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Inhibition of ligand-gated cation-selective channels by tamoxifen

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Abstract

The nonsteroidal antioestrogen tamoxifen has been shown to block a number of voltage-gated cation-selective channels but its effect on ligand-gated cation-selective channels has not been studied. We have investigated the action of tamoxifen and the related derivative toremifene on ligand-gated cationic nicotinic acetylcholine and 5-HT_3 receptor channels. Tamoxifen and toremifene both inhibited cationic currents of adult-type human muscle nicotinic acetylcholine receptors expressed in *Xenopus* oocytes with similar IC_{50} values of 1.2 ± 0.03 μM ($n_H = 0.84 \pm 0.02$) and 1.2 ± 0.1 μM ($n_H = 1.1 \pm 0.1$), respectively. Tamoxifen could also block native 5-HT_3 receptors in NG108-15 neuroblastoma/glioma hybrid cells with $IC_{50} = 0.81 \pm 0.15$ μM and n_H of 1.3 ± 0.3. The characteristics of block by tamoxifen at the 5-HT_3 receptor were voltage- and use-independent. The inhibition of the 5-HT-evoked currents were not overcome by increasing concentrations of 5-HT consistent with a noncompetitive mechanism of block.

Keywords: Nicotinic receptor; 5-HT_3 receptor; Tamoxifen

1. Introduction

The nonsteroidal antioestrogen tamoxifen has proved to be a valuable adjunct in the treatment of breast cancer. The therapeutic action is thought to be related to the inhibition of oestrogen-dependent cell proliferation subsequent to binding of the 4-hydroxy metabolite to the nuclear oestrogen receptor. Concerns over the clinical side effects of tamoxifen treatment, including nausea and gastrointestinal disturbances, have led to uncertainty about its prophylactic use. Neurological side effects, such as tremor and dysmetria have also been described in patients receiving high dose tamoxifen (Trump et al., 1992).

Tamoxifen has a wide variety of actions in vitro which cannot be easily reconciled with its antioestrogenic activity, including binding to calmodulin, inhibition of protein kinase C, stimulation of growth factor production and antioxidant effects (van den Koedijk et al., 1994, Grainger and Metcalfe, 1996). In contrast to these intracellular actions, tamoxifen has been shown to block the currents of a number of plasma membrane ion channels including volume-regulated anion channels and voltage-gated Na^+, Ca^{2+} and K^+ channels (Sartor et al., 1988; Valverde et al., 1993; Zhang et al., 1994; Hardy et al., 1995). The selectivity of tamoxifen is not, however, easy to predict because although it can inhibit a diverse range of plasma membrane ion channels, cyclic AMP- and calcium-activated chloride currents are not inhibited by 10 μM and neither is the resting membrane potassium conductance in CHO cells affected (Valverde et al., 1993, 1996).

In order to further identify the specificity of action of tamoxifen, we have investigated the cation-selective ligand-gated channels. Ligand-gated ion channel receptors are placed in one of three superfamilies: The nicotinic receptor family, the glutamate receptor family, and the adenosine 5′triphosphate receptor family. The nicotinic receptor family encompasses nicotinic acetylcholine receptors, ionotropic 5-HT receptors (5-HT_3), $GABA_A$ receptors and glycine receptors. We show that tamoxifen is able to inhibit the muscle type nicotinic receptors and 5-HT_3 receptors. The nature of the block of the 5-HT_3 receptor currents was examined further and the characteristics of the inhibition are discussed.

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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, aminopterine and thymidine (HAT) supplement and 50 μg/ml gentamicin. The undifferentiated cells were plated at a density of approximately 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 whole cells by use of standard patch clamp techniques (Hamill et al., 1981) using a PC501A patch clamp amplifier (Warners Instruments). Patch electrodes were fabricated from borosilicate glass tubing and when filled with the pipette solution detailed below had a resistance of between 2 and 5 MΩ. NG108-15 cells were viewed with an inverted microscope and 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. Perfusion of the experimental chamber was at a rate of approximately 1 ml/min, the volume of fluid within the chamber being held constant by continuous aspiration and the temperature of the perfusion fluid was maintained 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) (Docherty et al., 1991).

