

Inhibition of human mast cell proliferation and survival by tamoxifen in association with ion channel modulation

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Background: Human lung mast cells (HLMCs) and the human mast cell line HMC-1 express a strongly outwardly rectifying Cl⁻ current characteristic of that carried by the voltage-dependent Cl⁻ channel CIC-5. A similar but distinct current has been implicated in the control of cell proliferation in astrocytes.

Objective: In this study, we have examined the effects of the Cl⁻ channel blocker tamoxifen on ion channel activity and cell proliferation in both HMC-1 and HLMCs.

Methods: We used the whole-cell patch-clamp technique to characterize macroscopic ion currents in mast cells before and after addition of tamoxifen. HMC-1 proliferation was assessed by incorporation of tritiated thymidine, HLMC proliferation was determined by counting cells in long-term culture, and cell viability was assessed by annexin V binding and propidium iodide uptake.

Results: In HMC-1, tamoxifen reduced the outward Cl⁻ current at +130 mV by 73% ± 9% at a concentration of 3 μmol/L and simultaneously opened a novel inwardly rectifying nonselective cation current with a mean inward current of 153 ± 18 pA at -130 mV. Tamoxifen produced a dose-dependent inhibition of HMC-1 proliferation (90.3% ± 4.0% inhibition at 30 μmol/L) without altering cell viability. Tamoxifen inhibited the outward CIC-5-like current in HLMCs, did not open an inward current, and produced a dose-dependent inhibition of HLMC proliferation in long-term culture.

Conclusion: Tamoxifen inhibits HMC proliferation, possibly through ion channel modulation. This suggests that tamoxifen might be useful in the treatment of mast-cell-mediated diseases, including mastocytosis, asthma, and pulmonary fibrosis. (*J Allergy Clin Immunol* 2003;112:965-72.)

Key words: Human, mast cells, ion channels, cellular proliferation, tamoxifen

Mast cells play an important role in the pathophysiology of many diverse diseases, including asthma, rheumatoid arthritis, pulmonary fibrosis, and mastocytosis.¹ In these conditions, there is recruitment to and/or proliferation of mast cells within tissue structures such as bronchial smooth muscle,² synovium,³ alveolar tissue,⁴

Abbreviations used:

CIC-5:	Voltage-dependent Cl ⁻ current
DMEM:	Dulbecco's modified Eagle's medium
DMSO:	Dimethyl sulfoxide
HEPES:	4-(2-hydroxyethyl) piperazine-1-ethanesulfonic acid
HLMC:	Human lung mast cell
HMC-1:	Leukemic human mast cell line
IC ₅₀ :	Median inhibitory concentration
mAb:	Monoclonal antibody
NMDG:	N-methyl-D-glutamine
pAb:	Polyclonal antibody
PKC:	Protein kinase C
SCF:	Stem cell factor

and skin,⁵ respectively. The sustained secretion of a plethora of proinflammatory mediators, cytokines, and proteases contribute to disease pathogenesis.

Human mast cells require stem cell factor (SCF) to survive and proliferate.⁶ The cell surface receptor for SCF, CD117, is a receptor tyrosine kinase encoded by the proto-oncogene *c-kit*.⁷ The leukemic human mast cell line HMC-1 contains 2 mutations within the *c-kit* coding sequence,⁸ which cause constitutive receptor signaling via endogenous tyrosine kinase activity and result in unchecked cell proliferation.⁹ Similar mutations have also been reported in several other hematological proliferative disorders, including mastocytosis.¹⁰ Activation via CD117 also directly induces mediator release from mast cells and potentiates IgE-dependent mast cell secretion.¹¹ Because of this, CD117 is a potential target for the attenuation of mast cell disease. However, it is expressed on many hematopoietic progenitors, and its inhibition could have serious deleterious effects, so alternative mast-cell-specific targets need to be identified.

Ion channels, carrying either K⁺ or Cl⁻, play a regulatory role in the proliferation of many cell types, including T and B lymphocytes,^{12,13} hematopoietic progenitors,¹⁴ and glial cells.^{15,16} The mechanisms behind this are likely to include effects on Ca²⁺ signaling through regulation of cell membrane potential and control of cell volume. We have identified a unique voltage-dependent Cl⁻ current in both HMC-1 and ~10% of freshly isolated human lung mast cells (HLMCs),^{17,18} which has electrophysiologic properties characteristic of that carried by the voltage-dependent Cl⁻ channel CIC-5.¹⁹ However, CIC-5 currents

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TABLE I. Ionic composition of commonly used solutions

Solution	NaCl	KCl	Na-Meth	K-Meth	NMDG	CaCl ₂	MgCl ₂	EGTA
E1	140	5	—	—	—	2	1	—
E2	5	140	—	—	—	2	1	—
E3	—	—	140	5	—	2	1	—
E4	—	—	—	—	145	2	1	—
I1	—	140	—	—	—	—	2	2

