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Membrane impermeant antioestrogens discriminate between ligand- and voltage-gated cation channels in NG108-15 cells

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Abstract

Native 5-HT_3 and AChR ligand-gated cation channels can be inhibited (blocked) by the non-steroidal antioestrogen tamoxifen. However, the exact site and mechanism of inhibition by tamoxifen on these channels remain unclear. We have investigated the action of the membrane impermeant quaternary derivative, ethylbromide tamoxifen (EBT), on native ligand-gated 5-HT_3 receptor channels and voltage-gated K^+ channels in NG108-15 cells using whole cell patch clamp. Extracellular EBT inhibited whole cell cationic currents of 5-HT_3 receptors with IC_{50} of 0.22 ± 0.4 μM ($n_H = 1.05 \pm 0.2$). The channel block was characterised by voltage independent and use independent behaviour (similar to that of tamoxifen). EBT was unable to inhibit voltage-gated K^+ currents in NG108-15 cells. This was in contrast to the inhibition by tamoxifen which, at similar concentrations, accelerated the apparent inactivation of these outward K^+ currents. The inhibition of 5-HT_3 receptors by a membrane impermeant derivative of tamoxifen supports the view that the binding site for antioestrogens is extracellular and the inhibition is not mediated through genomic/transcriptional activity.

Keywords: 5-HT_3 receptor; Tamoxifen; Ethylbromide tamoxifen; Potassium current

1. Introduction

In addition to antioestrogenic activity, tamoxifen has been shown to interfere with a number of cellular targets including calmodulin and protein kinase C activity [1,2]. Several groups have shown that tamoxifen is able to inhibit various plasma membrane ion channels [3–7]. We previously described the action of tamoxifen on the ligand-gated 5-HT_3 and AChR channels [7]. The inhibition of the 5-HT_3 receptor currents was consistent with a non-competitive mechanism of block but the site could not be determined. It was postulated that non-steroidal antioestrogens interact directly with allosteric sites on ion channels [7].

To identify the site of action of non-steroidal antioestrogens we have investigated the channel blocking properties of tamoxifen and the membrane impermeant derivative ethylbromide tamoxifen (EBT). Both EBT and tamoxifen are oestrogen-receptor antagonists but the quaternary derivative (EBT) is unable to cross cell membranes and bind to intracellular targets, consequently tamoxifen but not EBT can inhibit oestrogen dependent proliferation in MCF-7 cells [8]. Membrane impermeant quaternary compounds are used to determine the site and mechanism of channel block [9]. In this study we investigate the action of tamoxifen and a quaternary derivative on

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voltage-gated and ligand-gated ion channel activity in the same cell type.

2. Materials and methods

2.1. Culture of neuroblastoma×glioma hybrid cells

Mouse neuroblastoma×rat glioma hybrid cells, NG108-15, were grown in Dulbecco's modified Eagle's medium (DMEM+Glutamax) supplemented with 10% foetal calf serum, 2% hypoxanthine, aminopterin and thymidine (HAT) supplement and 50 μg/ml gentamicin. The undifferentiated cells were plated at a density of approx. 5×10^4 cells per 35 mm diameter culture dish at least 24 h before use.

2.2. Whole cell patch clamp recording

Agonist activated macroscopic currents were recorded from single cells by whole-cell patch clamp techniques as described previously [7]. NG108-15 cells were continuously superfused with an extracellular solution containing (in mM): 136 NaCl, 2.6 $CaCl_2$, 2.4 KCl, 1.2 $MgCl_2$, 15 HEPES, 10 glucose, pH 7.4 at 23±2°C. Patch electrodes were filled with a solution containing (in mM): 110 KCl, 3.0 $MgCl_2$, 40 HEPES, 3 EGTA (titrated with KOH to pH 7.4) [10].

2.3. Agonist application

Agonist induced currents were recorded from NG108-15 cells voltage clamped at a membrane potential of −80 mV. The cell being recorded from was continuously superfused with extracellular bathing solution as described previously.

Application of agonist was for 5 s and the applications were repeated every 90 s to avoid desensitisation using a fast-step motored perfusion system (SF-77 Warner Instruments) [7]. Raw output signals from the patch clamp amplifier were filtered with a low bandpass filter at 5 kHz, and digitised at 1 kHz. Macroscopic currents recorded in response to the application of 5-HT were analysed off line. Records were not leak subtracted. The decaying phases of current responses during desensitisation were fitted to the sum of two exponential functions by minimisation of the sum of squared deviations (WCP v1.6; Strathclyde Electrophysiological Software).