2.3. Agonist application

Agonist-induced currents were recorded from NG108-15 cells voltage clamped at a membrane potential of −80

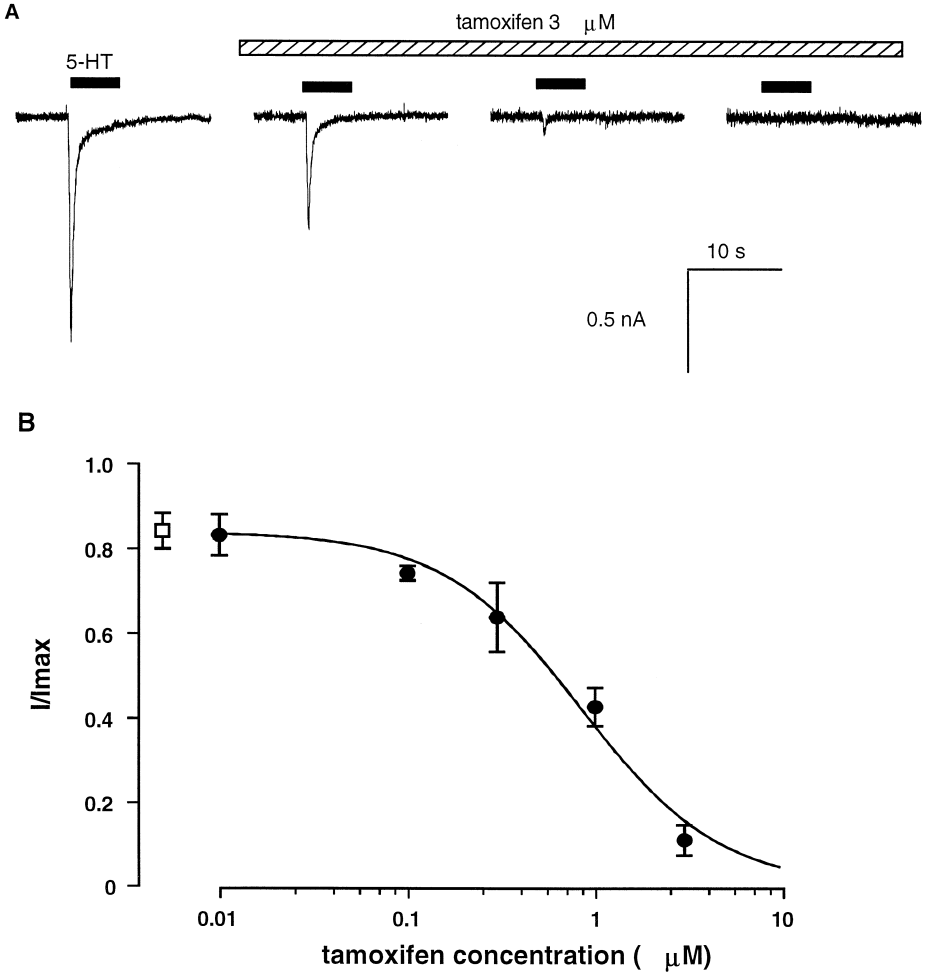

Fig. 1. Inhibition of 5-HT-evoked currents in NG108-15 cells. (A) Sequential whole cell recording showing the inward currents obtained by 5-s pulses of 10 μM 5-HT (black bars) followed by sequential inhibition by the currents in the continued presence of tamoxifen (hatched bar). (B) Concentration–inhibition curve. The IC_{50} was 0.81 ± 0.2 μM, $n_H = 1.3 \pm 0.3$. Each point represents the data obtained from four individual cells. Each cell was exposed to only one concentration of tamoxifen. The open square represents the currents obtained by the vehicle control (DMSO) at the highest concentration used. DMSO did not significantly reduce the 5-HT responses ($P > 0.05$)

mV. The cell being recorded from was continuously superfused with extracellular solution via one of the two square capillaries (width: 800 μm) glued together and mounted on a step motor-operated SF-77 fast-step perfusion system (Warner Instruments). Currents were evoked by stepping from one capillary with extracellular solution (±ligands) to the other capillary with agonist (±other ligands) containing extracellular solution. Application of agonist was for 5 s and the applications were repeated every 90 s to avoid desensitisation. Applications were computer controlled via a CED 1401 interface connected to a computer running WCP V1.6 patch clamp software (Strathclyde Electrophysiological Software). Raw output signals from the patch-clamp amplifier were filtered with a low-band-pass filter at 5 kHz, and digitised at 1 kHz. Macroscopic currents recorded from cells in response to the application of 5-HT were analysed off line using WCP 1.6 software. 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.

The speed of the solution exchange was tested with open pipette experiments. An open pipette voltage clamped at 0 mV was exposed to test solutions of different ionic composition from the control solution. The change in clamp current occurred in less than 20 ms, measuring the rise time as the time interval between 10 and 90% of the total response.