E, Extracellular bath solution; I, intracellular pipette solution; Meth, methanesulfonate. All solutions contained 10 mmol/L HEPES and were buffered to pH 7.3. Osmolality was adjusted to 300 mOsm using glucose as necessary.

have only otherwise been recorded in plasma membranes after heterologous expression in model cells,¹⁹ and no similar currents have ever been described in any other leukocyte. Thus, the presence and role of this current in HMC-1 and a subset of HLMCs are of great interest. A similar but distinct current, which is present in primary cultures of astrocytomas and astrocytoma cell lines, demonstrates cell-cycle-dependent expression and appears to regulate astrocyte proliferation.^{15,16} It is therefore our hypothesis that the mast cell CIC-5-like current is important for human mast cell proliferation. In the present study, we have investigated the ability of tamoxifen, a blocker of several Cl⁻ and K⁺ channels, to modulate the HMC-1 and HLMC CIC-5-like Cl⁻ current.^{20,21} If tamoxifen inhibits this current and our hypothesis is true, then tamoxifen will also inhibit mast cell proliferation.

MATERIALS AND METHODS

Reagents

The following reagents were purchased: physiologic salts, tamoxifen, monothioglycerol, goat polyclonal anti-IgE, A23187, Annexin V-FITC Apoptosis Detection Kit, and dimethyl sulfoxide (DMSO; Sigma, Poole, Dorset, UK); Dulbecco's modified Eagle's medium (DMEM) and Iscove's modified DMEM medium with Glutamax I, iron-supplemented acids, fetal calf serum, and phosphate-buffered saline (Life Technologies; Paisley, Scotland, UK); tritiated ([³H]) thymidine (Amersham, Little Chalfont, Bucks, UK); and 2× sodium dodecyl sulfate-polyacrylamide gel electrophoresis sample buffer and 4% to 12% Tris-glycine gradient-ready gels (Bio-Rad, Herts, UK).

Cells

The HMC-1 cell line was a generous gift from Dr J. Butterfield (Mayo Clinic, Rochester, Minn). The cells were maintained in Iscove's modified DMEM as described previously.¹⁷ HLMCs were purified from lung resection specimens as described previously,²² with final purity > 99%. HLMCs were cultured in DMEM/HEPES with Glutamax I containing 10% heat-inactivated fetal calf serum, 100 ng/mL SCF, 50 ng/mL interleukin-6, and 10 ng/mL interleukin-10. Half of the medium was replaced every 7 days. All subjects gave written, informed consent, and the study was approved by the Leicestershire Research Ethics Committee.

Electrophysiology

The whole-cell version of the patch-clamp technique was used.²³ Patch pipettes were made from borosilicate fiber-containing glass (Clark Electromedical Instruments, Reading, UK) and were heat-polished, resulting in a resistance of 4 to 6 M Ω . The standard pipette

solution contained (in mmol/L) KCl, 140; MgCl₂, 2; EGTA, 2; and HEPES, 10; pH 7.2. The standard external solution contained (in mmol/L) NaCl, 140; KCl, 5; CaCl₂, 2; MgCl₂, 1; and HEPES, 10; pH 7.3. These and other solutions used are shown in Table I. For recording, mast cells were placed in 35-mm dishes containing standard external solution.

Whole-cell currents were recorded using an Axoclamp 200A amplifier (Axon Instruments Inc, Foster City, Calif), evoked by applying voltage commands to a range of potentials in 10-mV steps from a holding potential of -20 mV, and analyzed as described previously.¹⁸ Junction potential changes during Cl⁻ substitution experiments were measured. These were no more than 3 mV and were ignored during analysis. Experiments were performed at 27°C, temperature being controlled by a Peltier device, and a perfusion system (Automate Scientific Inc, San Francisco, Calif) was used to allow solution changes. Drugs were added directly to the recording chamber.

Ca²⁺ imaging

Cells were loaded for 1 hour at 37°C in 2 μ mol/L fura-2-acetoxymethyl ester (Molecular Probes, Eugene, Ore). They were imaged in 35-mm wells containing normal extracellular recording solution (solution E1, Table I) at 24°C, on the stage of a Nikon TE200 microscope (Nikon UK, Kingston-upon-Thames, UK) equipped with a $\times$ 40 Plan Fluor objective (Nikon). Light from a mercury-arc lamp (Nikon) was passed alternately through excitation bandpass filters of 340 and 380 nm, which were exchanged by a computer-controlled filter wheel unit (Ludl Electronic Products, Hawthorne, NY). A 430-nm long-pass dichroic mirror and 510-nm long-pass emission filter supplied light to the Hamamatsu CCD camera (Hamamatsu Photonics, Hamamatsu City, Japan). All optical filters were from Omega Optical (Brattleboro, Vt). Data were collected using Openlab version 3.04 software (Improvision, Coventry, UK). Data acquisition occurred at a rate of 1 dual-wavelength image every 1 second and are presented as the 340-nm/380-nm ratio.

