



Tamoxifen metabolites acting *via* BK_{Ca} orchestrate the dynamics of K⁺ and Ca²⁺ in breast cancer cells

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The voltage- and calcium (Ca²⁺)-activated potassium (K⁺) channel of large conductance (BK_{Ca}) is aberrantly expressed in various breast cancer (BC) subtypes, including estrogen receptor (ER)-positive tumors. Increased proliferation of BC cells in response to tamoxifen (TAM) and its metabolites (TAM+M) has been shown to rely on the cell's BK_{Ca} status. However, the mechanism by which TAM+M impact on BK_{Ca} activity to promote malignancy is yet not clear. By examining murine MMTV-PyMT tumor-derived BC cells and human BC cell lines with a genetically encoded K⁺ ion indicator and electrophysiological recordings, we identified BK_{Ca}-dependent intracellular K⁺ signals and currents provoked by treatment with clinically relevant TAM+M in an ER-independent manner. In line with this, genetical or pharmacological blockade of BK_{Ca} significantly diminished the TAM+M-induced modulation of BK_{Ca} K⁺ currents and consequently also the drop of intracellular K⁺ ions in BC cells. Changes in the K⁺ balance subsequently triggered intra- and extracellular Ca²⁺ mobilization, which was in turn stimulated by the TAM+M-BK_{Ca} axis. Our results highlight that BK_{Ca} "onco-channels" may modulate the response of BC cells to TAM+M. Activation of the TAM+M-BK_{Ca} axis causes significant changes in K⁺ and Ca²⁺ ion homeostasis, which ultimately contributes to the outcome of endocrine-based BC pharmacotherapy.

Breast cancer (BC) is the most frequently diagnosed cancer and the leading cause of cancer-related death among women (1). BC comprises different subgroups based on the

expression of the human epidermal growth factor receptor (HER2⁺), the progesterone receptor (PR⁺) and/or the estrogen receptor (ER⁺) or triple-negative BC (TNBC) (2). Classical treatment often includes surgery, radiotherapy and, depending on the type of tumor, cytotoxic as well as endocrine therapy (3). In pursue of novel prognosticators, predictors, and putative treatment targets, molecular expression profiles defined four intrinsic BC subtypes (*i.e.* LumA, LumB, HER2-enriched, basal) (4), and more recently, a single-cell spatially resolved atlas revealed nine BC "ecotypes" (5). These recent insights into cell-based expression profiles enable better selection of therapeutic strategies and will promote target identification for novel personalized therapeutic approaches.

Ion channels have sparked interest as putative novel anti-cancer drug targets as they are frequently dysregulated in most cancer entities (6, 7). Of particular interest are potassium (K⁺) channels that contribute to malignant cell proliferation by perturbing the membrane potential, cell volume and calcium (Ca²⁺) signaling although K⁺ channels can also influence cell migration, invasion and apoptosis (8).

The voltage- and Ca²⁺-activated K⁺ channel of large conductance (9) alias BK_{Ca} or Slo1, is a transmembrane protein consisting of a tetramer of pore-forming α -subunits located in the plasma membrane (PM), Golgi apparatus, endoplasmic reticulum and mitochondria of BC cells (7, 10). Each α -subunit contains a voltage-sensing domain (S4) and two pore-forming loop segments (S5-S6) that cross the membrane. The cytoplasmic C-terminal tail of each α -subunit is composed of two high-affinity Ca²⁺-sensing sites located in the regulator of conductance (RCK)1 and RCK2 domains (10). In BC, BK_{Ca} promoted cell proliferation (7, 9, 11), metastatic spread, invasive growth in the brain (12) and was associated with high tumor stage and poor prognosis (11), hence, BK_{Ca} has been termed an "oncochannel" (13). Other hormone-sensitive cancers, such as cervical (14) and prostate cancer

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(15), and tumors that grow independently of hormones, such as glioma (16) and pancreatic ductal carcinoma (17), display altered BK_{Ca} channel expression together with a higher potential of malignancy. Interestingly, 17 β -Estradiol (E2) enhances the proliferation of BK_{Ca}-proficient murine and human BC cells (9), and particularly stimulates BK_{Ca} channel activity in human MCF-7 BC cells (18). Independent evidence for a link between E2 and an increase of the BK_{Ca} channel's open probability (P_O) was obtained from non-cancerous *Xenopus laevis* oocytes and smooth muscle cells in which these effects were mediated by the regulatory $\beta 1$ subunit that increases the sensitivity of the channel complex to Ca²⁺ (19–22).