2.4. Data analysis

Agonist responses from each cell were normalised to the 10-μM 5-HT response. Concentration–response curves

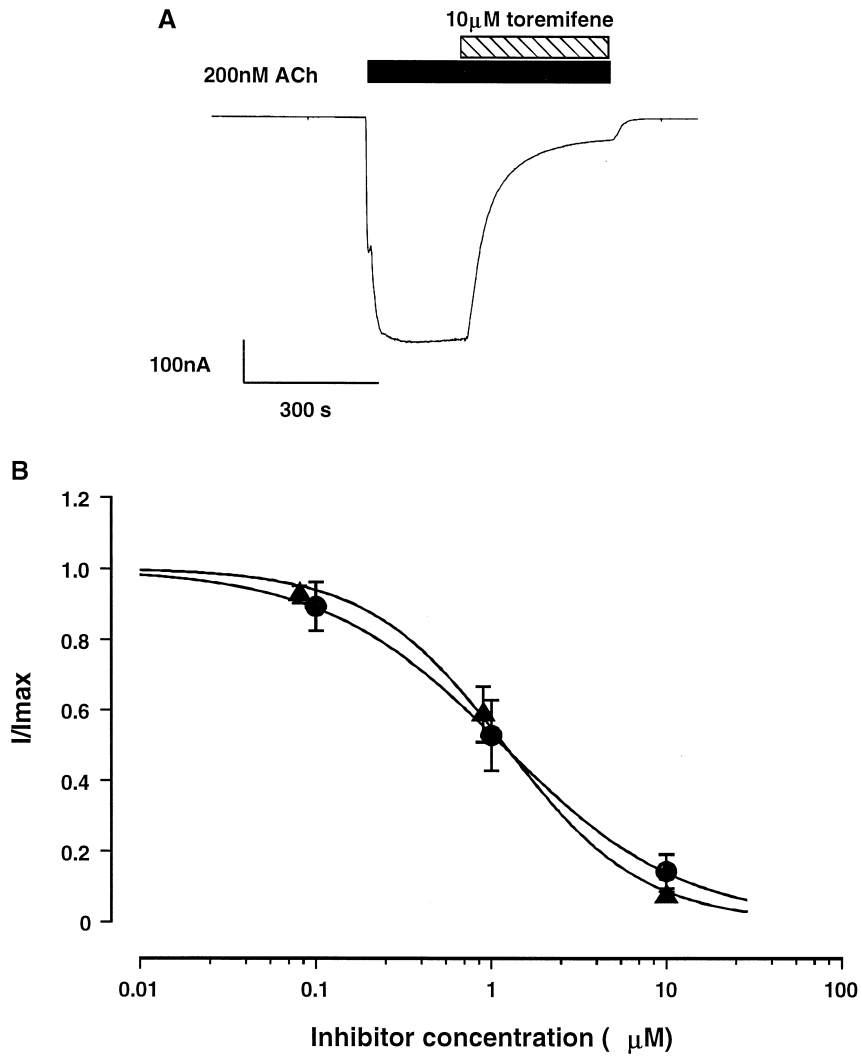

Fig. 2. Block of human muscle nicotinic acetylcholine receptor expressed in oocytes by tamoxifen and its analogues. (A) Inward currents in responses to 200 nM acetylcholine in the absence and presence of 10 μM toremifene. (B) Concentration-dependence of block of nicotinic acetylcholine receptor by tamoxifen (●) and toremifene (▲). Each point is mean ± S.E.M. of measurements made from 4–9 oocytes. Solid curves represent fits by the Hill equation in which maximum and minimum normalised currents were fixed to 1 and 0, respectively, yielding $IC_{50} = 1.2 \pm 0.03$ μM and $n_H = 0.84 \pm 0.02$ for tamoxifen and $IC_{50} = 1.2 \pm 0.13$ μM and $n_H = 1.1 \pm 0.14$ for toremifene.

for agonists were fitted iteratively using figure P software (Biosoft Cambridge) to the following equation:

$$I = \frac{I_{max}}{1 + \left(\frac{EC_{50}}{[A]}\right)^{n_H}},$$

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

Concentration–inhibition curves were fitted to the following equation:

$$I = 1 - \left(\frac{I_{max}}{1 + \left(\frac{IC_{50}}{[I]}\right)^{n_H}}\right)$$

where $[I]$ is the concentration of antagonist, IC_{50} is the concentration of antagonist to produce 50% inhibition.