HMC-1 proliferation assay

Assays were performed in 96-well plates with 5 $\times$ 10⁴ cells per well. Tamoxifen stock solutions in 100% DMSO were diluted with standard culture medium to give 0.1% DMSO per well and final concentrations of 0.3 to 30 μ mol/L tamoxifen. Controls containing either 0.1% DMSO or culture medium alone were also established. Each condition was tested in triplicate. [³H]thymidine (37 kBq) of was added to each well, and cells were cultured for 48 hours and then harvested onto glass-fiber filters. Radiation was monitored using a Wallac 1450 Microbeta scintillation counter (Wallac, Milton Keynes, UK). Assays were performed on 3 separate occasions.

HMC-1 apoptosis assay

Cells were seeded at a density of 1 $\times$ 10⁶ per well in standard culture medium with final concentrations of 0.3 to 30 μ mol/L tamoxifen. Controls containing either 0.1% DMSO or culture medium

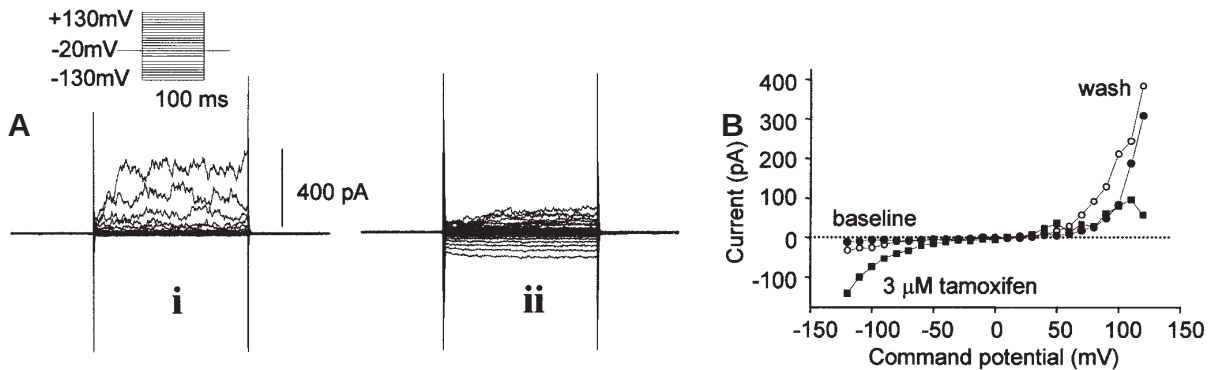


FIG 1. Whole-cell electrophysiologic recording of membrane currents in HMC-1 cells at rest and after addition of tamoxifen: **A**, current recordings from a single HMC-1 cell showing (i) baseline recording and (ii) currents recorded in the presence of 3 $\mu\text{mol/L}$ tamoxifen. Membrane currents were evoked from a holding potential of -20 mV to command potentials between $+130$ and -130 mV (illustrated inset). **B**, Data recorded from the same cell plotted as a current-voltage relationship.

alone were also established. Plates were incubated for either 6 or 18 hours before the percentage of apoptotic and dead cells was measured using an Annexin V-FITC Apoptosis Detection Kit according to the manufacturer's instructions.

HLMC proliferation

HLMCs for long-term culture were resuspended at a concentration of 0.25×10^6 cells/mL with tamoxifen added in the concentration range 1.0 to 30 $\mu\text{mol/L}$. Controls containing either 0.1% DMSO or culture medium alone were also established. Metachromatic cells were counted after 1 and 4 weeks using Kimura stain. The mast cell phenotype of these cells was further confirmed by staining with a monoclonal antibody (mAb) to CD117 and tryptase assessed by flow cytometry and measurement of intracellular content of histamine. HLMC apoptosis in response to tamoxifen was measured as for HMC-1 above.

HLMC activation

For analysis of histamine release, 1×10^4 mast cells were warmed to 37°C in duplicate, and an equal volume of medium containing goat polyclonal anti-human IgE was added (1:1000 dilution, which gives maximal histamine release). After a 30-minute incubation at 37°C , the cells were centrifuged at $250g$ for 4 minutes, the supernatant was decanted, and control cell pellets were lysed in sterile, deionized water for measurement of total histamine content, as described previously.²² Tamoxifen was added in the concentration range 0.1 to 3 $\mu\text{mol/L}$ as appropriate, with a final DMSO concentration of 0.1%.

Western blotting for the presence of phosphorylated CD117

HMC-1 cells were incubated for 24 hours in the presence of tamoxifen at a final concentration of 10 $\mu\text{mol/L}$ in a T-175 flask. CD117 was immunoprecipitated using 25 μg rabbit anti-human CD117 agarose-conjugated polyclonal antibody (pAb; Santa Cruz Biotechnology; Autogen Bioclear UK, Calne, Wiltshire, UK) from cell lysates, prepared according to the manufacturer's instructions. Samples were electrophoresed on 4% to 12% gradient-ready gels for 60 minutes at 200 V and analyzed for the presence of CD117 using either goat anti-human CD117 pAb (R&D Systems, Abingdon, Oxon, UK) or mouse anti-human phosphotyrosine mAb (PY99; Santa Cruz Biotechnology Ltd). Bands were detected using an enhanced chemiluminescence detection kit (Amersham International, Maidstone, Kent, UK) according to the manufacturer's instructions.