In patients with BC having ER-positive tumors endocrine treatment with tamoxifen (TAM), high BK_{Ca} α -subunit expression levels modified their outcome in an unfavorable manner. This was recapitulated in *in vitro* experiments with TAM and its metabolites (TAM+M) that stimulated the growth of human and murine BC cell lines in a BK_{Ca}-dependent manner (9) as well as BK_{Ca}'s P_O in human MCF-7 cells (23). Both effects, *i.e.* a TAM-mediated increase in BK_{Ca} activity and the modulation of P_O , have also been observed in other model systems such as murine and canine colonic myocytes, and they were again dependent on the presence of the BK_{Ca} α and $\beta 1$ subunits (24, 25). Similarly, the P_O of avian BK_{Ca} α - and β -subunit complexes can be modulated by TAM (26).

Mechanistically, hyperpolarization of the PM induced by K⁺ channels such as BK_{Ca} increases the driving force for Ca²⁺ influx, for example, by store-operated Ca²⁺ entry (SOCE) or constitutive Ca²⁺ entry (CCE) (8). The resulting Ca²⁺ signaling in BC cells has been fundamentally linked to processes underlying proliferation, invasion, metastasis and apoptosis (27) and can be potentiated with TAM as shown in MCF-7 cells exposed to agonists such as ATP (28). The effect to release Ca²⁺ from intracellular stores such as the endoplasmic reticulum as well as the TAM-modulated Ca²⁺ entry through PM channels is likely ER-independent as it has been commonly observed in non-ER positive tumor cells such as human glioma (28), oral cancer cells (29), non-melanoma skin cancer (30), HepG2 human hepatoblastoma cells and bladder cancer cells (31), but also under physiological conditions in intact Madin-Darby canine kidney (MDCK) cells and human neutrophils (32) and platelets (33).

It remains, however, unclear whether and to what extent the effects of TAM on tumor-promoting BK_{Ca} channel activity and on Ca²⁺ signaling events depend on the same pathway in BC although such interactions between BK_{Ca} and the cytosolic Ca²⁺ are known from other cell systems (34–37).

Here, we investigated the impact of TAM+M on BK_{Ca} channel activity and Ca²⁺ signaling in murine and human BC cells using an integrated approach that on the one hand combined pharmacological tools and gene knockout models and on the other hand electrophysiological and imaging-based approaches for the visualization of Ca²⁺ and K⁺ ions in living cells with molecular docking strategies. These studies

aimed to uncover the underlying molecular mechanisms by which the TAM+M-BK_{Ca} axis contributes to the Ca²⁺ homeostasis of BC cells and thus to malignant cell behaviors that promote cancer progression.

Results

BK_{Ca} is expressed in clinically relevant BC biopsies and functionally relevant in human BC cells

Bulk gene expression analysis of 551 human derived BC specimen revealed different K⁺ channel transcript levels in ER⁺ BC. Consistent with a previous report (7), *KCNMA1* mRNA was significantly higher expressed in these BC specimens compared to other cancer-associated K⁺ channels (*KCNA3*, *KCNH1*, *KCNH2*, *KCNK5*, *KCNK9*, *KCNN3*, and *KCNN4*). Interestingly, *KCNMA1*-derived transcripts were also present in all individual samples analyzed (Fig. 1A). Transcript levels of *KCNMA1* in the ER⁺ human BC cell lines MCF-7 (hBK_{Ca}^{low}) and CAMA-1 (hBK_{Ca}^{medium}) and the ER⁻ human BC cell line MDA-MB-453 (hBK_{Ca}^{high}) confirmed highest expression level of BK_{Ca} channels in hBK_{Ca}^{high} BC cells (Fig. 1B). An investigation of BK_{Ca} channel subunits confirmed β -, γ -, and Lingo1 transcripts in the BC cell lines, which, however, showed significantly lower expression levels in hBK_{Ca}^{high} compared to the pore-forming α -subunit of BK_{Ca} (Fig. S1A).

To verify the functionality of BK_{Ca} channels in these human BC cell lines, whole-cell patch-clamp recordings were performed to obtain current density-voltage relationships in hBK_{Ca}^{low}, hBK_{Ca}^{medium} and hBK_{Ca}^{high} BC cells. At +120 mV and physiological intracellular Ca²⁺ concentrations ([Ca²⁺]_i), K⁺ outward currents of hBK_{Ca}^{high} BC cells were significantly increased over hBK_{Ca}^{medium} and hBK_{Ca}^{low} BC cells (Fig. 1C). Representative current-over-time traces of hBK_{Ca}^{low}, hBK_{Ca}^{medium} and hBK_{Ca}^{high} (Fig. 1, D–I left) were normalized to cell size in current density calculations (Fig. 1, D–I right). K⁺ outward currents that were reliably detected in response to depolarization steps in hBK_{Ca}^{low} cells were insensitive to the specific BK_{Ca} blocker paxilline (PAX) (Fig. 1D). In contrast, peak K⁺ outward currents were significantly inhibited by PAX treatment in hBK_{Ca}^{medium} and hBK_{Ca}^{high} cells (Fig. 1, E and F), verifying previously reported patch-clamp recordings on hBK_{Ca} localized in the PM of MCF-7 and MDA-MB-453 cells (7, 9).