2.4. Data analysis

Agonist responses from each cell were normalised to the 10 μM 5-HT response. Concentration-inhibition curves were fitted iteratively using Fig P software (Biosoft, Cambridge) to the following equation:

$$I = 1-I_{max}/(1 + ((IC_{50})/[A])^{n_H})) \quad (1)$$

where [A] is the concentration of antagonist, I_{max} is the maximum normalised current response (in the absence of antagonists), IC_{50} is the concentration of antagonist to produce 50% inhibition and n_H is the Hill coefficient.

2.5. Voltage-gated potassium channels

Voltage-gated potassium channels were recorded from undifferentiated NG108-15 cells clamped at a holding potential of −80 mV and outward K^+ currents were evoked by stepwise changes in membrane potential lasting for 0.5 or 5 s. Raw output currents were filtered with a low bandpass filter at 5 kHz, and digitised at 2 kHz using a CED 1401 interface connected to a PC 486 computer running WCP v1.6 patch clamp software.

2.6. Drugs

Tamoxifen base was obtained from Sigma-Aldrich and EBT was synthesised as described below based on a method of Jarman [8].

2.6.1. Quaternisation of tamoxifen

Tamoxifen (1 g) was added to ethylbromide (35 ml) and stirred overnight at room temperature. The reaction mixture was filtered and the white solid collected was crystallised from ethanol in toluene as colourless needles to give the quaternary salt m.p. 166–168°C ([8], 158–159°C); (found C, 67.59; H, 7.27; N, 2.80 $C_{28}H_{34}ONBr.\ H_2O$ calc. for C, 67.72; H, 7.10; N, 2.75); δ_H (360 MHz; $CDCl_3$): 0.94 (3H, t, *J* 7.4 Hz, $C\underline{H}_3CH_2C$), 1.41 (3H, t, *J* 7.4 Hz, CH_3CH_2N), 2.47 (2H, q, *J* 7.4 Hz, $CH_3C\underline{H}_2C$), 3.42 [6H, s, $N(CH_3)_2$] 3.77 (2H, q, $CH_3C\underline{H}_2N$), 6.55 (2H, Abq, *J* 8.5 Hz, $C_6H_4OCH_2$, *ortho* H),

6.83 (2H, Abq, J 8.5 Hz, $C_6H_4OCH_2$, *meta* H) 7.11–7.39 (10H, m, 2Ph). The ^1H NMR spectrum was determined in deuterochloroform with tetramethylsilane as an internal standard, at 360 MHz with a Bruker WM 360 spectrometer. The melting point was determined on an electrothermal melting point apparatus and is uncorrected for temperature and pressure. The purity of EBT was greater than 98% as determined by elemental analysis. All of the chemicals used were obtained from Aldrich.

Tamoxifen and EBT stock solutions were dissolved in dimethyl sulphoxide at a concentration of 10 mM, and subsequently diluted in extracellular solution to give final concentrations of 0.01–10 μM. The concentration of dimethyl sulphoxide in the perfusion medium did not exceed 0.1%: these concentrations had no significant effect on 5-HT gated currents in NG108-15 cells expressing native 5-HT_3 receptors and delayed rectifier type potassium currents.

2.7. Statistics

Values reported are mean ± S.E.M. Mean values were compared using paired and unpaired two tailed t-tests as appropriate. $P < 0.05$ was considered sig-