2.5. Expression and recording from human muscle nicotinic acetylcholine receptor expressed in xenopus oocytes

Nicotinic receptor expression in oocytes and electrophysiological recording were as previously described (Newland et al., 1995; Croxen et al., 1997). Briefly, in vitro transcribed cRNAs encoding the human muscle nicotinic acetylcholine receptor α, β, δ, and ε subunits were coinjected into defolliculated *Xenopus laevis* oocytes. Whole-cell currents were measured with a two-microelectrode voltage-clamp, with 3 M KCl in the electrode, and oocytes were continuously perfused with Ringer (in mM: 115 NaCl, 2.5 KCl, 10 HEPES, 1.8 $CaCl_2$, pH 7.2) with or without appropriate drugs. Recordings were at −60 mV and at room temperature (19–22°C). Nicotinic acetylcholine receptor were activated by a low concentration of acetylcholine (200 nM), and to allow complete recovery from the action of acetylcholine or tamoxifen and its analogues, at least 5 min of washout was used between drug applications. Due to the slow rate of desensitisation, inhibition was determined at a fixed time point, i.e., 220 s after application of the antioestrogen.

2.6. Drugs

Tamoxifen and toremifene 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 acetylcholine-gated currents in oocytes expressing nicotinic acetylcholine receptors ($n = 4$) or 5-HT-gated currents in NG108-15 cells expressing native 5-HT_3 receptors. All chemical and drugs were obtained from Sigma, Poole, UK, except toremifene which was kindly donated by L. Kangas, Orion-Farmos Pharmaceuticals, Finland.

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 significant.

3. Results

3.1. Inhibition of 5-HT_3 receptor currents by tamoxifen

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 largely blocked by 10 nM d-tubocurarine (88% ±1.3%, $n = 6$) and completely blocked by tropisetron at 10 nM ($n = 4$) consistent with the activity of 5-HT_3 ligand-gated channels. Typically 5-HT-induced currents had a rapid onset and a slower biphasic decay. During the first 10–15 min of recording, there was an increase in amplitude and slowing of the rate of decay of the 5-HT responses. These changes are probably due to changes in cytoplasmic milieu that occur during whole-cell recording and leads to a decreased rate of desensitisation (Yakel et al., 1991). All experiments were undertaken after this initial period of stabilisation. Fig. 1A shows that 3-μM tamoxifen completely inhibits the 5-HT-induced current by the third application of 5-HT (representing 250-s exposure to tamoxifen). The inhibition was concentration-dependent, with an IC_{50} of 0.81 ± 0.15 μM and had a Hill coefficient of 1.3 ± 0.3 (Fig. 1B). The inhibition due to low concentrations (≤ 1 μM) could be reversed but at higher concentrations reversibility was incomplete during the course of the experiment.

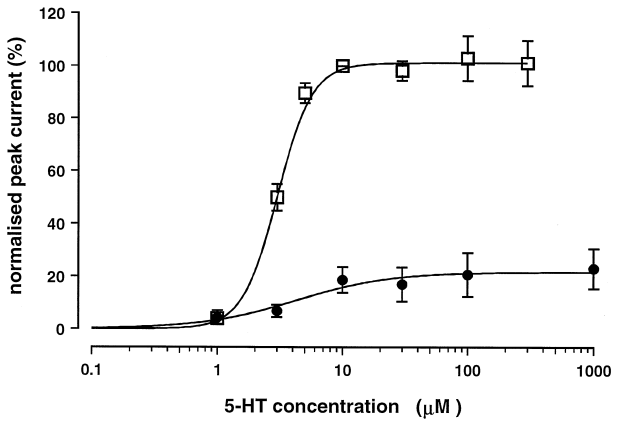

Fig. 3. Noncompetitive nature of inhibition of 5-HT_3 receptors by tamoxifen. (□) represents the current obtained by increasing concentration of 5-HT in the absence of tamoxifen. (●) represents the currents obtained with increasing concentrations of 5-HT in the presence of 3 μM tamoxifen. Each point is the mean ± S.E.M. of data from four individual cells.

The inhibition of muscle type nicotinic acetylcholine receptor currents was also tested using an oocyte expression system (see Section 2). Tamoxifen was also able to inhibit currents evoked by 200 nM acetylcholine with a similar potency as derived from the concentration–inhibition curve shown in Fig. 2B. The IC_{50} obtained was 1.2 ± 0.03 μM and the Hill coefficient was 0.84 ± 0.02. The related antioestrogen toremifene also blocked the nicotinic acetylcholine receptor in a concentration-dependent manner, with a similar IC_{50} of 1.2 ± 0.1 μM and n_H was 1.1 ± 0.14. Fig. 2A shows a representative trace of the acetylcholine-induced currents before and during the coapplication of toremifene. The block by tamoxifen and toremifene was completely reversible within 5–6 min of washout.