Data presentation and statistical analysis

Data are expressed as mean $\pm$ SEM unless otherwise stated. Differences between groups of data were explored using Student's paired or unpaired t test (2-tailed) as appropriate. Frequency of ClC-5-like channel expression under different culture conditions was compared using the χ^2 test. A P value $<.05$ was considered statistically significant.

RESULTS

Effects of tamoxifen on HMC-1 whole-cell currents

As described previously,¹⁷ all HMC-1 expressed the outwardly rectifying, voltage-dependent ClC-5-like Cl^- current at rest (Fig 1). Mean resting current at $+130$ mV was 861 ± 21 pA ($n = 47$), and resting membrane potential was 4.3 ± 0.3 mV ($n = 28$). Addition of tamoxifen produced a dose-dependent block of this current in the range 1 to 10 $\mu\text{mol/L}$ (Fig 1). At $+130$ mV in 1 $\mu\text{mol/L}$ tamoxifen, the outward current was reduced from 782 ± 35 to 729 ± 36 pA ($n = 10$, $P = .483$); in 3 $\mu\text{mol/L}$ tamoxifen, from 816 ± 28 to 221 ± 77 pA at $+130$ mV ($n = 14$; $P = .007$); and in 10 $\mu\text{mol/L}$ tamoxifen, from 796 ± 32 to 208 ± 43 pA ($n = 8$, $P = .001$). The median inhibitory concentration (IC_{50}) for Cl^- channel blockade was estimated to be 2.0 $\mu\text{mol/L}$. Block was neither time nor voltage dependent.

Interestingly, tamoxifen simultaneously opened a strongly inwardly rectifying current (Fig 1), which has not been seen previously in HMC-1. At -130 mV in 3 $\mu\text{mol/L}$ tamoxifen, inward current increased from -11 ± 3 to -153 ± 18 pA ($n = 14$, $P = .0006$). Membrane potential remained around 0 mV (Fig 1). In 3 $\mu\text{mol/L}$ tamoxifen, substitution of extracellular Cl^- by methanesulfonate (solution E3, Table I) reduced the partially blocked outward current at $+130$ mV from 248 ± 50 to 105 ± 21 pA ($n = 4$, $P = .01$), confirming the presence of residual outward Cl^- current, but was without effect on the inward current, suggesting that this is not carried by Cl^- (Fig 2, A). Replacing extracellular Na^+ with K^+ (solution E2, Table I) was without effect on either current ($n = 4$) (data not shown). In contrast, replacement of extracel-

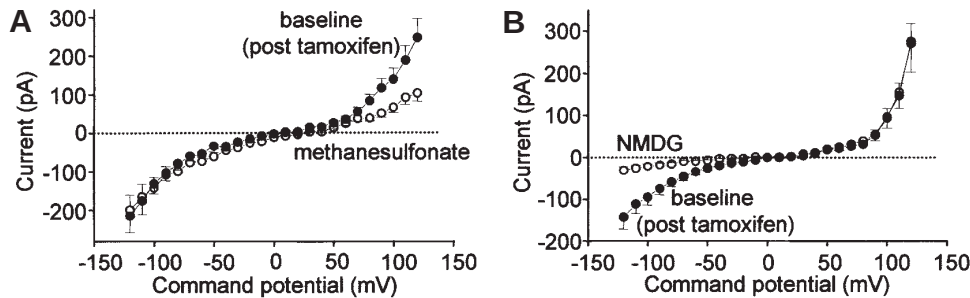


FIG 2. Whole-cell electrophysiologic recordings in HMC-1 cells showing the effects of altering extracellular ionic composition on membrane currents recorded in the presence of tamoxifen: **A**, mean current-voltage curve from 4 cells, showing the effect of substituting extracellular Cl^- by methanesulfonate (solution E3, Table I). **B**, Mean current-voltage curve from 5 cells, showing the effect of substituting extracellular Na^+ and K^+ by NMDG (solution E4).