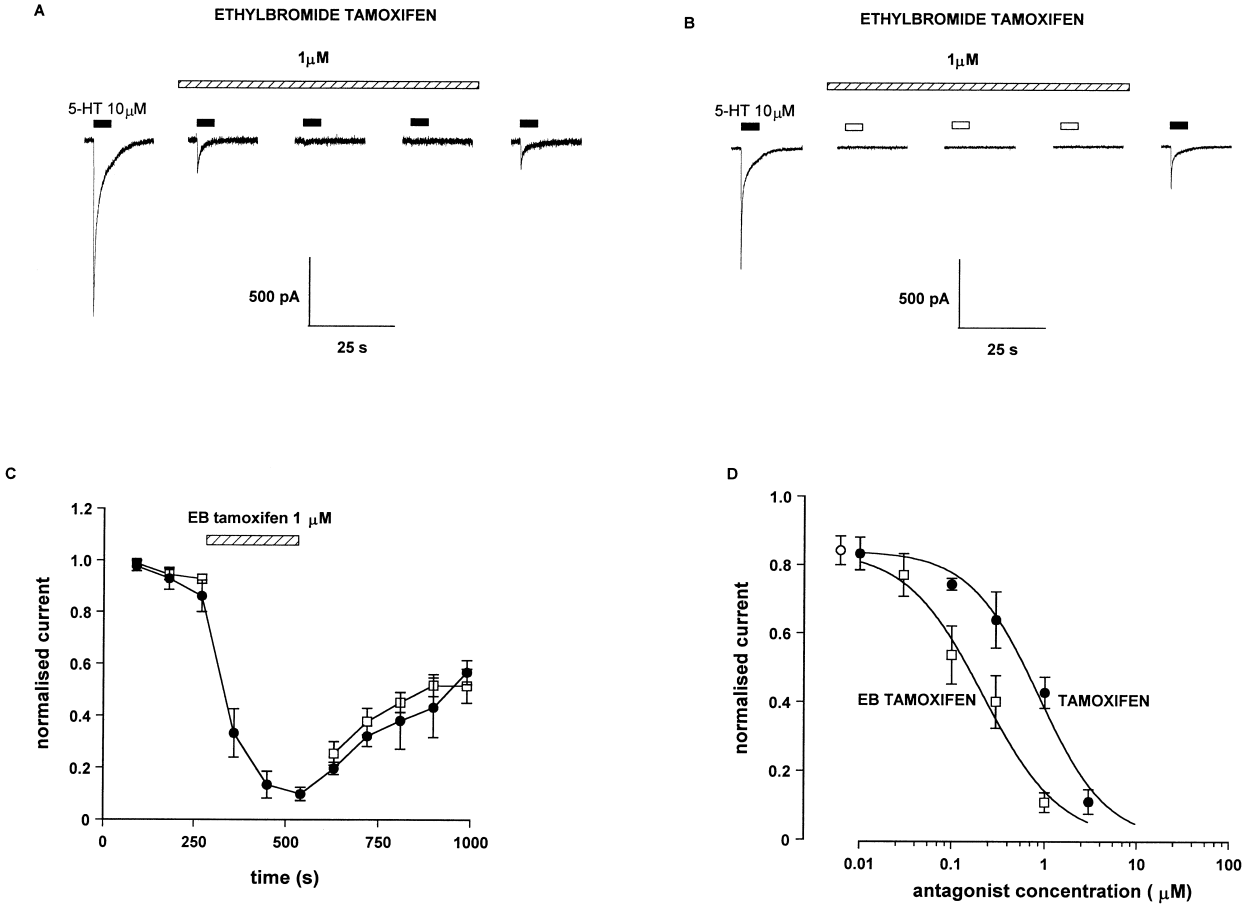


Fig. 1. Inhibition of 5-HT responses by non-steroidal antioestrogens. (A) Inhibition of responses to brief application of 5-HT (10 μM, black bars, ■) during and after exposure to EBT (1 μM) for 4.5 min (hatched bar). (B) Inhibition of 5-HT (10 μM, ■) responses after exposure to EBT (1 μM, 4.5 min, hatched bar) with sham exposure of 5-HT (□). (C) Summary of data obtained in A (●) and B (□) during exposure to EBT ($n = 4$ cells for both conditions). (D) Concentration-inhibition curve. The IC_{50} was 0.81 ± 0.2 μM, $n_H = 1.3 \pm 0.3$ for tamoxifen (●) and 0.22 ± 0.4 μM, $n_H = 1.05 \pm 0.2$ for EBT (□). Each point represents the data obtained from four individual cells. Each cell was exposed to only one concentration of tamoxifen. The open circle (○) represents the currents obtained by the vehicle control (DMSO) at the highest concentration used. DMSO did not significantly reduce the 5-HT responses ($P > 0.05$).

nificant. Current-voltage (IV) relationships were compared using two-way analysis of variance with replication.