Additionally, immunofluorescence (IF) staining shows a higher abundance of BK_{Ca} in hBK_{Ca}^{high} compared to hBK_{Ca}^{low} BC cells (Fig. S1B), confirming whole-cell patch-clamp experiments. Importantly, phalloidin staining indicates that the cell size of hBK_{Ca}^{low} and hBK_{Ca}^{high} BC cells varies (Fig. S1B), explaining the differences in the conductance and the resulting current densities over time in the whole-cell patch-clamp recordings.

To further evaluate whether hBK_{Ca}^{high} activity is sensitive to the BK_{Ca} specific activator NS11021, the compound was added to the bath solution (Fig. 1G), resulting in higher K⁺ outward currents during patch-clamp recordings. Further, we

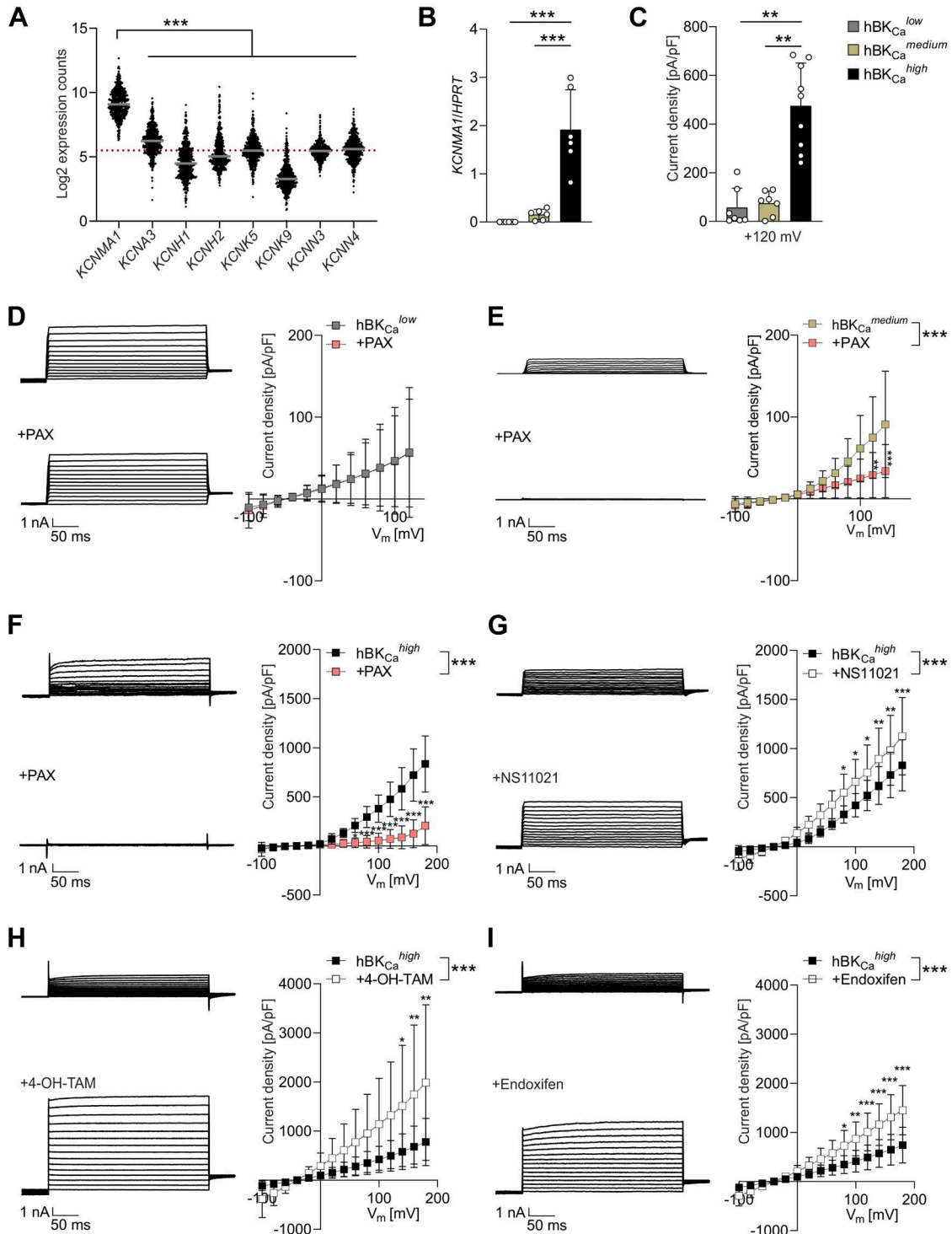


Figure 1. BK_{Ca} expression in human BC biopsies and electrophysio-/pharmacological properties of human BC cell BK_{Ca} channels. mRNA expression levels of (A) *KCNMA1* and selected cancer-associated K⁺ channels from human BC biopsies (partly adapted from (7)) and (B) *KCNMA1* in human BC cell lines hBK_{Ca}^{low}, hBK_{Ca}^{medium} and hBK_{Ca}^{high}. C, current-density-voltage relationships of hBK_{Ca}^{low}, hBK_{Ca}^{medium} and hBK_{Ca}^{high} compared at +120 mV (data of selected voltage was adapted from the recordings in D–F). D–I, representative whole-cell current recordings (left) and current density-voltage relationships (right) of hBK_{Ca}^{low}, hBK_{Ca}^{medium} and hBK_{Ca}^{high} BC cells, (D, E, F) before and during paxilline (PAX), (G) NS11021, (H) 4-OH-TAM, and (I) endoxifen treatment. Data represents average ± SD. n(biopsies) = (A) 551; n(independent experiments) = (B) 6; n(cells) = (C) 7–9, (D) 7, (E) 7, (F) 9, (G) 11, (H) 8, (I) 8, with *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001. A and C, Kruskal-Wallis test followed by Dunn’s multiple comparisons test. B, one-way ANOVA followed by Tukey’s multiple comparisons test. D–I two-way ANOVA followed by Šidák’s multiple comparisons test. Statistical values, probe and primer sequences are described in detail in Table S1, Table S10, and Table S11, respectively.