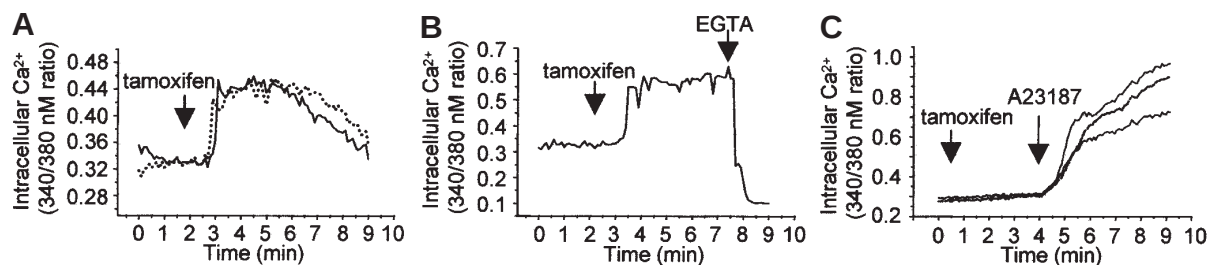


FIG 3. Tamoxifen induces Ca^{2+} influx in HMC-1 cells but not HLMCs: **A**, Two HMC-1 cells recorded before and after addition of 10 $\mu\text{mol/L}$ tamoxifen, showing a rapid and sustained rise in intracellular Ca^{2+} concentration. Representative of 17 cells recorded. **B**, Another HMC-1 cell recorded during application of 10 $\mu\text{mol/L}$ tamoxifen, demonstrating an immediate fall in intracellular Ca^{2+} concentration after chelation of extracellular Ca^{2+} with 5 mmol/L EGTA. Representative of data from 8 cells. **C**, Three HLMCs recorded during application of 10 $\mu\text{mol/L}$ tamoxifen, demonstrating no response, and then later during addition of the calcium ionophore A23187 at a concentration of 500 nmol/L, which produced a rapid and sustained increase in intracellular Ca^{2+} . Representative of data from 38 cells.

lular cations with *N*-methyl-D-glutamine (NMDG; solution E4, Table I) reduced the inward current at -130 mV from 142 ± 18 to 31 ± 4 pA ($n = 5$, $P = .003$) but did not affect the outward current (Fig 2, B). Similar responses to tamoxifen were seen in the absence of external Ca^{2+} ($n = 4$), and addition of 2 mmol/L EGTA to the external recording solution did not alter the inward current ($n = 2$, data not shown). Taken together, this indicates that the inward current is carried by mixed cations with equal permeability to Na^+ and K^+ and that tamoxifen opens a nonselective cation channel in HMC-1.

The Cl^- channel block and nonselective cation channel activation both appeared within 1 minute of adding tamoxifen to the bath and reversed within 1 minute of washing tamoxifen out, suggesting a local membrane effect (Fig 1).

Effect of tamoxifen on HLMC currents

In freshly isolated HLMCs cultured overnight in the absence of SCF, the CIC-5-like current was present in 3/59 HLMC cells, with an outward current at $+120$ mV of 277 ± 97 pA. In contrast, the current was present in 14/71 freshly isolated HLMCs cultured overnight in 100

ng/mL SCF ($P = .014$ compared with no SCF; outward current, 222 ± 62.5 pA at $+120$ mV) and in 12/79 long-term cultured mast cells ($P = .059$ compared with no SCF; outward current, 369 ± 145 pA at $+120$ mV). Addition of SCF to freshly isolated HLMCs cultured in the absence of SCF did not induce any outward current ($n = 7$), in keeping with previous observations that SCF did not directly affect current activity in HMC-1.¹⁷

In 2 freshly isolated HLMCs expressing the CIC-5-like current, 3 $\mu\text{mol/L}$ tamoxifen produced >75% reduction in outward current but failed to open any inward current (not shown). In an additional 6 freshly isolated HLMCs that did not express the CIC-5-like current, tamoxifen failed to open any inward current. Addition of 3 $\mu\text{mol/L}$ tamoxifen to 5 long-term cultured HLMCs expressing the outward current produced a noticeable block of outward current, but membrane seals were repeatedly lost during recording of data. No inward current was observed.

Tamoxifen induces Ca^{2+} influx in HMC-1 but not HLMCs

To assess whether the HMC-1 nonselective cation current carries Ca^{2+} , intracellular Ca^{2+} concentrations were monitored using ratiometric Ca^{2+} imaging. Within 1

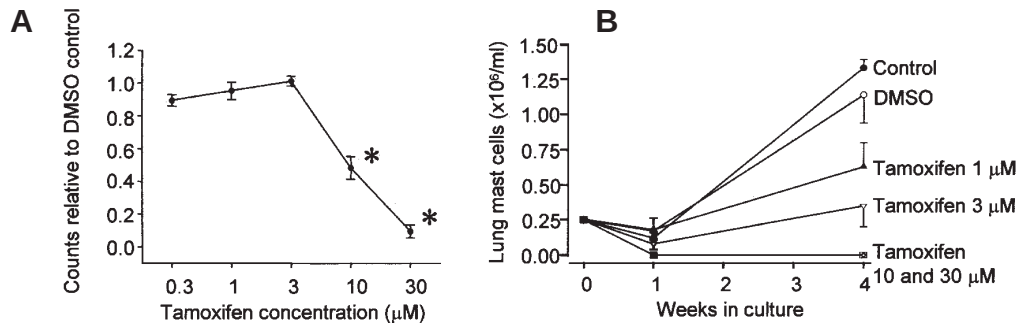


FIG 4. Tamoxifen inhibits proliferation in HMC-1 cells and HLMCs: **A**, thymidine incorporation relative to DMSO control in HMC-1 cells cultured in the presence of tamoxifen (mean of 3 experiments); * $P < .05$ compared with control. **B**, Lung mast cell numbers during 4 weeks of culture in the presence of tamoxifen. Data from 3 separate lung donors are presented. $P < .05$ for all concentrations of tamoxifen compared with DMSO control.

