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Inhibition of voltage-gated cationic channels in rat embryonic hypothalamic neurones and C1300 neuroblastoma cells by triphenylethylene antioestrogens

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Abstract The effect of the non-steroidal antioestrogens tamoxifen and toremifene on voltage-gated cationic currents was examined in primary cultures of rat hypothalamic neurones and the C1300 mouse neuroblastoma cell line using the whole-cell patch clamp technique. When applied to the external bathing solution both tamoxifen and toremifene were able to inhibit TTX-sensitive sodium currents with IC_{50} values of 1–2 μM and delayed rectifier type potassium currents (IC_{50}, 2–3 μM). However, only toremifene showed a significant inhibition of the I_A current (IC_{50} 3 μM). Inhibition of voltage-gated cationic currents was significantly impaired when tamoxifen was applied in a serum-containing solution. The steroidal antioestrogen ICI 182,780 did not inhibit any of the currents at 10 μM.

Key words: Hypothalamus; Tamoxifen; Toremifene; ICI 182,780; Potassium current; Sodium current

1. Introduction

Tamoxifen is a non-steroidal triphenylethylene antioestrogen [29] which has become a valuable adjunct in the treatment of breast cancer. However, tamoxifen has been reported to inhibit the function of a number of proteins located in the plasma membrane, including multidrug resistance P-glycoprotein [3], protein kinase C [17], calmodulin [9], Na^+/Ca^{2+} exchanger and Ca^{2+}-ATPase [14]. In addition, antioestrogens have been shown to modulate both anionic and cationic membrane conductances in a variety of cell types including potassium currents in neuroblastoma cells [20], Ca^{2+} currents in a pituitary cell line [22] and chloride currents in epithelial cells [24], fibroblasts [10], lens fibres [30] and neuroblastoma cells [4,32].

Tamoxifen and other antioestrogens, such as toremifene [12] and ICI 182,780 [5], are being evaluated for their efficacy as chemoprophylactics for breast cancer in women [12]. Given the diversity of putative cellular targets it is remarkable that the number of clinical side effects reported for antioestrogens is so low [7,26]. In particular, a study of patients on high dose tamoxifen treatment reported reversible neurological toxicity [23].

The purpose of our study was to examine the selectivity of action of non-steroidal antioestrogens on voltage-gated sodium and potassium channels in neurones from the hypothalamus, a region of the brain known to be sensitive to regulation by oestrogens. Preliminary results have been presented previously in abstract form [11].

2. Materials and methods

2.1. Cell culture

The hypothalamic neurones were removed from 15-day rat embryos (Sprague-Dawley) into L-15 medium (Gibco BRL, Paisley, UK). A single cell suspension was obtained by trituration through a fire-polished Pasteur pipette (10–12 passages). Cells were rinsed in MEM without phenol red (Gibco) plus 10% foetal calf serum and 10% v/v horse serum and plated (approx. 2×10^5 cells/ml) onto 35 mm petri dishes precoated with polyornithine (100 μg/ml) and laminin (5 μg/ml). After 24 h cells were bathed in MEM defined medium [1] supplemented with glucose (6%), glutamine (2 mM) and HEPES buffer (5 mM). Cultures were maintained at 37°C in 7.5% CO_2 for up to 7 days. Experiments were performed 5–6 days after plating. Neurones were identified by cell morphology and immunofluorescence staining with neurone-specific MAP2 antibody. In the absence of mitotic inhibitors a mixture of astrocytes, neurones and occasional fibroblasts formed a monolayer of 50–70% confluence within 5 days.

C1300 neuroblastoma cells [27] were maintained in culture using Dulbecco's modified Eagle's medium (DMEM) without phenol red supplemented with 10% (v/v) foetal calf serum and 2 mM L-glutamine. Cells were grown at 37°C in a humidified atmosphere of 5% CO_2 in air. Cells were subcultured into 35 mm culture dishes when confluent and used for patch clamp experiments within 48 h.