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observed that TAM+M (4-OH-TAM and endoxifen) elevated K⁺ outward currents in hBK_{Ca}^{high} cells massively, and even more than NS11021 (Fig. 1, H and I), suggesting that TAM+M impacts the ion homeostasis of BC cells by modulating the subcellular K⁺ homeostasis *via* BK_{Ca} channel activation. Importantly, we observed that the majority of successfully measured hBK_{Ca}^{high} cells indeed responded to 4-OH-TAM (72.73%) and endoxifen (61.54%) with increased K⁺ currents (Fig. S1C), which is in line with the variability of BK_{Ca} protein levels in individual hBK_{Ca}^{high} cells (Fig. S1B). Potential reasons for this “patchy” BK_{Ca} expression profile were not investigated, but might be explained by the previously demonstrated cell cycle-dependent variation of K⁺ channel expression in non-cancerous and cancer cells (6, 38, 39), as our IF and patch-clamp experiments were carried out in non-synchronized cells.

Together, these findings confirm the presence of BK_{Ca} channels in ER⁺ human BC tissue and hBK_{Ca}^{low/medium/high} BC cells. In addition, functional BK_{Ca} channel expression in hBK_{Ca}^{high} correlated with TAM+M-mediated effects on K⁺ outward currents.

TAM+M modulate K⁺ dynamics through BK_{Ca} independently of ER

Based on the observed effects on whole-cell currents by TAM+M (Fig. 1), we investigated K⁺ homeostasis utilizing IcyM GEPII1.0, a well-established cytosolic FRET-based K⁺ sensor (7, 40). Next to hBK_{Ca}^{low}, hBK_{Ca}^{medium} and hBK_{Ca}^{high} BC cell models, we additionally studied SKBr-3 (ER⁻) as hBK_{Ca}^{low} cell line (Fig. 2). The latter was selected based on the RNAseq data in Cancer Cell Line Encyclopedia (CCLE) library and included in our analysis to address how the absence of ER (and low BK_{Ca}) in human BC cells affect the response to TAM+M.