minute of adding 3 or 10 μmol/L tamoxifen, there was a rapid increase in intracellular Ca^{2+} concentration, which remained stable for 2 to >8 minutes (Fig 3, A) and then slowly declined ($n = 17$; Fig 3, A). No effect was seen in the absence of external Ca^{2+} , suggesting that there was no release of Ca^{2+} from internal stores ($n = 12$), and addition of 5 mmol/L EGTA to the bath immediately reduced intracellular Ca^{2+} concentrations to background levels ($n = 8$; Fig 3, B). This indicates that Ca^{2+} concentration increased via the influx of extracellular Ca^{2+} , most likely through the nonselective cation channel. There was no response to 0.1% DMSO control ($n = 8$).

In contrast to the effect on HMC-1, tamoxifen did not produce a Ca^{2+} response in HLMCs up to a concentration of 10 μmol/L ($n = 38$ cells from 2 donors) but did produce a clear response to the calcium ionophore A23187 (Fig 3, C).

Tamoxifen inhibits HMC-1 proliferation without inducing apoptosis or cell death

In HMC-1, tamoxifen produced a profound dose-dependent inhibition of thymidine incorporation, with proliferation reduced by $90.3\% \pm 4.0\%$ at 30 μmol/L tamoxifen ($n = 3$, $P = .0019$; Fig 4, A). The calculated IC_{50} for inhibition of proliferation was 9.8 μmol/L. Tamoxifen did not initiate apoptosis or necrosis based on flow cytometric analysis of annexin V and propidium iodide binding (10.4% apoptosis, 5.0% necrosis in control cells compared with 8.2% apoptosis, 4.8% necrosis in cells treated with 30 μmol/L tamoxifen) or trypan blue exclusion (>95% under all conditions). Therefore, tamoxifen inhibits HMC-1 proliferation without inducing cell death. In contrast, proliferation of the A549 and H460 lung cancer cell lines, which do not express the ClC-5 -like current, was not affected by tamoxifen (data not shown).

Tamoxifen inhibits HLHC proliferation

During the first week of culture, HLHC numbers decreased slightly in control wells and at concentrations of tamoxifen up to 3 μmol/L but all died in tamoxifen at 10 and 30 μmol/L ($n = 3$ donors; Fig 4, B). However,

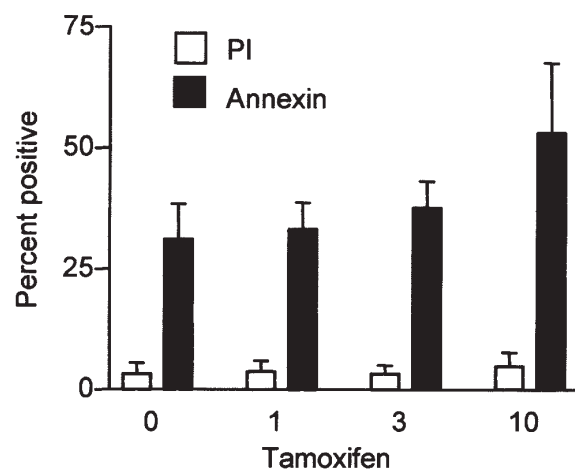


FIG 5. Annexin V binding and propidium iodide uptake by HLMCs cultured in the presence of tamoxifen. At 10 μmol/L tamoxifen, $P = .09$, for annexin compared with control.

addition of tamoxifen for 24 hours only induced apoptosis in a small proportion of HLMCs at 10 μmol/L, as analyzed by annexin V binding, and no acute cell death was monitored by propidium iodide uptake (Fig 5) and trypan blue exclusion (100% viable under all conditions). After 4 weeks in culture, mast cells remained 100% pure and in control wells increased in number ~5-fold over baseline. Tamoxifen produced a significant dose-dependent inhibition of this increase in mast cell number (Fig 4, B). These cells after 4 weeks in culture contained 2.75 ± 0.04 pg histamine per cell and expressed intracellular tryptase and extracellular CD117 (not shown).

Tamoxifen does not modulate mast cell histamine release

Tamoxifen has previously been reported to inhibit histamine release from rat peritoneal mast cells.²⁴ In 2 experiments with freshly isolated HLMCs isolated from different donors, 3 μmol/L tamoxifen did not modulate background histamine release (control, $1.9\% \pm 0.3\%$; tamoxifen, $2.3\% \pm 0.4\%$) and did not inhibit or potentiate IgE-dependent histamine release (net control, $6.2\% \pm 0.4\%$; net tamoxifen,