3. Results

3.1. Inhibition of 5-HT_3 receptor currents by EBT

A typical trace of the currents evoked in NG108-15 cells by a brief (5 s) exposure to 10 μM 5-HT is shown in Fig. 1. Currents induced by 10 μM 5-HT were blocked by 10 nM *d*-tubocurarine and tropisetron (10 nM) as reported previously [7]. Likewise, we previously demonstrated that these currents are also blocked by tamoxifen in a concentration dependent, non-competitive manner [7]. Fig. 1A shows that 1 μM EBT also completely inhibits the 5-HT induced current by the third application of 5-HT (representing 250 s exposure to EBT). This inhibition was concentration dependent, with an IC_{50} of 0.22 ± 0.38 μM and had a Hill coefficient of 1.1 ± 0.2 (Fig. 1D). The inhibition at low concentrations (≤ 1 μM) could be reversed but at higher concentrations reversibility was incomplete during the course of the experiment.

3.2. Absence of use dependence of block of 5-HT_3 receptors by EBT

A feature of inhibition of 5-HT currents by tamoxifen is the lack of use dependent blockade [7]. Exposure to EBT (1 μM) progressively inhibited the responses to 5-HT (10 μM) with steady state inhibition obtained after two applications of 5-HT (Fig. 1A,C). Fig. 1B shows a control experiment with sham application of 5-HT (extracellular solution alone) during exposure to 1 μM EBT. After three sham applications of the agonist the EBT was removed and recovery from blockade followed; it can be seen that the inhibition of 5-HT evoked currents was similar to that obtained in Fig. 1A. Fig. 1C shows the inhibition of the currents by EBT, which appears time dependent rather than use dependent as exposure to EBT alone can inhibit subsequent 5-HT responses. Recovery from EBT (1 μM) during these long experiments was incomplete during the 5 min washout.

3.3. Lack of voltage dependence of block of 5-HT_3 receptor currents by EBT

Previously we showed that tamoxifen blocks 5-HT responses in a voltage independent manner [7]. Consequently, the inhibitory effect of 0.1 μM EBT on 5-HT responses at a range of holding potentials was examined. The inhibition observed did not appear to be voltage dependent over the range of −80 to +40 mV as illustrated by the representative traces in Fig. 2A. The mean current-voltage relationship with and without EBT is shown in Fig. 2B, and proportional reductions of current at different holding potentials are shown in Fig. 2C. The slope of the line was not significantly different from zero ($P > 0.05$).

3.4. The effect of EBT on 5-HT_3 receptor desensitisation

Other non-competitive inhibitors of 5-HT_3 receptors in neuronal cell lines have been shown to increase the rate of desensitisation [7,11,12]. The effect of EBT on the 5-HT evoked currents was investigated accordingly. After an initial period of stabilisation upon achieving whole cell recording conditions, stable 5-HT responses could be recorded and the desensitisation of the inward current could be described by a bi-exponential decay process (Fig. 2D). The time course of desensitisation was quantified by curve fitting of the decaying phases of responses to the sum of two exponentials. Under control conditions the kinetic parameters obtained from the double exponential fits gave a fast time constant (τ_1) of 595 ± 150 ms and a slow time constant (τ_2) of 5.74 ± 1.7 s ($n = 5$). The mean values for the fast desensitisation component were significantly reduced following exposure to 100 nM EBT for 270 s ($\tau_1 = 326 \pm 65$ ms, $n = 5$, $P < 0.05$ versus control); the mean values for the slow desensitisation component were unaffected by exposure to 100 nM EBT for 270 s ($\tau_2 = 4.0 \pm 1.3$ s, $n = 5$; $P > 0.05$). EBT, like tamoxifen, did not significantly affect the rise time (10–90%) of the response to 5-HT (control = 135 ± 34 ms; EBT = 131 ± 50 ms; $n = 5$; $P > 0.05$).