3.2. Noncompetitive block of 5-HT currents by tamoxifen

The nature of the block of 5-HT currents by tamoxifen was further investigated in the NG108-15 cells. The ability of cells to respond to increasing 5-HT concentrations was examined in the presence of 3 μM tamoxifen. Fig. 3

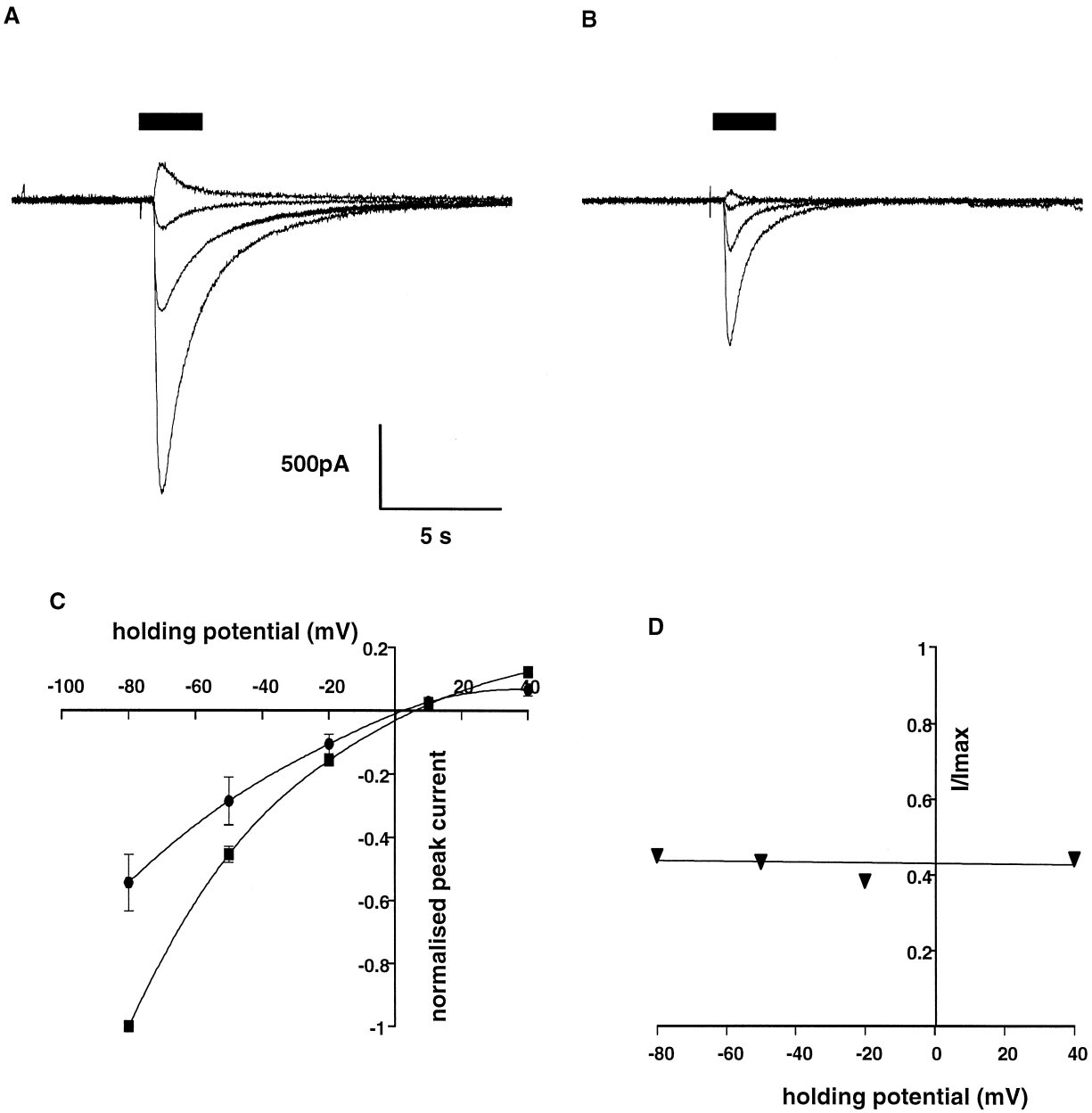

Fig. 4. Lack of effect of tamoxifen on the voltage-dependence of 5-HT-induced currents. (A) Representative traces of currents evoked by 10 μM 5-HT at different holding potentials in one cell before, and (B) during a 10-min application of 1 μM tamoxifen. (C) 5-HT response amplitude plotted as a function of membrane potential before (■) and during (●) tamoxifen (1 μM) application ($n = 5$). (D) Shows the lack of voltage-dependent inhibition (▼) observed with tamoxifen (1 μM) at different holding potentials.