2.2. Electrophysiology

The intracellular solution contained (in mM) 140 KCl, 10 NaCl, 1 EGTA, 1.2 $MgCl_2$, 10 HEPES, pH 7.4. and cells were bathed in Hanks' solution containing 140 NaCl, 10 HEPES, 5 KCl, 0.5 $MgCl_2$, 1.2 $CaCl_2$, pH 7.4. Whole-cell patch clamp recordings [8] were carried out at room temperature. Drugs were applied to the bathing solution using a U-tube applicator as described elsewhere [2]. Data were recorded and analysed using VGEN and WCP software (J. Dempster, Strathclyde University, UK). All data were analysed without leak subtraction. Remaining capacitative transients are removed from certain figures to aid clarity. IC_{50} values were taken from the following formula which was used to fit the curves to the inhibition plots: $y=[(a-d)/(1+(X/C)b)]+d$ where y is the percentage of the initial response caused by a blocker at concentration X; d is the estimated maximal inhibition by the blocker; a is the response at $X=0$; C is the concentration of blocker required to cause 50% inhibition and b is the Hill coefficient.

Statistical comparisons were made using paired Student's t-test using $P<0.05$ as significant.

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Abbreviations: 4-AP, 4-aminopyridine; DR, delayed rectifier; EGTA, ethylene glycol-bis-(β-aminoethyl ether) *N*,*N*,*N*′,*N*′-tetraacetic acid; HEPES, *N*-[2-hydroxyethyl] piperazine-*N*′-[2-ethanesulphonic acid]; MEM, minimal essential medium; TEA, tetraethylammonium

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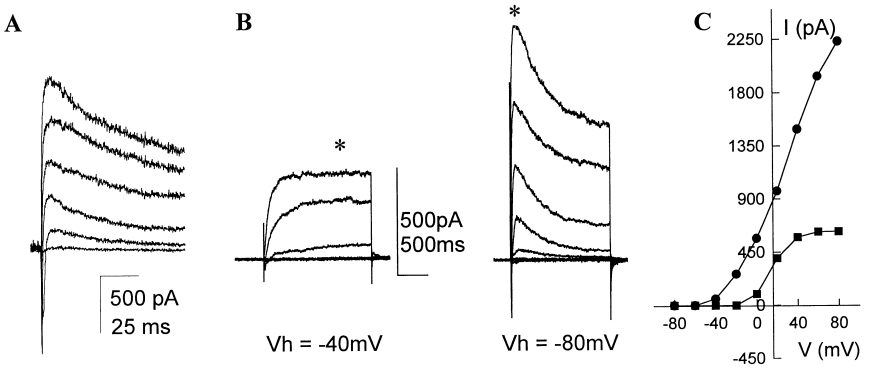

Fig. 1. Voltage-activated currents recorded under whole-cell patch clamp conditions from embryonic rat hypothalamic neurones (E15 days). A: Typical trace of currents elicited by pulsing from −80 mV up to +60 mV in steps of 20 mV. B: Separation of I_A and DR currents. Whole cell currents obtained from a hypothalamic neurone hold at $V_h = -40$ mV (left) or −80 mV (right). C: The corresponding current-voltage (I-V) relationship obtained from the steady-state and peak responses as indicated by * in B. C. ●, $V_h = -80$ mV; ■, $V_h = -40$ mV. Note that the IA currents obtained at −20 mV were uncontaminated by DR currents.

3. Results

3.1. Sodium and potassium currents in embryonic hypothalamic neurones

Fig. 1A illustrates typical Na^+ and K^+ currents in a 15-day embryonic rat hypothalamic neurone recorded using quasi-physiological conditions. The inward current was instantaneously and reversibly blocked by 0.1 μM tetrodotoxin (TTX, $n = 5$; results not shown) without affecting the outward current. The use of 10 mM tetraethylammonium (TEA, $n = 4$) and 2 mM 4-aminopyridine (4-AP, $n = 5$) revealed two potassium currents: a 4-AP-sensitive I_A current and a TEA-sensitive delayed rectifier current (results not shown). The activity of the I_A current could be markedly suppressed by alteration of the holding voltage from −80 mV to −40 mV as shown in Fig. 1B. From the I/V curve in Fig. 1C it can be seen that the peak outward current obtained at −20 mV was exclusively I_A, without activation of DR-like current. The I_A component showed a voltage-independent inactivation time constant of 22 ms and a half-inactivation voltage of −75 mV (results not shown).