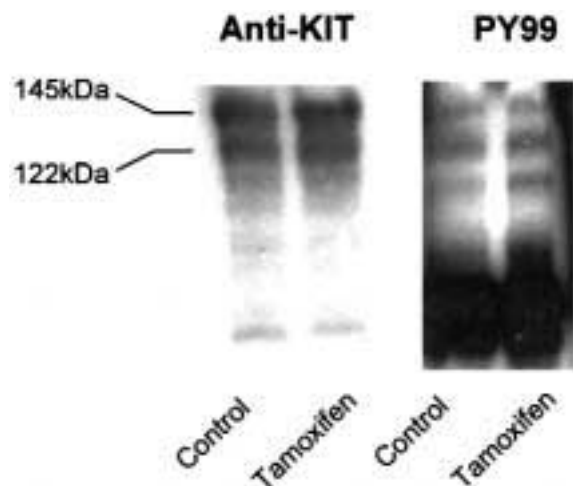


FIG 6. Tamoxifen does not act on the CD117 signaling pathway: **A**, detection with a CD117 pAb identified a doublet of approximate size 145 kDa and 122 kDa. **B**, Detection with an anti-phosphotyrosine mAb (PY99) identified the same doublet and additional smaller but more intense bands, which were not detected by the CD117 pAb.

5.9% $\pm$ 0.3%). In 2 experiments using long-term HLMCs, 3 μ mol/L tamoxifen did not alter background release (control, 9.3% $\pm$ 1.45%; tamoxifen, 9.5% $\pm$ 1.0%) or IgE-dependent histamine release (net control, 54.0% $\pm$ 7.7%; net tamoxifen, 52.7% $\pm$ 7.2%).

Tamoxifen does not interfere with CD117 autophosphorylation

CD117 was immunoprecipitated from HMC-1 cell lysates after incubation with 10 μ mol/L tamoxifen (Fig 6, A and B). Blotting with anti-CD117 pAb detected a characteristic CD117 doublet of 145 kDa and 122 kDa in samples treated with tamoxifen and untreated controls (Fig 6, A). Blotting with PY99 anti-phosphotyrosine mAb detected a weakly phosphorylated doublet as well as several more strongly phosphorylated smaller bands (Fig 6, B). These bands most likely represent signaling pathway proteins that coprecipitate with phosphorylated CD117, as reported previously (reviewed in Linnekin²⁵). Neither the intensity of CD117 phosphorylation nor the pattern of additional banding differed between tamoxifen-treated samples and untreated controls. Furthermore, tamoxifen did not alter the surface expression of CD117 on HMC-1 or HLMCs (not shown). Tamoxifen is therefore unlikely to act on the proximal CD117 signaling pathway, although in the absence of site-specific anti-phosphotyrosine antibodies, a localized inhibition might not be identified.

DISCUSSION

We have demonstrated for the first time that tamoxifen is a potent inhibitor of human mast cell proliferation at low micromolar concentrations. This occurs in association with ion channel modulation, which might explain in part how this antiproliferative effect is produced.

Several Cl⁻ and K⁺ currents have been implicated in the regulation of cell proliferation.^{13,20,26} HMC-1 consistently expresses an unusual strongly outwardly, rectifying Cl⁻ conductance, which has electrophysiologic properties similar to those of the voltage-dependent Cl⁻ channel CIC-5, and they also express mRNA for this channel.¹⁷ A similar but distinct current has been implicated in the regulation of astrocyte proliferation.¹⁶ Because this current is seen in only ~10% of "mature" HLMCs,¹⁸ a subset of which have the potential to proliferate,²⁷ we developed the hypothesis that this unusual CIC-5-like current is involved in the regulation of human mast cell proliferation. Tamoxifen is a nonsteroidal triphenylethylene antagonist of the estrogen receptor, which inhibits several volume-sensitive Cl⁻ channels and K⁺ channels with an IC₅₀ of ~1 to 4 μ mol/L.^{20,28,29} In the present study, tamoxifen markedly inhibited the HMC-1 CIC-5-like Cl⁻ current, with an IC₅₀ for channel block of ~2 μ mol/L and 80% channel inhibition at 3 μ mol/L. This suggests that tamoxifen is a useful tool for studying the physiologic role of this channel. The exact mechanism(s) by which tamoxifen blocks Cl⁻ channel activity is not clear but could include direct block of channel pores, conformational changes because of channel binding, nonspecific effects because of intercalation with the plasma membrane,³⁰ or modulation of intracellular signaling pathways. The ability of tamoxifen to block a wide variety of cation and anion channels at low micromolar concentrations, often in a rapidly reversible manner, suggests that a local membrane effect is perhaps most important.

Interestingly, in parallel with Cl⁻ channel blockade, tamoxifen simultaneously opened an inwardly rectifying current in HMC-1, which we had never seen previously in these cells. Ion-substitution experiments confirmed that this was carried by mixed cations with equal permeability to Na⁺ and K⁺. Ratiometric Ca²⁺ imaging indicated that tamoxifen also induces influx of extracellular Ca²⁺, suggesting that the nonselective cation current also carries Ca²⁺. Opening of this nonselective cation current has never been reported in other cell types during electrophysiologic recording, and it is beyond the scope of this study to determine how tamoxifen opens this channel in HMC-1. Nevertheless, the simultaneous effect on the Cl⁻ current and rapid reversibility again suggest that a local membrane effect is perhaps most important.

