British Journal of Pharmacology (2002) 136, 961–964

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SPECIAL REPORT

The pure anti-oestrogen ICI 182,780 (Faslodex™) activates large conductance Ca^{2+}-activated K^+ channels in smooth muscle

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Oestrogen and tamoxifen activate large conductance Ca^{2+}-activated K^+ (BK_{Ca}) channels in smooth muscle through a non-genomic mechanism that depends on the regulatory β1 subunit and an extracellular binding site. It is unknown whether a 'pure' anti-oestrogen such as ICI 182,780 (Faslodex™), that has no known oestrogenic properties, would have any effect on BK_{Ca} channels. Using single channel patch clamp techniques on canine colonic myocytes, the hypothesis that ICI 182,780 would activate BK_{Ca} channels was tested. ICI 182,780 increased the open probability of BK_{Ca} channels in inside-out patches with an EC_{50} of 1 μM. These data suggest that molecules with the ability to bind nuclear oestrogen receptors, regardless of oestrogenic or anti-oestrogenic nature, activate BK_{Ca} channels through this nongenomic, membrane-delimited mechanism. The identity and characteristics of this putative binding site remain unclear; however, it has pharmacological similarity to oestrogen receptors α and β, as ICI 182,780 interacts with it.
British Journal of Pharmacology (2002) **136,** 961–964

Keywords: 17-β oestradiol; tamoxifen; *Slo*; MaxiK channel; β1 subunit

Abbreviations: BK_{Ca}, large conductance Ca^{2+}-activated K^+ channel; tamoxifen, [Z]-1-[p-dimethylaminoethoxy-phenyl]-1,2-diphenyl-1-butene; ICI 182,780 (7α-[9-[(4,4,5,5,5-pentafluoropentyl)sulphinyl]nonyl]-estra-1,3,5(10)-triene-3,17β-diol); HEDTA, N-[2-hydroxyethyl]-ethylenediamine-triacetic acid

Introduction Oestrogen (17-β oestradiol) has nongenomic, or alternative, effects unrelated to the nuclear events (Falkenstein *et al*., 2000) and some anti-oestrogens share this property. These compounds exert effects outside the classical signalling pathway of altering gene transcription. For example, oestrogen and tamoxifen activate BK_{Ca} channels in smooth muscle (Valverde *et al*., 1999; Dick *et al*., 2001). BK_{Ca} channels have a large unitary conductance and are Ca^{2+}/voltage-sensitive. In smooth muscle cells, BK_{Ca} channels contain a regulatory β1 subunit that alters channel function in many ways (McManus *et al*., 1995). Appreciation for the physiological importance of the β1 subunit has recently increased, as genetically engineered mice lacking this regulatory subunit are hypertensive and demonstrate altered smooth muscle reactivity (Brenner *et al*., 2000). An extracellular binding site and the β1 subunit are essential for sensitivity of BK_{Ca} channels to oestrogen and tamoxifen (Valverde *et al*., 1999; Dick *et al*., 2001; 2002; Dick & Sanders, 2001). The identity of the extracellular binding site is not known (Nadal *et al*., 2001).

Tamoxifen is used clinically as an oestrogen receptor antagonist for the treatment and prevention of breast cancer. However, tamoxifen has mixed oestrogenic and anti-oestrogenic properties, ranging from full agonist to full antagonist depending on the tissue and endpoint examined. Tamoxifen inhibits the binding of oestrogen to nuclear receptors preventing cell proliferation. In contrast, tamoxifen has oestrogenic properties in liver, bone, and uterus, where it alters blood lipids, bone density, and endometrial thickness (Jordan, 2000). Because of oestrogenic properties, tamoxifen is associated with adverse chemotherapeutic reactions including tumour flare, hot flashes, and the stimulation of liver and myometrial carcinomas (Jordan, 2000; Howell *et al*., 2000). Thus, the search for oestrogen receptor antagonists without oestrogenic properties has been an area of intense interest. ICI 182,780 is a highly specific oestrogen receptor antagonist marketed under the trade name Faslodex (Howell *et al*., 2000). ICI 182,780 is used for treating oestrogen receptor-dependent tumours, and, in contrast to tamoxifen, has no known oestrogenic properties.