3.2. Effect of antioestrogens on the potassium and sodium currents in embryonic hypothalamic neurones

Two non-steroidal antioestrogens, tamoxifen and toremifene, were tested for their ability to block the DR and I_A currents. Fig. 2A shows an example of the effect of tamoxifen (5 μM for 2 min) on Na^+ and K^+ currents recorded in a hypothalamic neurone. Tamoxifen induced a substantial reduction of the steady-state outward current (mainly DR) without a significant decrease in the transient component (I_A). The reduction in current amplitude occurred rapidly upon the addition of the drug (inhibition reaching steady state in 1–3 min) with only partial and delayed recovery of the initial currents following withdrawal of the compounds (results not shown). The peak current was not significantly different from the control current recorded in the absence of tamoxifen. The concentration-inhibition curves for toremifene and tamoxifen obtained at the +20 mV peak and +60 mV steady-state value (monitoring the I_A and DR currents respectively) are given in Fig. 2B,C. Tamoxifen and toremifene both at 5 μM inhibited the steady-state currents (DR) by 74% and 89% respectively but no significant inhibition of the I_A current at +20 mV could be observed by tamoxifen at 1 μM and only by 25% at 5 μM (Fig. 2C). Toremifene, in contrast, was able to reduce the I_A current by 75% at 5 μM (Fig. 2B). The IC_{50} values for toremifene were 3 μM for the +20 mV currents and 1 μM for the +60 mV currents.

The effects of the antioestrogens on the sodium currents are shown in Fig. 3. The I_{Na} current was elicited by a voltage pulse from $V_h = -80$ mV to 0 mV before and after the addition of 0.1 μM toremifene (Fig. 3A). The dose-response curves obtained for tamoxifen and toremifene (Fig. 4B) show that toremifene ($IC_{50} = 1$ μM) was a slightly more potent blocker of the inward sodium current than tamoxifen ($IC_{50} = 2$ μM).

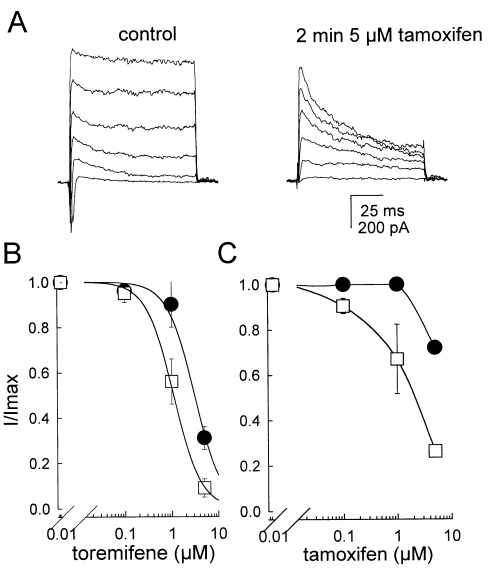

Fig. 2. Effect of tamoxifen on whole-cell currents of hypothalamic neurones. A: Currents recorded using the voltage protocol as given in Fig. 1A in control bathing solution and 2 min after the addition of 5 μM tamoxifen. Note the relative sparing from inhibition of the I_A-like current. B and C: Concentration-response curves for block of outward currents from hypothalamic neurones by (B) toremifene and (C) tamoxifen ($n = 3$ for each point). □, +60 mV steady state; ●, +20 mV, peak.

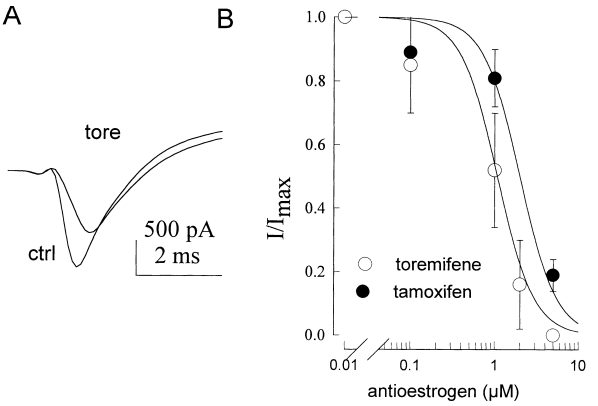

Fig. 3. Effect of antioestrogens on sodium currents. A: A trace obtained by pulsing from −80 mV to 0 mV before and after 2 min in 0.1 μM toremifene. B: Concentration-inhibition curve for the two antioestrogens on the sodium currents. Values were taken after 2 min exposure to the compounds. $N=3$ for each point and only one concentration was tested per cell.