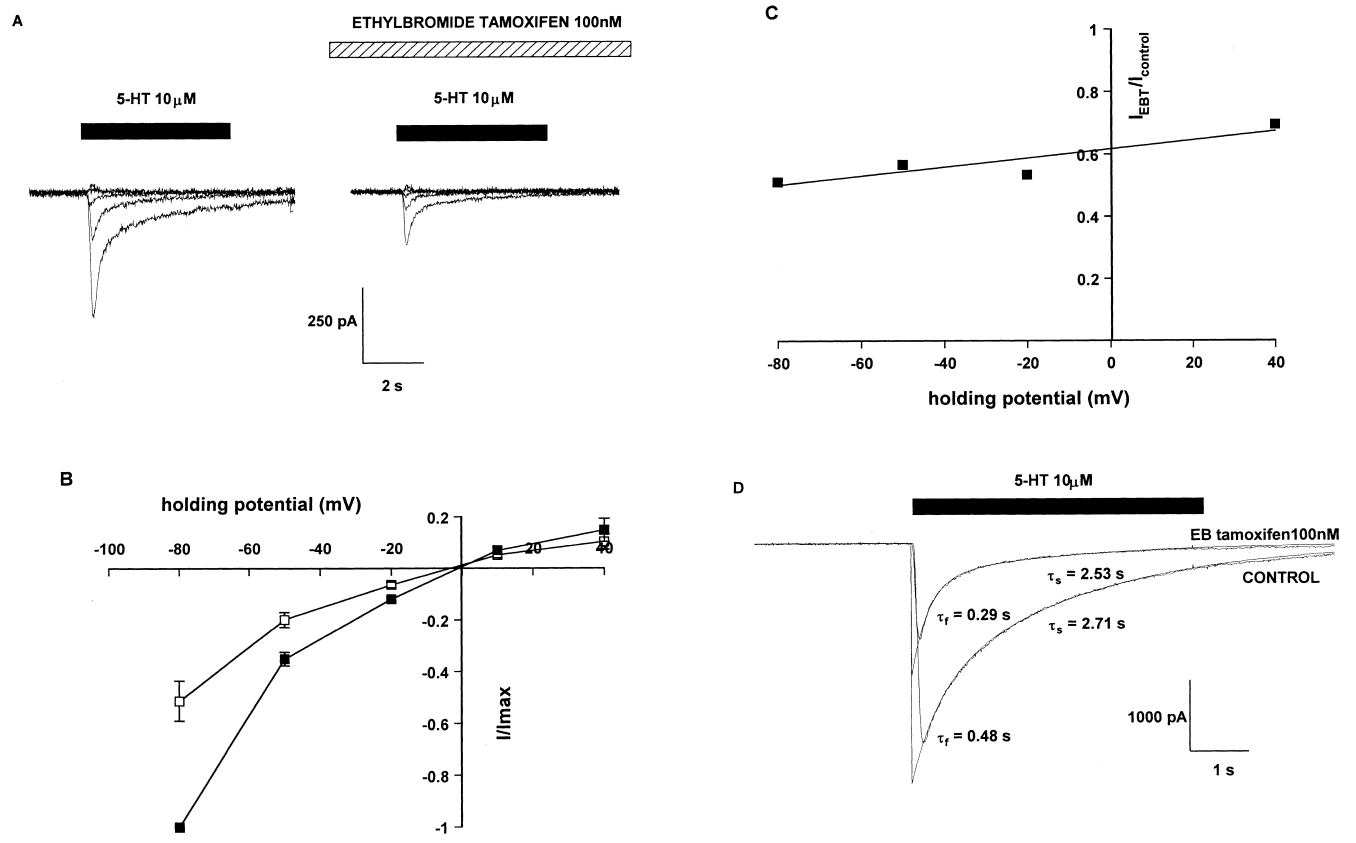
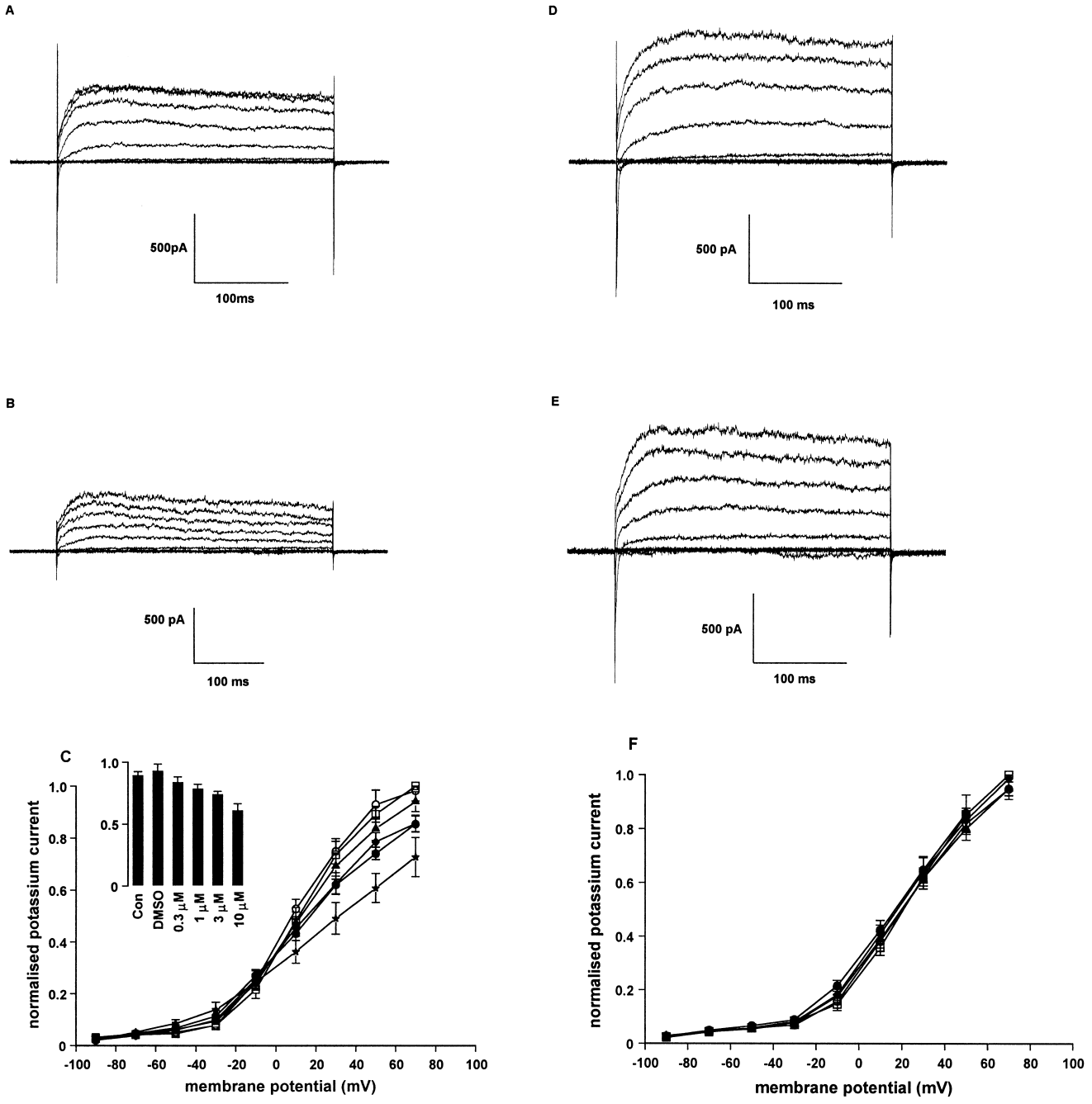