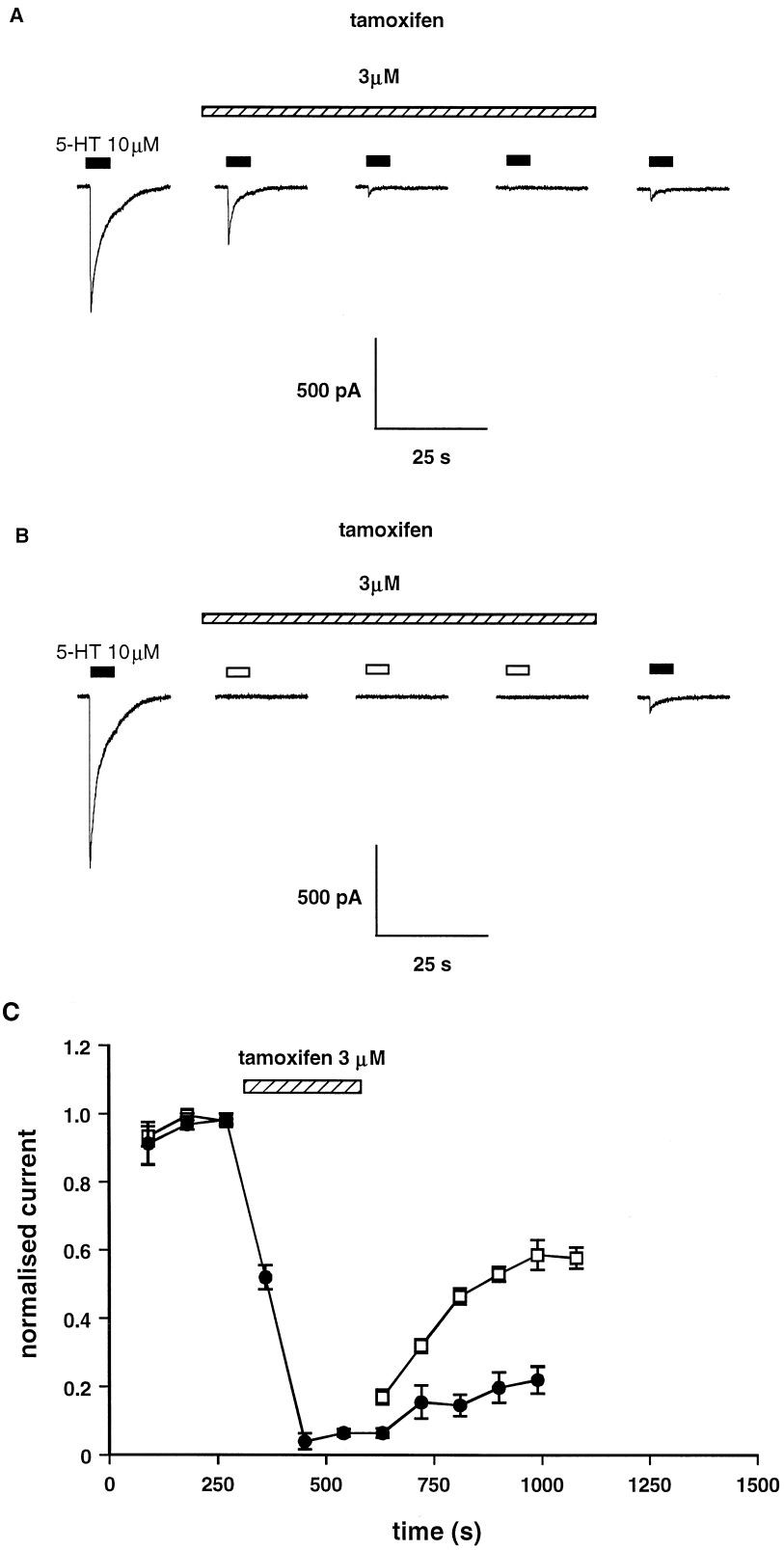

Fig. 5. Absence of use-dependence of inhibition of 5-HT responses by tamoxifen. (A) Inhibition of responses to brief application of 5-HT (10 μM, black bars, ■) during and after exposure to tamoxifen (3 μM) for 4.5 min (hatched bar). (B) Inhibition of 5-HT (10 μM, ■) responses after exposure to tamoxifen (3 μM, 4.5 min, hatched bar) with sham exposure of 5-HT (□). (C) Summary of data obtained in A (■) and B (□) during exposure to tamoxifen ($n = 4$ cells for both conditions).

shows the concentration–response relationship in the presence and absence of 3 μM tamoxifen. 5-HT-induced currents showed saturation with increasing concentration and displayed an EC_{50} of 3.0 ± 0.1 μM and a Hill coefficient (n_H) of 3.1 ± 0.5 ($n = 4$ per data point). In the presence of tamoxifen the currents were reduced and nonsurmountable with increasing concentrations of 5-HT which gave a similar a EC_{50} value but reduced Hill coefficient (4.3 ± 1.7 μM; n_H 1.2 ± 0.5 with tamoxifen) consistent with a noncompetitive type of block.