Alterations in cytosolic K⁺ concentration ([K⁺]_i) were first induced by the BK_{Ca} channel opener NS11021 in MCF-7 hBK_{Ca}^{low} (ER⁺), CAMA-1 hBK_{Ca}^{medium} (ER⁺) and MDA-MB-453 hBK_{Ca}^{high} (ER⁻) (Fig. 2, A and B). BK_{Ca} abundance of these cells correlated with the [K⁺]_i loss, which was significantly different in hBK_{Ca}^{high} (ER⁻) compared to the [K⁺]_i response seen in hBK_{Ca}^{medium} (ER⁺) and hBK_{Ca}^{low} (ER⁺), strongly suggesting that these [K⁺]_i alterations occurred irrespective of the ER status of the cells (Fig. 2B). Importantly, in hBK_{Ca}^{low} cells expressing RFP-tagged BK_{Ca} (+BK_{Ca}-RFP^{rescue}) we consistently observed a massive drop in [K⁺]_i compared to respective control hBK_{Ca}^{low} (ER⁺) cells, regardless of whether RFP (+RFP) was present or not (Fig. 2A). In addition, we made use of a previously mapped panel of patient-derived BK_{Ca} loss- and gain-of-function mutations (41). Interestingly, one of the loss-of-function channel variants carries the G354S mutation (41), a site residing within the selectivity filter of BK_{Ca}, which is highly conserved among K⁺ selective ion channels (42). This loss-of-function mutant results in a “pore-death” variant of BK_{Ca} with dramatically reduced K⁺ conductance and a lower selectivity to K⁺, which causes symptoms like ataxia, dyskinesias and cognitive

impairment in a young patient carrying the BK_{Ca} G354S mutation (43). We induced the G354S mutation in our established BK_{Ca}-RFP construct by site-directed mutagenesis. Importantly, the expression of BK_{Ca}^{G354S}-RFP (+BK_{Ca}^{G354S}) in hBK_{Ca}^{low} (ER⁺) BC cells demonstrated levels comparable to the empty vector control (+RFP) and “wild type” BK_{Ca}-RFP^{rescue} (Fig. S1D). FRET-based K⁺ recordings evoked by NS11021 in hBK_{Ca}^{low} (ER⁺) BC cells expressing BK_{Ca}^{G354S} were, however, significantly impaired compared to functional “wild type” BK_{Ca}-RFP^{rescue} channels (Fig. 2A). Employing a related BK_{Ca} channel opener, NS1619, showed similar effects on [K⁺]_i in hBK_{Ca}^{low} (ER⁺) and hBK_{Ca}^{high} (ER⁻) BC cells (Fig. S1E).

To test if 4-OH-TAM and endoxifen impact the cell's K⁺ dynamics in a BK_{Ca}- and/or concentration-dependent manner, we analyzed [K⁺]_i in hBK_{Ca}^{low} (ER⁺) and hBK_{Ca}^{high} (ER⁻) cell lines. Indeed, 4-OH-TAM induced a significantly higher drop of [K⁺]_i in hBK_{Ca}^{high} (ER⁻) compared to hBK_{Ca}^{low} (ER⁺) cells, an effect that was consistent for all concentrations tested (Figs. 2C and S1F). Furthermore, administration of endoxifen resulted in a concentration-dependent K⁺ loss in hBK_{Ca}^{high} (ER⁻) as indicated by the decreasing FRET ratio signal. At the maximal concentration tested, differences in [K⁺]_i in hBK_{Ca}^{low} (ER⁺) and hBK_{Ca}^{high} (ER⁻) reached the level of significance (Fig. 2D). In addition, we compared the FRET ratio signals recorded from SKBR-3 hBK_{Ca}^{low} (ER⁻) and hBK_{Ca}^{medium} (ER⁺) to hBK_{Ca}^{low} (ER⁺) and hBK_{Ca}^{high} (ER⁻) BC cells treated with endoxifen. This analysis validated that the TAM+M-mediated K⁺ loss correlated with the BK_{Ca} expression level but occurred irrespective of the cell's ER status (Figs. 2E and S1G). Subsequently, we excluded the potential interference caused by growth factors and steroid hormones. Therefore, cells were cultured in charcoal-stripped bovine serum (CCS), a reagent leading to significant hormone deprivation, before monitoring live-cell K⁺ changes by FRET imaging. In hBK_{Ca}^{low} and hBK_{Ca}^{high} BC cells, we reproduced previous findings on TAM+M-induced [K⁺]_i alterations, suggesting that the response to TAM+M is independent of media supplements, *i.e.* of endocrine pathways (Fig. S2, A and B).

To more directly evaluate whether steroid hormones influence the K⁺ homeostasis in a manner comparable to the anti-estrogenic TAM+M, we investigated changes in the K⁺ FRET ratio signal in response to E2. Pro-proliferative effects of E2 on human BC cells and stimulatory effects on BK_{Ca} channels have been reported (9, 18) but strikingly, at least in non-cancerous tissue, may exclusively depend on BK_{Ca}'s auxiliary subunit β1 (21, 22). In the observed timeframe, however, live-cell K⁺ imaging revealed only minor effects of E2 on the K⁺ balance in hBK_{Ca}^{low} and hBK_{Ca}^{high}, and no differences between both BC cell models, suggesting E2 is linked to BK_{Ca} by a different mode of action (Fig. 2F).

