −30 mV. This effect was probably due to inhibition of I_{K1} because this current is primarily activated over this voltage range in guinea-pig ventricular myocytes (Backx & Marban, 1993), and MTO had no influence on the residual background currents when I_{K1} was blocked by 1 mM $BaCl_2$. During the plateau of the cardiac action potential, channels conducting I_{K1} allow either no or very little current to pass. However, when the cell repolarizes and especially during the final repolarization, I_{K1} becomes important and is responsible for the regenerative increase in repolarization rate (Shimoni *et al*., 1992; Zeng *et al*., 1995). Whether blockade of I_{K1} induces action potential prolongation is an unsettled issue because 'pure' I_{K1} blockers such as the benzopyran compounds RP 58866 and its active enantiomer terikalant, turned out to be also potent blockers of I_{Kr} (Jurkiewicz *et al*., 1996). Interestingly, in the rat cardiomyocyte where rapid repolarization of action potential is due to activation of the transient outward current, I_{to}, (Josephson *et al*., 1984; Dukes & Morad, 1991) MTO had no influence on APD as long as this outward current was dominant. During late repolarization, however, when I_{K1} becomes activated, MTO markedly delayed final repolarization which eventually resulted in early afterdepolrizations. To what extent inhibition of I_{K1} by MTO contributes to APD prolongation in guinea-pig myocytes is presently not known.

A striking property of I_{Kr} is its unusual sensitivity to changes in $[K^+]_o$ (Yang & Roden, 1996; Yang *et al*., 1997). Thus I_{Kr} plays a major role causing shortening of APD when $[K^+]_o$ is elevated or, conversely, prolongation of APD when $[K^+]_o$ is reduced. Accumulation of $[K^+]_o$ in extracellular clefts of contracting guinea-pig papillary muscles may therefore be responsible for the smaller effect of MTO on APD in multicellular preparations as compared with single cells (see Figures 1 and 5). APD prolongation by block of I_{Kr} is an important factor in arrhythmogenesis (for review, see Sanguinetti & Salata, 1996). Although early afterdepolarizations by MTO were only observed in single cardiomyocytes, I_{Kr} block may also cause undesirable proarrhythmic effects in multicellular heart preparations, especially when $[K^+]_o$ is low.

Normally the heart is under the steady control of the sympathetic and parasympathetic autonomic nervous system. Vagal activation inhibits release and antagonises the effects of released catecholamines on force of contraction, heart rate and AV-conduction. One important mechanism by which catecholamines and acetylchline exert their antagonistic effects is by modulating $I_{Ca(L)}$. Although MTO had no direct effect on $I_{Ca(L)}$, it influenced the current indirectly by reversing the inhibitory action of carbamylcholine on isoprenaline-stimulated $I_{Ca(L)}$. This effect could have been due to inhibition of muscarinic receptors or to a mechanism downstream to receptor activation. The A_1-adenosine receptor agonist R-PIA utilizes the same pertussis toxin-sensitive intracellular signal transduction pathway as carbamylcholine to inhibit the catecholamine-enhanced $I_{Ca(L)}$ (for review see Belardinelli *et al*., 1989). Since MTO did not affect $I_{Ca(L)}$ in the presence of R-PIA plus isoprenaline, inhibition of muscarinic receptors was very likely the mechanism of MTO's indirect effect on $I_{Ca(L)}$. In atrial cells as well as in sinoatrial- and atrioventricular node, activation of K_{ACh} channels by an increased vagal tone plays an important role in slowing sinus rate, prolonging A-V conduction and shortening atrial refractoriness. K_{ACh} channels are linked to muscarinic or adenosine receptors through pertussis-toxin sensitive GTP-binding proteins (Pfaffinger *et al*., 1985) and it has been shown that GTPγS, a nonhydrolysable GTP analogue, can activate GTP-binding proteins irreversibly, resulting in persistent activation of K_{ACh} channels (Kurachi *et al*., 1986). MTO (30 μM) decreased the carbamylcholine-activated $I_{K,ACh}$ in control cells by more than 90% but reversed the $I_{K,ACh}$ activated by GTPγS by only 12%. This finding suggests that MTO may act primarily through its blocking action on muscarinic receptors and only to a small extent by blocking the K_{ACh} channel itself and/or by interfering with the regulatory functions of GTP-binding proteins coupled to the channel. The blocking effect of MTO on cardiac muscarinic receptors was further confirmed by radioligand binding experiments which showed that MTO displaced [^{3}H]-QNB binding concentration-dependently (1–1000 μM). The slightly different K_i values obtained at various [^{3}H]-QNB concentrations indicate that this antagonistic effect was not strictly competitive. The heart rate is normally under tonic parasympathetic control. Thus administration of MTO which blocks the muscarinic receptors of the parasympathetic transmitter acetylcholine, can be expected to raise the heart rate. This effect should on the one hand alleviate APD prolongation of ventricular myocytes due to reverse rate-dependence but could on the other hand induce early afterdepolarizations due to the dominance of catecholamines released from sympathetic nerve endings.

In summary, MTO is a potent blocker of inward rectifier- and of delayed rectifier K^+ channels. The resulting APD prolongation might cause undesirable proarrhythmic effects which may be alleviated or accentuated by the anticholinergic action of MTO.

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