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

The inhibitory effect of 1 μM tamoxifen 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 shown by the representative traces in Fig. 4A, B. The reduction in the magnitude of currents obtained at all voltages are shown in Fig. 4C. The similar inhibition at different voltages is detailed in Fig. 4D.

3.4. Absence of use-dependence of block of 5-HT_3 receptors by tamoxifen

The inhibition by tamoxifen of 5-HT currents was examined for use-dependence. Exposure to tamoxifen (3 μM) progressively inhibited the responses to 5-HT (10 μM) with steady state inhibition obtained after two applications of 5-HT (Fig. 5A, C). Fig. 5B shows a control experiment with sham application of 5-HT (extracellular solution alone) during exposure to 3 μM tamoxifen. After three sham applications of the agonist, the tamoxifen 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. 5A. Fig. 5C shows the inhibition of the currents by tamoxifen, which appears to be time-dependent rather than use-dependent as exposure to tamoxifen alone can inhibit subsequent 5-HT responses. It is of interest to note that the 5-HT_3 receptor currents recovered more quickly from tamoxifen exposure in cells undergoing sham agonist application (Fig. 5C) than cells exposed to 5-HT. Recovery from tamoxifen (3 μM) during these long experiments was incomplete during the 5-min washout.

3.5. The effect of tamoxifen on 5-HT_3 receptor desensitisation

Many noncompetitive inhibitors of 5-HT_3 receptors in neuronal cell lines have been shown to increase the rate of desensitisation (Fan, 1994a,b). The effect of tamoxifen 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 biexponential decay process (Fig. 6). 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 fast time constant (τ_1) of 1.01 ± 0.18 s and a slow time constant (τ_2) of 7.54 ± 1.6 s ($n = 5$). Both mean values were significantly reduced following exposure to 1 μM tamoxifen for 270 s ($\tau_1 = 0.48 \pm 0.1$ s, $P < 0.02$ vs. control; $\tau_2 = 2.81 \pm 0.36$ s; $n = 5$; $P < 0.05$ vs. control). Tamoxifen did not significantly

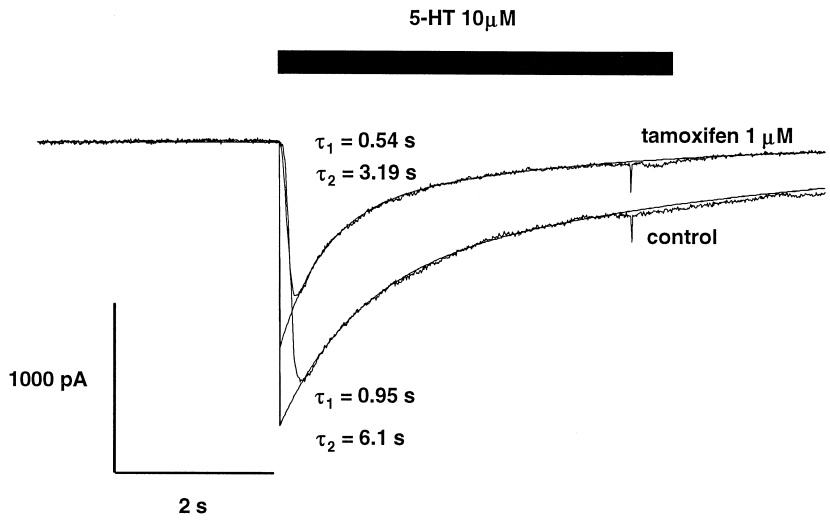

Fig. 6. Representative current responses evoked by 10 μM 5-HT in the presence (upper trace) and absence (lower trace) of 1 μM tamoxifen. The decaying phase was fitted to the sum of two exponentials and the time constants for both conditions are shown. Tamoxifen inhibited the peak response and increased the desensitisation rate as shown by the reduction in both the fast and slow time constants.

effect the rise time (10–90%) of the response to 5-HT (control = 134 ± 9 ms; tamoxifen = 152 ± 18 ms; $n = 5$; $P > 0.05$).

4. Discussion

We have shown that tamoxifen is able to inhibit both the nicotinic acetylcholine receptor and neuronal 5-HT_3 receptor currents in separate experimental systems. Both receptors were blocked by similar concentrations of tamoxifen when applied to the extracellular bathing solution. The reduction in both 5-HT and acetylcholine-evoked currents by tamoxifen was concentration-dependent. The inhibition was fully reversible in the oocyte experiments and at least partially in the NG108-15 cells.