To additionally validate and broaden our data on TAM+M-mediated [K⁺]_i, endoxifen was administered to hBK_{Ca}^{low}-expressing BK_{Ca}-RFP^{rescue} or loss-of-function mutant BK_{Ca}^{G354S} cells or cells solely expressing RFP as a control. Compared to hBK_{Ca}^{low}, BK_{Ca}-RFP^{rescue} expressing BC cells showed a massive decrease of [K⁺]_i, whereas cells expressing

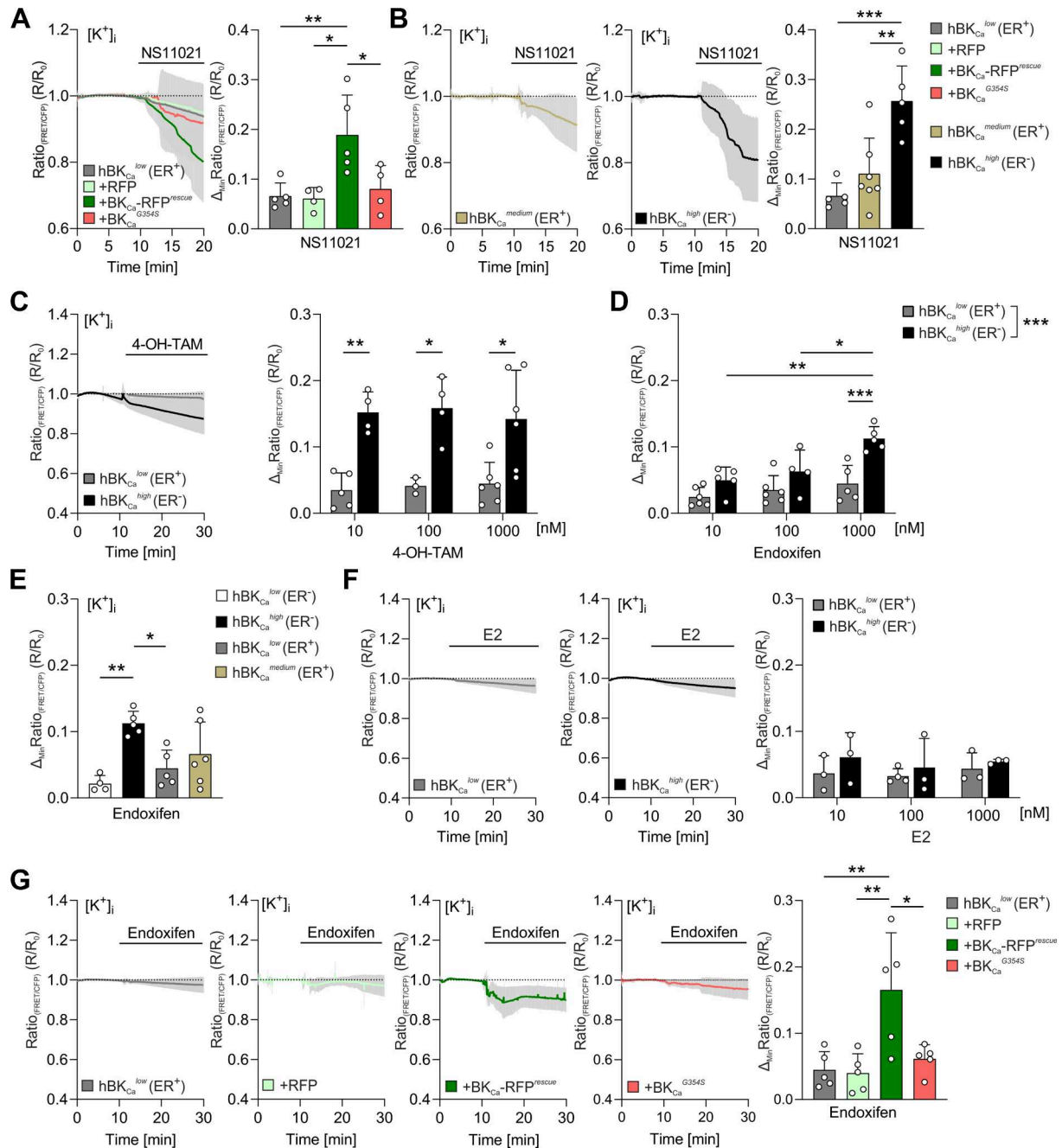


Figure 2. TAM+M trigger cytosolic K⁺ dynamics in BC cells independently of ER. (A, B, C, F, and G), K⁺ FRET-ratio signals obtained by live-cell imaging of cytosolic K⁺ concentrations ([K⁺]_i) over time of hBK_{Ca}^{low} (ER⁺ or ER⁻), hBK_{Ca}^{medium} (ER⁺) and hBK_{Ca}^{high} (ER⁻) BC cells and (A–G) corresponding bar charts with individual data points for statistical analysis, which is based on the difference of the basal (R₀) to the minimal (R) K⁺ FRET/CFP ratio signals and expressed as Δ_{min}(R/R₀). Alterations of [K⁺]_i were induced by bath-administration of (A, B) BK_{Ca} activator NS11021 (hBK_{Ca}^{low} from A adapted in B), active TAM metabolites (C) 4-OH-TAM and (D, E, G) endoxifen or (F) E2 following 10 min of FRET recording at basal conditions. ER⁺ hBK_{Ca}^{low} cells expressed in addition either (A, G) RFP (+RFP), RFP-tagged BK_{Ca} (+BK_{Ca}-RFP^{rescue}) or RFP-tagged “pore-death” BK_{Ca}^{G354S} (+BK_{Ca}^{G354S}) as indicated. FRET-ratio signal responses are displayed for (C, E, G) 1 μM 4-OH-TAM or endoxifen and (F) 1 μM E2. Data represents average ± SD. n(independent experiments) = (A) 4–5, (B) 5–7, (C) 3–6, (D) 4–6, (E) 4–6, (F) 3–4, (G) 5 with *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001. (A, B, E, G) One-way ANOVA followed by Tukey’s multiple comparisons test and (C, D, F) Two-way ANOVA followed by Tukey’s multiple comparisons test with significant group differences in (C, D). Statistical values are described in detail in Table S2.

only RFP did not exhibit a drop in [K⁺]_i, *i.e.* FRET signals remained at the level of hBK_{Ca}^{low} (Fig. 2G and S2C). “Pore-death” BK_{Ca}^{G354S} expression in hBK_{Ca}^{low} prevented the endoxifen-induced [K⁺]_i loss compared to BK_{Ca}-RFP^{rescue} channels (Fig. 2G), supporting the hypothesis of a TAM+M-promoted BK_{Ca} activity.