Fig. 2. Lack of effect of EBT on the voltage dependence of 5-HT induced currents. (A) Representative traces of currents evoked by 10 μM 5-HT at different holding potentials in one cell before, and during a 10 min application of 100 nM EBT. (B) 5-HT response-amplitude plotted as a function of membrane potential before (■) and during (□) EBT (100 nM) application ($n=5$). (C) The ratio of the mean currents shown in B are plotted against holding potential and illustrate the lack of voltage dependent inhibition (■) by EBT (100 nM). (D) Representative current responses evoked by 10 μM 5-HT in the presence (upper trace) and absence (lower trace) of 100 nM EBT. The decaying phase was fitted to the sum of two exponentials and the time constants for both conditions are shown. Tamoxifen inhibited the peak response and increased the desensitisation rate.

3.5. *The effect of tamoxifen and EBT on voltage-gated potassium channels in NG108-15 cells*

Tamoxifen is known to inhibit delayed rectifier type potassium channels and this inhibition has been demonstrated in several cell types [6,13]. We have examined the effects of both tamoxifen and EBT on voltage-gated potassium channels in NG108-15 cells (Fig. 3). Tamoxifen (0.1–10 μM) slowly (>2 min) inhibited voltage-gated potassium currents in a concentration dependent manner (Fig. 3A,B, data summarised in Fig. 3C). EBT (0.1–3 μM), however, was without effect on these currents at concentrations of EBT up to 5 μM (Fig. 3D,E, data summarised in Fig. 3F). The inactivation rates of the potassium currents were significantly increased by tamoxifen (control = 1872 ± 245 ms; tamoxifen = 643 ± 120 ms; $n=6$; $P<0.01$), whereas there was no significant effect with EBT (control = 1895 ± 115 ms; EBT = 1734 ± 72 ms; $n=5$; $P>0.05$) (Fig. 4).