The question remains as to whether the ability of tamoxifen to activate BK_{Ca} channels is due to its partial oestrogenic properties. This was determined by assessing the effect of a pure anti-oestrogen, ICI 182,780. The results delineate a novel mechanism of action and indicate that the putative extracellular binding site has pharmacology in common with oestrogen receptors α or β, as a pure anti-oestrogen interacts with it. This interaction is translated, by an unknown mechanism, into changes in BK_{Ca} channel activity.

Methods Canine colonic smooth muscle cells were isolated by enzymatic dispersion described previously (Dick *et al*., 2001; 2002). The methods were approved by an Animal Care and Use Committee and were consistent with NIH guidelines. Myocytes were suffused at 3 ml min^{-1} with (mM) KCl 140, HEPES 10, Tris 5; pH 7.1. Additionally, this solution contained either 1 mM EGTA or 1 mM HEDTA (N-[2-hydroxy ethyl]-ethylenediamine-triacetic acid) and between 0.01–10 μM free Ca^{2+} (Maxchelator 2.05; Pacific Grove, CA, U.S.A.).

Data were analysed with the Analysis of Single Channel Data program (Guy Droogmans; University of Leuven,

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Belgium). NP_o (number of channels multiplied by the open probability) was measured before, during, and after exposing the patches to ICI 182,780 (Tocris Cookson Inc., Ballwin, MO, U.S.A.). ICI 182,780 was made as a 10 mM stock in DMSO and diluted to the final concentration in bathing solutions. DMSO, at concentrations of 0.1% and less, has no effect on BK_{Ca} channels (Dick *et al*., 2001). NP_o and unitary conductance were determined from all-points amplitude histograms. Data are presented as the mean ± s.e. from *n* cells. Statistical analyses were made with Student's paired *t*-test or one-way analysis of variance (with repeated measures and Bonferroni *post-hoc* analysis as necessary). The threshold for statistical significance was $P<0.05$. Statistical tests were run in SigmaStat (version 2; Jandel Scientific Software; San Rafael, CA, U.S.A.).

Results *ICI 182,780 increases the NP_o of BK_{Ca} channels by a nongenomic mechanism* BK_{Ca} channels were studied in inside-out patches of membrane taken from canine colonic myocytes as described previously (Dick & Sanders, 2001; Dick *et al*., 2001, 2002). ICI 182,780 (10 μM) increased NP_o when applied to the bath (Figure 1A–D). In contrast to the effect of tamoxifen, ICI 182,780 increased NP_o without affecting the unitary conductance of BK_{Ca} channels (Figure 1E).

Effects of ICI 182,780 on BK_{Ca} channels are rapid, but not readily reversible The increase of BK_{Ca} channel NP_o by ICI 182,780 was observed in cell-free patches of membrane, indicating it is a nongenomic effect. Additionally, a hallmark of nongenomic steroid effects is the speed of the effect. Figure 2 shows that ICI 182,780 (10 μM) rapidly increased BK_{Ca} channel NP_o. The time from ICI 182,780 (10 μM) application to reach a steady-state NP_o was 4.8 ± 0.7 min ($n=13$). The effect of ICI 182,780, however, was not readily reversible. As can be seen in Figure 2A, brief exposure to ICI 182,780 caused an increase in NP_o that persisted for approximately 30 min. In six cells, NP_o was 306 ± 38% higher than control 21 ± 2 min after 10 μM ICI 182,780 was washed out of the bath.

The increase in NP_o by ICI 182,780 is concentration-dependent and saturable Increasing concentrations of ICI 182,780 (0.1–10 μM) were added to solutions bathing the cytoplasmic face of inside-out patches. ICI 182,780 increased NP_o in a concentration-dependent manner. The EC_{50} was 1.0 ± 0.2 μM ($n=10$; Figure 3). The unitary