Finally, loss of [K⁺]_i should lead to an increased extracellular K⁺ concentration ([K⁺]_{ex}). To examine [K⁺]_{ex}, we applied recombinant purified GEPII1.0 as previously described (40, 44). Gramicidin, a K⁺ ionophore, was used to induce maximal K⁺ release from cells (Fig. S2D). Also, we observed a clear trend for 4-OH-TAM and a significant increase of [K⁺]_{ex}

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in hBK_{Ca}^{high} BC cells treated with endoxifen (Fig. S2E), confirming that the decrease in [K⁺]_i ultimately results in an efflux of K⁺ to the extracellular space.

In sum, TAM+M treatment initiates a cascade causing a drop of [K⁺]_i and consequently elevated [K⁺]_{ex} levels. The K⁺ alterations observed are closely related to the cell's BK_{Ca} channel abundance but occur independently of the ER status of all BC cell lines studied.

TAM+M-mediated effects on K⁺ dynamics are abolished by genetic or pharmacological inactivation of endogenous BK_{Ca} channels

In a BC mouse model based on the mouse mammary tumor virus (MMTV) polyoma middle T antigen (PyMT), genetic ablation of the BK_{Ca} α -subunit showed beneficial outcomes regarding tumor-free and overall survival in comparison to respective BK_{Ca}-proficient control littermates. Interestingly, and contrary to expectations from clinical use of the anti-estrogenic compounds, TAM+M treatment promoted the proliferative behavior of isolated murine BC cells obtained from the MMTV-PyMT model only in the presence of BK_{Ca} (9). Based on these previous studies, murine MMTV-PyMT^{tg/+} WT (mBK_{Ca}^{WT}) and BKKO (mBK_{Ca}^{KO}) derived BC cells were isolated from primary tumors and the cytosol-targeted K⁺ sensor Ic-LysM GEPII1.0 was expressed to study basal [K⁺]_i and changes induced by TAM+M. In agreement with the

live-cell imaging experiments in human BC cells, endoxifen and 4-OH-TAM induced a BK_{Ca}-dependent drop in [K⁺]_i (Fig. 3, A and B and Fig. S2F). Further, K⁺ efflux in mBK_{Ca}^{WT} was sensitive to PAX, resulting in [K⁺]_i loss equalizing with the one observed in mBK_{Ca}^{KO} (Fig. 3A). Accordingly, TAM+M-induced BK_{Ca}-mediated [K⁺]_i changes in hBK_{Ca}^{high} (ER⁻) (hereafter referred to as hBK_{Ca}^{high}) were sensitive to PAX, whereas hBK_{Ca}^{low} (ER⁺) (hereafter referred to as hBK_{Ca}^{low}) did not respond to pharmacological BK_{Ca} channel inhibition (Fig. 3, C and D and Fig. S2G).