The lipophilicity of tamoxifen could result in channel block occurring via the cytosolic face of the channel, having diffused through the plasma membrane. However, intracellular application of EBT (via the pipette solution) at 10 μM for up to 15 min had no obvious effect on the size of the currents nor the inactivation rates ($n=5$), indicating that antioestrogens do not have to gain access to the cytoplasm in order to inhibit delayed rectifier (DR) type potassium currents.



4. Discussion

Previously, we reported that tamoxifen was able to inhibit 5-HT_3 currents in NG108-15 cells by a non-competitive mechanism [7]. We were unable to distinguish between a direct effect on the channel or an intracellular effect on second messenger systems (such as modulation of protein kinase C [14]). The fact that we have shown that both tamoxifen and the membrane impermeant EBT can inhibit neuronal 5-HT_3 currents at similar concentrations makes it unlikely that non-steroidal antioestrogens inhibit 5-HT_3 channels after crossing the membrane. Again, we cannot exclude the possibility that the impermeant derivative does not trigger a signalling event through binding to a membrane receptor. The data presented

Fig. 3. The effects of antioestrogens on voltage-gated delayed rectifier type potassium currents. The cells were clamped at a V_{hold} −80 mV and 300 ms depolarising steps from −70 to 70 mV were applied every 5 s to evoke delayed rectifier type potassium currents. Control potassium currents are illustrated in A, whilst the effect of 3 min application of 3 μM tamoxifen to the same cell is illustrated in B. These experiments were repeated in four different cells for each concentration of tamoxifen investigated and the results summarised in C. C shows a normalised peak current-voltage (IV) relationship under control conditions and in the presence of differing concentrations of tamoxifen ($n=4$): □, in HEPES Ringer; ○, vehicle control; ▲, tamoxifen 0.3 μM; ♦, tamoxifen 1 μM; ●, tamoxifen 3 μM; ★, tamoxifen 10 μM. The DMSO vehicle control and tamoxifen 0.3 μM did not significantly inhibit the voltage activated currents, but there was a significant inhibition with tamoxifen 1 μM, 3 μM and 10 μM tamoxifen ($P<0.01$). The insert illustrates the inhibition of DR currents by increasing concentrations of tamoxifen. Mean values were taken from currents evoked at +50 mV. Control potassium currents are illustrated in D, whilst the effect of 3 min application of 3 μM EBT to the same cell is illustrated in E. These experiments were repeated in seven different cells for each concentration of EBT investigated and the results summarised in F. F shows a normalised peak current-voltage (IV) relationship under control conditions or in the presence of differing concentrations of EBT ($n=7$): □, in HEPES Ringer; ○, vehicle control; ▲, EBT 0.3 μM; ♦, EBT 1 μM; ●, EBT 3 μM. The DMSO vehicle control and all concentrations of EBT did not significantly inhibit the voltage activated currents ($P>0.05$).
←

here do exclude a direct blocking effect on the cytosolic face of the membrane.

The inhibition curves for tamoxifen and EBT gave similar Hill coefficients of approx. 1 for the 5-HT_3 receptors which indicates a single binding site on the channel. As we have shown that tamoxifen exhibits time dependent and not use dependent block, we suspected that the inhibitory site on the channel was accessed from within the lipid bilayer [7]. EBT, however, is unlikely to fully partition into a lipid bilayer because of its positively charged quaternary group. Consequently, the binding site for non-steroidal antioestrogens is most likely on the extracellular domain of the channel rather than the intracellular or hydrophobic membrane spanning region.

The effect of tamoxifen on DR type potassium currents is to reduce peak outward current and accelerate the inactivation rate whereas intracellular or extracellular application of EBT was without detectable effect. Small hydrophilic quaternary ammonium compounds have been shown to accelerate the inactivation rate of DR currents with minimal effect on rise times and peak currents [9]. The results obtained with EBT do not follow this pattern but instead indicate that the site of action of these antioestrogens on the DR type potassium channel is within the lipid membrane.