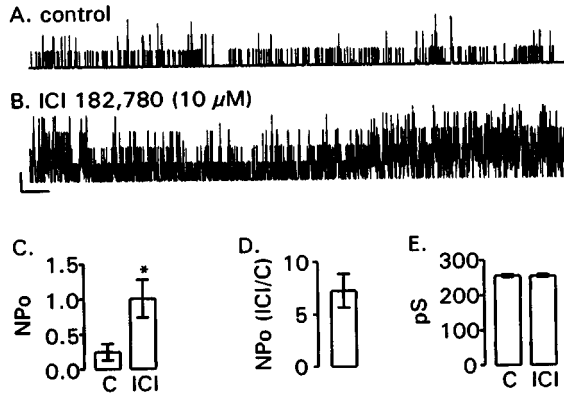

Figure 1 ICI 182,780 increases BK_{Ca} channel NP_o in excised membrane patches. Representative 1 min recordings of BK_{Ca} channel activity in an inside-out patch before (panel A; control; C) and after a 5 min exposure to 10 μM ICI 182,780 (ICI; panel B). Patch potential was +80 mV; 100 nM free Ca^{2+}. ICI 182,780 increased NP_o without effect on the unitary conductance. Scale bar represents 20 pA and 3 s. Panel C group data ($n=13$) demonstrating the effect of 10 μM ICI 182,780 on BK_{Ca} channel NP_o. The asterisk indicates a significant effect of ICI 182,780 on NP_o ($P=0.001$ by Student's paired *t*-test). Panel D NP_o in the presence of 10 μM ICI 182,780 normalized to control. Panel E ICI 182,780 had no effect on the unitary conductance of BK_{Ca} channels ($P=0.60$ by Student's paired *t*-test).

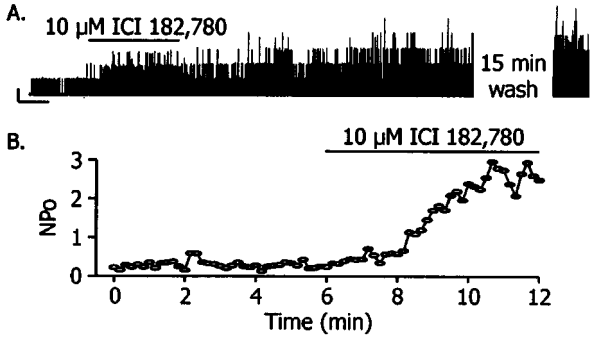

Figure 2 Rapid, but not readily reversible, effect of ICI 182,780 on BK_{Ca} NP_o. Panel A shows a representative recording of BK_{Ca} channel activity in an inside-out patch. Patch potential was +100 mV; 100 nM free Ca^{2+}. Current was recorded for 2 min prior to exposing the patch to 10 μM ICI 182,780 for 3 min, which increased NP_o. NP_o remained elevated after ICI 182,780 was washed out; an effect that persisted after a 15 min wash. Scale bar represents 20 pA and 1 min Panel B is a plot of NP_o vs time from another patch under the same recording conditions. NP_o was steady after patch excision and increased with the application of 10 μM ICI 182,780.

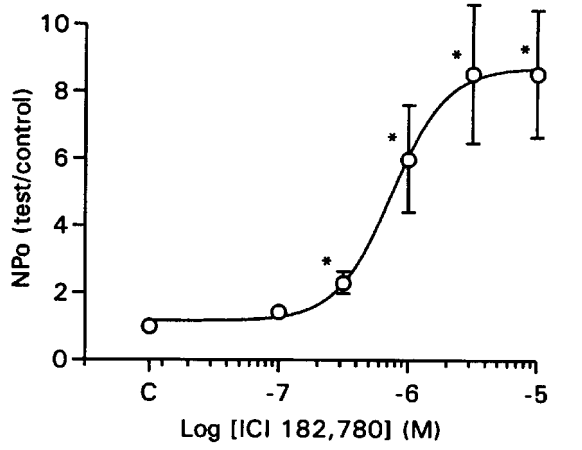

Figure 3 Concentration-dependent effect of ICI 182,780 on BK_{Ca} channel NP_o. Group data ($n=10$) showing the concentration-dependent effect of ICI 182,780 on BK_{Ca} channel NP_o at +80 mV and 100 nM free Ca^{2+}. NP_o was measured at least 5 min after each concentration of ICI 182,780 was applied. The EC_{50} was 1 μM. Asterisks indicate a difference from control (C) by one-way repeated measures analysis of variance.

conductance under control conditions was 260 ± 4 pS ($n=10$) and did not change with exposure to ICI 182,780 ($P=0.69$ by one-way repeated measures analysis of variance).