3.3. Effect of antioestrogens in the sodium and potassium currents in C1300 neuroblastoma cells

To examine further the effects of steroidal and non-steroidal antioestrogens on neuronal voltage-gated currents, the C1300 neuroblastoma cell line was chosen. Previous studies [6,15,16] have shown that the outward potassium currents in derivatives of the C1300 neuroblastoma cell line are almost exclusively DR and, thus, would facilitate the analysis of the effect of antioestrogens on DR currents in isolation. Using the same voltage protocols as above, currents could be elicited from the C1300 cells of similar characteristics to the voltage-gated sodium currents observed in the embryonic hypothalamic neurones along with outward currents resembling those carried by DR potassium channels. Inward Na^+ currents were completely blocked by 0.1 μM TTX (data not shown) and outward K^+ currents markedly reduced by 10 mM TEA (results not shown). Both sodium and potassium currents were significantly blocked within 2 min following exposure to extracellular tamoxifen or toremifene (see Fig. 4A). The IC_{50} values obtained for the sodium currents were 4.5 μM for tamoxifen and 2.9 μM for toremifene (Fig. 4B). The IC_{50} value for both of the antioestrogens was 2.2 μM against the potassium currents (Fig. 4C).

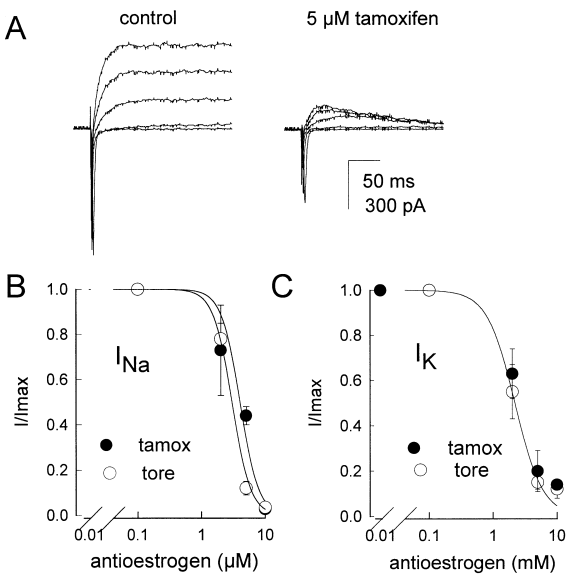

Fig. 4. Effect of antioestrogens on voltage-dependent outward currents in C1300 neuroblastoma cells. A: Representative family of traces showing DR and sodium currents obtained using the voltage protocol given in Fig. 1, before (control) and after exposure to extracellular 5 μM tamoxifen. Concentration-inhibition curves for the inward sodium currents (B) and outward potassium currents (C) in C1300 cells. Values were taken after 2 min exposure to antioestrogen. $N=3$ for each data point and only one concentration was tested per cell.

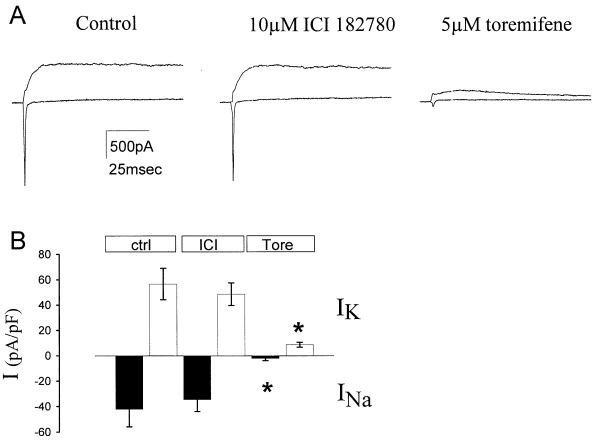

Fig. 5. Effect of steroidal and non-steroidal antioestrogens on C1300 cells. A: A typical current observed in response to voltage steps from V_h −80 to −20 and +60 mV in a C1300 cell. Addition of 10 μM ICI 182,780 to the external bathing solution for up to 3 min had no significant effect on the inward or outward currents whereas subsequent addition of 5 μM toremifene markedly reduced both currents by 2 min. B: Summary of the effects of ICI 182,780 and toremeifene on the I_{Na} and I_K currents in C1300 cells. The mean values (±S.E.M.) are given for the inward sodium currents (black columns, $n=4$) and outward potassium currents (blank columns, $n=4$) in control (ctl) solution, after 3 min in 10 μM ICI 182,780 (ICI) and then, after washing in control solution, subsequent exposure to 5 μM toremifene for 2 min (tore). The asterisk indicates that the values were significantly reduced compared with the controls.