The inhibition by two antioestrogens in two different cell systems, oocytes expressing cloned nicotinic receptors and hybrid cells expressing native 5-HT_3 receptors, may indicate that the effect of the antioestrogens is a direct effect on the membrane channel protein, although we have not excluded a second messenger mechanism [such as modulation of protein kinase C (Zhang et al., 1995)] or genomic pathway.

The mechanism by which tamoxifen is able to block the nicotinic and 5-HT_3 receptors is not clear and the data do not clearly identify a single mode of action. A number of compounds have been identified which can block the cation-selective ligand-gated channels at low micromolar concentrations. Fan (1994a) has shown that mepacrine inhibits 5-HT_3 receptors in a noncompetitive manner. Chlorpromazine is an uncompetitive inhibitor of the nicotinic receptor channel pore (Revah et al., 1990) and is also able to inhibit 5-HT_3 receptors in murine neuroblastoma cells (Sepulveda et al., 1994). Our results are consistent with an insurmountable, noncompetitive block of the 5-HT_3 receptor currents. It is possible, however, that our results could be explained in terms of competitive antagonism if it was assumed that tamoxifen slowly equilibrated with the 5-HT_3 receptor. The peak response to 5-HT is achieved within 200 ms and this may be too fast for a new equilibrium between bound tamoxifen and applied 5-HT to be established. Consequently, a nonsurmountable antagonism would be observed. This phenomena has been described for other 5-HT_3 receptor antagonists in PC12 cells, N1E-115 cells and nodose ganglia (Neijt et al., 1988; Furukawa et al., 1992; Peters et al., 1993), so that while depression of the maximum response to increasing concentration of 5-HT is consistent with a noncompetitive inhibition it does not completely rule out a competitive mechanism. Nevertheless, the effects of tamoxifen on 5-HT_3 receptor desensitisation argue against a simple competitive mechanism because competitive antagonists would not be expected to increase the rate of desensitisation.

The block of the 5-HT_3 receptor currents had features consistent with uncompetitive inhibition (open channel block), i.e., increased rate of desensitisation coupled with no change in rise time (Bufler et al., 1996). There are, however, several features that argue against such a mechanism of action. Open channel blockers usually show use-dependent block where the antagonist relies on the agonist opening the channel to gain access to its binding site within the pore (Gurney and Rang, 1984) and may demonstrate a bell-shaped concentration–response curve with increasing concentrations of agonist. Our data did not show any use-dependence of action nor a bell-shaped concentration–response curve. Tamoxifen inhibited 5-HT responses consistent with a time-dependent inhibition rather than a use-dependent blockade which indicates that tamoxifen has access to its binding site while the channel is in the closed conformation. When testing for use-dependence in cells exposed to tamoxifen alone (without pulses of 5-HT) the subsequent responses to 5-HT appeared to reverse more quickly than in cells that were exposed to tamoxifen and pulses of 5-HT. This difference in recovery could indicate that tamoxifen stabilises the desensitised state of the 5-HT_3 receptor, an action that is not consistent with a simple competitive inhibition. The apparent lack of voltage-dependence also argues against tamoxifen acting as a pure open channel blocker because charged pore blockers usually demonstrate voltage-dependent binding to the open channel (Prior et al., 1995).

Lipophilic tertiary amines, such as tamoxifen can partition into the lipid bilayer and may gain access to the channel in the closed state and this may account for the lack of obvious voltage- and use-dependence. The pronounced lipid solubility of tamoxifen suggests binding to a strongly hydrophobic region which may be common to both receptors. The inhibition curves for tamoxifen gave Hill coefficients of approximately 1 for both nicotinic and 5-HT_3 receptors which indicates a single binding site on the channel. The application system used to apply tamoxifen to the NG108 neuroblastoma cells will change the bathing medium within 5 s, whereas the inhibition of the 5-HT currents took approximately 160 s. As we have shown that tamoxifen exhibits time-dependent and not use-dependent block, we suspect that the inhibitory site on the channel may be accessed from within the lipid bilayer.

The effect of tamoxifen was investigated against the ligand-gated cation selective channels in light of the reported neurological side effects and activity against a number of separate ion channels. While inhibition of nicotinic acetylcholine receptors may theoretically be implicated in the neurotoxicity of high-dosage tamoxifen, the antagonism of 5-HT_3 receptors appears to be at odds with its ability to cause nausea.

In conclusion, tamoxifen inhibits both muscle type nicotinic and 5-HT_3 receptors. The inhibition in 5-HT_3 receptors appears to be noncompetitive, voltage-independent and use-independent. Hill-coefficient values indicate that binding is probably to a single hydrophobic site to which tamoxifen has access while the channel is in the closed

conformation and, once bound, appears to stabilise the desensitised state.

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