ICI 182,780 shifts the voltage-dependence of BK_{Ca} gating to more negative potentials In all the experiments described thus far, the bath free Ca^{2+} concentration was 100 nM, near the lower limit where the influence of the $\beta 1$ subunit on BK_{Ca} channel gating is thought to decline. To determine the effect of ICI 182,780 on channel gating over a wider range of free Ca^{2+} concentrations, and thus NP_o, two different solutions were used. In the first, the bath contained 1 mM EGTA and no added Ca^{2+}, setting the free Ca^{2+} concentration near 10 nM. Under these low Ca^{2+} conditions, ICI 182,780 increased NP_o $420 \pm 95\%$ ($n=8$). The second solution contained 1 mM HEDTA and 10 μM free Ca^{2+}. Under these high Ca^{2+} conditions, ICI 182,780 increased NP_o $431 \pm 107\%$ ($n=7$). Additionally, an activation curve was made in 100 nM free Ca^{2+}. ICI 182,780 (10 μM) shifted the voltage of half activation from 106 ± 6 to 86 ± 5 mV ($n=7$; $P<0.0001$ by Student's paired t-test; Figure 4).

Discussion ICI 182,780 activates BK_{Ca} channels in smooth muscle, a previously unrecognized action that is rapid, concentration-dependent, and similar to that of oestrogen and tamoxifen (Valverde *et al*., 1999; Dick & Sanders, 2001; Dick *et al*., 2001). This is a nongenomic, or alternative, effect as it does not involve changes in gene transcription (Falkenstein *et al*., 2000). The activation of BK_{Ca} channels by [xeno]oestrogens is not strictly due to oestrogenic properties, as a pure anti-oestrogen, ICI 182,780, has similar effects. The extracellular binding site has pharmacology in common with oestrogen receptor α and β, as oestradiol, tamoxifen, and ICI 182,780 interact with it. This binding is translated, by an unknown mechanism, into changes in BK_{Ca} channel activity.

ICI 182,780 (AstraZeneca, Cheshire, U.K.) is a steroidal oestrogen receptor antagonist (Howell *et al*., 2000). ICI 182,780 is thought to be a pure anti-oestrogen (i.e., it does not have any known oestrogen agonist activity). There are few data regarding nongenomic mechanisms of action for ICI 182,780. One example is inhibition of L-type Ca^{2+} channels (i.e., Ba^{2+} current) by ICI 182,780 in A7r5 cells (Ruehlmann *et al*., 1998). ICI 182,780 also increased K^+ current in three of four cells tested; however, the nature of the current, and the concentration-dependence and magnitude of the ICI 182,780 effect were not determined. ICI 182,780 also reduces coronary vascular tone in isolated perfused rat hearts (Ruehlmann *et al*., 1998). This could be due, in part, to inhibition of L-type Ca^{2+} channels or activation of K^+ channels. Increased outward current could be due to diminished inward current, and not the activation *per se*, of an outward current. These results suggest that previous observations could be explained by activation of BK_{Ca} channels.

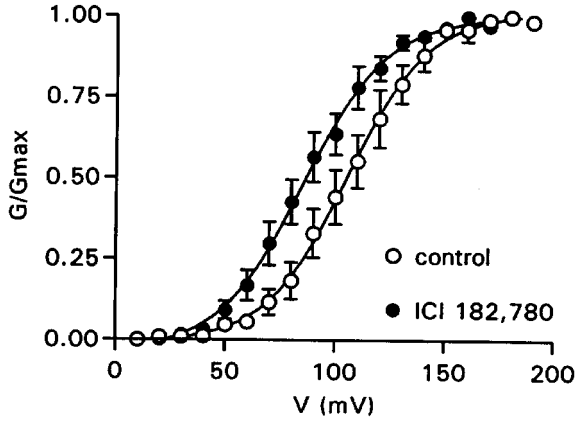

Figure 4 ICI 182,780 shifts the voltage-dependence of BK_{Ca} gating. Group data ($n=7$) demonstrating a negative shift in the voltage of half activation by 10 μM ICI 182,780. The patch potential was held at 0 mV and stepped to potentials from +10 to +200 mV; 100 nM free Ca^{2+}. Conductance was calculated from current, normalized to the maximum, and fit with a Boltzmann sigmoidal function.