Tamoxifen and toremifene are triphenylethylene antioestrogens that are structurally unrelated to oestrogen. The effect of the steroidal analogue of oestrogen, the 7α-alkylamide derivative ICI 182,780, on the cationic currents in C1300 neuroblastoma cells was also investigated. Neither inward nor outward currents were significantly altered by exposure to 10 μM ICI 182,780 for up to 2–3 min (Fig. 5A). Following 2–3 min washing in Hanks, subsequent exposure to tamoxifen significantly blocked both I_{Na} and I_K currents at 5 μM. Mean sodium and potassium currents measured in the absence and in the presence of ICI 182,780 or tamoxifen are shown in Fig. 5B.

3.4. Effect of serum on the channel blocking action of tamoxifen in C1300 neuroblastoma cells

Tamoxifen has been reported to bind to rat serum low-density lipoprotein [28]. The addition of 10% (v/v) foetal calf serum to the external bathing solution containing 5 μM tamoxifen prevented the inhibition of the inward sodium currents by tamoxifen (5.8±5.3% compared with 86.5±7.8% block in the absence of serum; $P=0.01$; $n=3$). Similarly, serum significantly reduced the blocking action of tamoxifen

against potassium currents from 61.6 ± 5.4% in the absence of serum to 19.4 ± 8.7% in the presence of serum ($P = 0.002$; $n = 4$; values taken at 2 min).

4. Discussion

The data presented here show that tamoxifen and toremifene, both non-steroidal antioestrogens, are able to inhibit macroscopic voltage-gated cationic currents in two different types of neuronal cells, primary cultures of hypothalamic neurones and C1300 neuroblastoma cells. Tamoxifen and toremifene (1) are equally potent blocking INa in hypothalamic neurones and C1300 cells and (2) exhibit differing affinities for two types of potassium currents, i.e. tamoxifen preferentially inhibits DR currents while toremifene inhibits both DR and I_A currents. (3) The inhibitory effect of tamoxifen was impaired in the presence of serum.

It has been previously shown that tamoxifen blocks both cationic and anionic currents [22,20,23,24,4,31,32]. Despite this apparent wide spectrum of action, tamoxifen also shows discrimination between different channels. It does not inhibit resting potassium currents in non-neuronal cells [25] or cAMP- and Ca^{2+}-dependent epithelial chloride channels [24].

Other tricyclic compounds such as chlorpromazine have been shown to block calcium and potassium currents in excitable cells [18,19,30]. It is interesting to note that the tricyclic compounds demonstrated selectivity for different channel types at low micromolar (10 μM) but not at higher concentrations [30]. Tricyclic antioestrogens are highly lipophilic and will readily bind to plasma membranes [3]. Taking into account that the only structural difference between tamoxifen and toremifene is the presence of a chloride substitution on one of the phenolic rings in toremifene, the extent to which non-steroidal antioestrogens show selectivity for membrane channels will require further studies.

Steroidal antioestrogens (e.g. ICI 182,780) had no discernible effect on the sodium or potassium currents recorded in hypothalamic neurones and C1300 cells (this study) nor on volume-activated chloride channels in C1300 neuroblastoma cells [32]. However, a recent study by Ruehlmann et al. [21] reported the ability of ICI 182,780 to modulate the activity of potassium and calcium channels in vascular smooth muscle cells, mimicking the effect of oestradiol.

The rapid effects of antioestrogens upon voltage-gated cationic currents suggest their interaction with either the channel protein, directly or indirectly, rather than through a genomic pathway. The fact that the inhibitory effects of antioestrogens took place in the absence of ATP or GTP in the pipette solution supports the view that intracellular signalling is not significant.

Neurological side effects have been described in patients receiving high dosage tamoxifen [23] but generally the toxicity is low. Several reasons can be put forward to account for the apparent discrepancy between in vitro and in vivo data. The concentrations of free (unbound) drug are likely to be different in different tissues such that insufficient concentrations will reach the neuronal membranes in vivo. Furthermore, tamoxifen will be protein bound in serum and, as is shown in this study, the presence of serum reduces the effectiveness of tamoxifen as a channel blocker. Nevertheless accumulation will occur with time which may reach appropriate concentrations.

The electrical activity of hypothalamic neurones has been shown to be modulated by oestrogens [13,15]. However, little is known about the effects of antioestrogens on hypothalamic neurones. Although no side effects that may be related to impairment of hypothalamic function have been reported, it is possible that long-term treatments may result in concentrations high enough to induce symptoms.

The interference of tamoxifen and toremifene, but not ICI 182,780, with hypothalamic neuronal channels may have important implications for the design of antioestrogens devoid of neuronal toxicity.

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