Our hypothesis states that blockade of the CIC-5-like current will attenuate cell proliferation, so if true, tamoxifen will produce this effect. Indeed, tamoxifen proved to be a potent inhibitor of both HLMCs and HMC-1 proliferation. In HMC-1 this occurred in the absence of cell death or apoptosis, suggesting that it was not a nonspecific, toxic effect. HLMCs, however, appeared more sensitive than HMC-1 to the effects of this drug, in that inhibition of proliferation was seen at lower doses, whereas at the higher doses, all HLMCs died within the first week of culture. This would be expected because the drive for proliferation will be much higher in the neoplastic HMC-1 line than in the slowly proliferating, mature HLMCs. With 1 μ mol/L tamoxifen, HLMC numbers were unaffected at 1 week

compared with control but were significantly reduced at 4 weeks, providing strong evidence that tamoxifen inhibits proliferation in these cells. The observation that 3 $\mu\text{mol/L}$ tamoxifen did not induce apoptosis or acute cell death in HLMCs but that more cells died with this concentration during the first week of culture might suggest that the proliferating cell population helps maintain cell numbers at 1 week. Even at a dose of 10 $\mu\text{mol/L}$, tamoxifen induced apoptosis in only a small proportion of HLMCs, suggesting that its predominant influence on HLMC number is through inhibition of proliferation.

The similar dose range with tamoxifen for Cl^- channel modulation and the antiproliferative effects suggest that the two might be related, although this cannot be proved in the present study. The opening of the nonselective cation channel in parallel with Cl^- channel blockade in HMC-1 also complicates the interpretation of the role of Cl^- channel blockade on cell proliferation, but the fact that this channel did not open in HLMCs in response to tamoxifen suggests it is not involved in the mast cell proliferative response. It is entirely plausible that channel modulation will affect proliferation, because this will interfere with the regulation of cell membrane potential, Ca^{2+} signaling, and regulation of cell volume. The fact that a half-log increase in concentration was required for the antiproliferative effects of tamoxifen compared with channel modulation in HMC-1 is understandable, because to negate the influence of a channel on cell membrane potential, nearly all channels must be blocked.¹³ This is in close agreement with our observations regarding Cl^- channel blockade and inhibition of proliferation by tamoxifen in HMC-1.

Other potential mechanisms for the antiproliferative effect of tamoxifen, independent of ion channel modulation, include antagonism of estrogen-receptor-mediated signaling, inhibition of protein kinase C (PKC), or perhaps inhibition of the *c-kit* tyrosine kinase pathway. The first is unlikely, because HMC-1 and HLMCs do not require estrogen to proliferate. Inhibition of PKC is a possibility, although this requires tamoxifen concentrations of 40 to 100 $\mu\text{mol/L}$,³¹ and if we were inhibiting PKC, IgE-dependent histamine release from HLMCs would be expected to diminish. Because the SCF receptor CD117 is constitutively activated in HMC-1 cells and is the driving force for cell proliferation, it was also important to determine that tamoxifen was not acting directly on the CD117 signaling pathway. Fig 6 suggests that incubation with tamoxifen did not affect the activation state of CD117.

In several cell types, CIC-5 is located predominantly in the membranes of intracellular vesicles, where it colocalizes with H^+ -ATPase.^{32,33} Here, it is believed to provide the electrical shunt for the efficient acidification of vesicles, so its inhibition would be predicted to prevent this. An interesting property of tamoxifen is its ability to inhibit acidification of intracellular organelles, causing the redistribution of weak-base chemotherapeutics from these structures to the nuclei of drug-resistant neoplastic cells, thus reversing drug resistance.³⁴ The ability of

tamoxifen to block the mast cell CIC-5-like current might therefore explain how this is achieved.

Finally, irrespective of the mechanism(s) by which tamoxifen inhibits mast cell proliferation, this effect clearly has potential clinical implications. In doses used for patients with breast cancer, treatment with 40 mg tamoxifen daily gives a serum concentration of $\sim 1 \mu\text{mol/L}$, and at 320 mg daily, a serum concentration of $\sim 4.5 \mu\text{mol/L}$.³⁵ However, tissue concentrations are between 10 and 60 times higher than serum concentrations, with particularly high levels achieved in liver and lung.³⁶ Thus, tissue concentrations at regular clinical doses might be between 10 and 60 $\mu\text{mol/L}$, which is above the concentration required for modulation of mast cell Cl^- channel activity and in the range at which we observed profound inhibition of HMC-1 proliferation. Tamoxifen has some potentially serious side effects, including an increased risk of thromboembolism and endometrial cancer. Nevertheless, our data suggest that tamoxifen might be a useful adjunct in the treatment of serious diseases in which mast cell hyperplasia is evident, such as refractory asthma, pulmonary fibrosis, and mastocytosis, and is worthy of further clinical trials.

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