The activation of smooth muscle BK_{Ca} channels by [xeno]oestrogens depends on the presence of the regulatory $\beta 1$ subunit (Dick & Sanders, 2001; Dick *et al*., 2001; Valverde *et al*., 1999). There are four known regulatory β subunits, but only $\beta 1$ and $\beta 4$ (Behrens et al., 2000) confer oestrogen sensitivity upon BK_{Ca} channels. Studies using membrane impermeant [xeno]oestrogens suggest an extracellular binding site exists, perhaps within the extracellular loop of the BK_{Ca} channel $\beta 1$ subunit (Valverde *et al*., 1999; Dick *et al*., 2002). However, preliminary data indicate that a chimeric β subunit containing the $\beta 1$ extracellular loop and the $\beta 2$ intracellular and transmembrane portions does not respond to oestrogen or tamoxifen (Orio *et al*., 2002). It remains to be determined whether this chimeric $\beta 1/2$ subunit binds [xeno]oestrogens or whether additional portions of the $\beta 1$ subunit are necessary for transducing the response to BK_{Ca} channels. It is possible that a yet unidentified mediator, such as oestrogen receptor α or β, may be a necessary molecular component.

Gene transcripts for both the α and β isoforms of the oestrogen receptor are present in smooth muscle (Hodges *et al*., 2000); however, the hypothesis that one or both of these receptor isoforms is involved in this signalling pathway to BK_{Ca} channels has not been tested. As attractive as the possibility seems, there are difficulties with suggesting that either oestrogen receptor α or β is a transducing element in the membrane (Nadal *et al*., 2001). Neither oestrogen receptor α nor β has a predicted transmembrane spanning domain. This limitation might be overcome by post-translational modification; however, neither isoform has an obvious site. Additionally, while *Xenopus* oocytes (Valverde *et al*., 1999) and human embryonic kidney cells (Dick *et al*., 2001) serve as competent expression systems for studying the $\beta 1$-dependent effects of [xeno]oestrogens on BK_{Ca} channels, neither is thought to possess endogenous oestrogen receptors, and are commonly used to express them (Kahlert *et al*., 2000; Watson, 1991). Whether the plasma membrane receptor for [xeno]oestrogens is identical to, related to, or completely distinct from oestrogen receptor α or β remains a matter of debate.

Regardless of the molecular machinery involved, ICI 182,780 activates smooth muscle BK_{Ca} channels in a non-genomic fashion with an EC_{50} near 1 μM. This effect on BK_{Ca} channels in smooth muscle would not be expected to have clinical significance, as the therapeutic serum concentration in humans is approximately 12.6 ng ml^{-1} ($\approx$21 nM; (Howell *et al*., 1996)). Thus, the effects of ICI 182,780 on BK_{Ca} channel NP_o, and L-type Ca^{2+} channels (Ruehlmann *et*

al., 1998), *in vitro* are achieved only at much higher concentrations. However, the data suggest that molecules with the ability to bind nuclear oestrogen receptors, regardless of oestrogenic or anti-oestrogenic nature, activate BK_{Ca} channels through this membrane-delimited mechanism. Further research is required to identify the putative extracellular binding site for [xeno]oestrogens.

I thank Nancy Horowitz for preparing the cells used in this study. I appreciate the comments of Drs. Kenton M. Sanders and James L. Kenyon regarding this manuscript. Dr. Dick is the recipient of a postdoctoral National Research Service Award (F32 DK09947). NIH DK41315 supported this work.

References

BEHRENS, R., NOLTING, A., REIMANN, F., SCHWARZ, M., WALDSCHUTZ, R. & PONGS, O. (2000). hKCNMB3 and hKCNMB4, cloning and characterization of two members of the large-conductance calcium-activated potassium channel β subunit family. *FEBS Lett.,* **474,** 99–106.

BRENNER, R., PEREZ, G.J., BONEV, A.D., ECKMAN, D.M., KOSEK, J.C., WILER, S.W., PATTERSON, A.J., NELSON, M.T. & ALDRICH, R.W. (2000). Vasoregulation by the β1 subunit of the calcium-activated potassium channel. *Nature,* **407,** 870–876.