In conclusion, membrane impermeant non-steroidal antioestrogens inhibit 5-HT_3 receptors but not delayed rectifier type potassium currents in NG108-15 cells. The inhibition of 5-HT_3 receptors appears to be non-competitive, voltage independent and use independent. Hill coefficient values indicate that binding is probably to a single extracellular site to which antioestrogens have access while the channel is in the closed conformation. The use of a quaternary tamoxifen in this series of experiments eliminates an intracellular binding location for both 5-HT_3 and DR type potassium channels. The rapid inhibition of the 5-HT_3 receptor currents by EBT is not mediated via nuclear oestrogen receptor binding or direct modulation of intracellular enzymes.

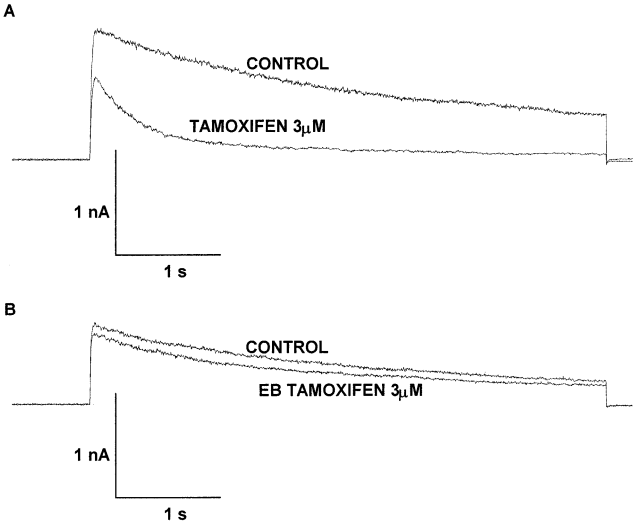


Fig. 4. The effects of antioestrogens on voltage-gated DR type potassium current inactivation. Cells were held at a membrane potential of −80 mV and the potential changed to +60 mV for 5 s to activate the potassium currents. (A) The effects of 3 μM tamoxifen on potassium current inactivation. (B) The effect of 3 μM EBT on potassium current inactivation. A and B were measured after 3 min exposure to antioestrogens.

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References

[1] C.D.M.A. Van Den Koedijk, M.A. Blankenstein, J.H.H. Thijssen, Biochem. Pharmacol. 47 (1994) 1927–1937.
[2] D.J. Grainger, J.C. Metcalfe, Nat. Med. 2 (1996) 381–385.
[3] P. Sartor, P. Vacher, P. Mollard, B. Dufy, Endocrinology 123 (1988) 534–540.
[4] M.A. Valverde, G.M. Mintenig, F.V. Sepulveda, Pflug. Arch. 425 (1993) 552–554.
[5] J.J. Zhang, T.J.C. Jacob, M.A. Valverde, S.P. Hardy, G.M. Mintenig, F.V. Sepulveda, D.R. Gill, S.C. Hyde, A.E.O. Trezise, C.F. Higgins, J. Clin. Invest. 94 (1994) 1690–1697.
[6] S.P. Hardy, C. deFelipe, M.A. Valverde, FEBS Lett. 434 (1998) 236–240.
[7] M.C. Allen, C. Newland, M.A. Valverde, S.P. Hardy, Eur. J. Pharmacol. 354 (1998) 261–269.
[8] M. Jarman, O.T. Leung, G. Leclerq, D. Devleeschouer, S. Stoessel, R.C. Coombs, R.A. Skilton, Anti-Cancer Drug Des. 1 (1986) 259–268.
[9] B. Hille, in: Ionic Channels of Excitable Membranes, 2nd edn., Sinauer, 1992, Ch. 15.
[10] R.J. Docherty, R. Robbins, D.A. Brown, in: J. Chad, H. Wheal (Eds.), Cellular Neurobiology, IRL Press, Oxford, 1991, pp. 75–95.
[11] P. Fan, Br. J. Pharmacol. 112 (1994) 745–748.
[12] P. Fan, Br. J. Pharmacol. 112 (1994) 741–744.
[13] B. Dubois, J.M. Dubois, Cell. Signal. 2 (1990) 387–393.
[14] L. Zhang, M. Oz, F. Weight, Neuroreport 6 (1995) 1336–1340.