DICK, G.M., HUNTER, A.C. & SANDERS, K.M. (2002). Ethylbromide tamoxifen, a membrane-impermeant antiestrogen, activates smooth muscle calcium-activated large conductance potassium channels from the extracellular side. *Mol. Pharmacol.,* **61,** 1105–1113.

DICK, G.M., ROSSOW, C.F., SMIRNOV, S., HOROWITZ, B. & SANDERS, K.M. (2001). Tamoxifen activates smooth muscle BK channels through the regulatory β1 subunit. *J. Biol. Chem.,* **267,** 34594–34599.

DICK, G.M. & SANDERS, K.M. (2001). (Xeno)estrogen-sensitivity of smooth muscle BK channels conferred by the regulatory β1 subunit: A study of β1 knockout mice. *J. Biol. Chem.,* **276,** 44835–44840.

FALKENSTEIN, E., TILLMANN, H.C., CHRIST, M., FEURING, M. & WEHLING, M. (2000). Multiple actions of steroid hormones – a focus on rapid, nongenomic effects. *Pharmacol. Rev.,* **52,** 513–556.

HODGES, Y.K., TUNG, L., YAN, X.D., GRAHAM, J.D., HORWITZ, K.B. & HORWITZ, L.D. (2000). Estrogen receptors α and β: prevalence of estrogen receptor β mRNA in human vascular smooth muscle and transcriptional effects. *Circulation,* **101,** 1792–1798.

HOWELL, A., DEFRIEND, D.J., ROBERTSON, J.F., BLAMEY, R.W., ANDERSON, L., ANDERSON, E., SUTCLIFFE, F.A. & WALTON, P. (1996). Pharmacokinetics, pharmacological and anti-tumour effects of the specific anti-oestrogen ICI 182780 in women with advanced breast cancer. *Br. J. Cancer,* **74,** 300–308.

HOWELL, A., OSBORNE, C.K., MORRIS, C. & WAKELING, A.E. (2000). ICI 182,780 (Faslodex): development of a novel, 'pure' antiestrogen. *Cancer,* **89,** 817–825.

JORDAN, V.C. (2000). Progress in the prevention of breast cancer: concept to reality. *J. Steroid Biochem. Mol. Biol.,* **74,** 269–277.

KAHLERT, S., NUEDLING, S., VAN EICKELS, M., VETTER, H., MEYER, R. & GROHE, C. (2000). Estrogen receptor α rapidly activates the IGF-1 receptor pathway. *J. Biol. Chem.,* **275,** 18447–18453.

MCMANUS, O.B., HELMS, L.M., PALLANCK, L., GANETZKY, B., SWANSON, R. & LEONARD, R.J. (1995). Functional role of the β subunit of high conductance calcium-activated potassium channels. *Neuron,* **14,** 645–650.

NADAL, A., ROPERO, A.B., FUENTES, E. & SORIA, B. (2001). The plasma membrane estrogen receptor: nuclear or unclear? *Trends Pharmacol. Sci.,* **22,** 597–599.

ORIO, P., ROJAS, P., TORO, L. & LATORRE, R. (2002). MaxiK channel activation by estradiol and tamoxifen: role of the extracellular domain of the β1 subunit. *Biophys. J.,* **82,** 205A (abstract).

RUEHLMANN, D.O., STEINERT, J.R., VALVERDE, M.A., JACOB, R. & MANN, G.E. (1998). Environmental estrogenic pollutants induce acute vascular relaxation by inhibiting L-type Ca^{2+} channels in smooth muscle cells. *FASEB J.,* **12,** 613–619.

VALVERDE, M.A., ROJAS, P., AMIGO, J., COSMELLI, D., ORIO, P., BAHAMONDE, M.I., MANN, G.E., VERGARA, C. & LATORRE, R. (1999). Acute activation of Maxi-K channels (*hSlo*) by estradiol binding to the β subunit. *Science,* **285,** 1929–1931.

WATSON, C.S. (1991). Human estrogen receptor introduced into the Xenopus oocyte represses expression from an artificial frog estrogen response element. *J. Steroid Biochem. Mol. Biol.,* **38,** 423–431.

(Received March 12, 2002
Revised April 22, 2002
Accepted May 21, 2002)