These findings support the notion that murine and human BK_{Ca} channels respond similarly to TAM+M with respect to the effect of these compounds on the cell's K⁺ balance. Further, genetic and pharmacological inhibition of endogenous BK_{Ca} prevented the observed alterations in [K⁺]_i pointing towards a common TAM+M-BK_{Ca} axis.

TAM+M stimulate a rise in [Ca²⁺]_i levels through BK_{Ca}

Multiple hallmarks of cancer are influenced by alterations in K⁺ channels expression and Ca²⁺ ion dynamics including cell cycle control, migration and adhesion, as well as apoptosis (45, 46). Previous work indicated that cancer cell Ca²⁺ signaling is affected by aberrant K⁺ channel expression (47). Mechanistically, K⁺ channel activation and the resulting K⁺ efflux provide a driving force for Ca²⁺ influx (8, 48). Therefore, we examined the potential influence of TAM+M on

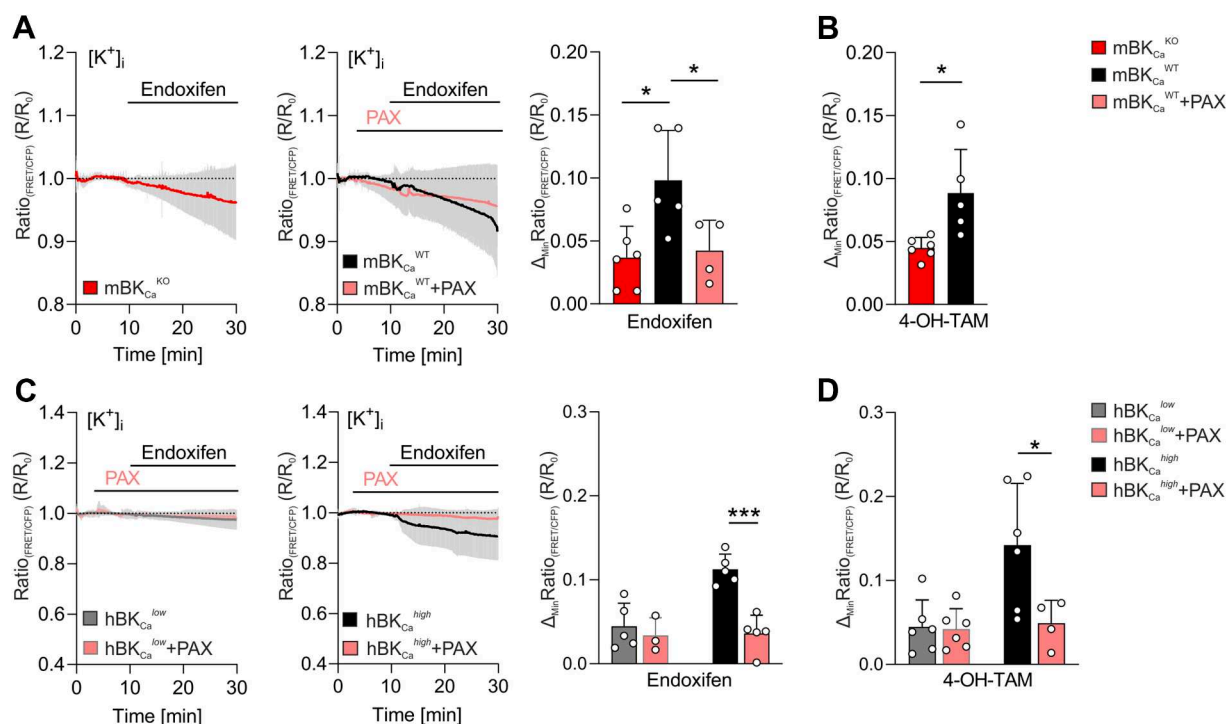


Figure 3. TAM+M-mediated effects on murine and human BC cell K⁺ homeostasis require functional BK_{Ca} channels. A–D, FRET-ratio signals and corresponding statistics of cytosolic K⁺ ([K⁺]_i) dynamics over time in murine MMTV-PyMT BK_{Ca} WT (mBK_{Ca}^{WT}) and KO (mBK_{Ca}^{KO}) and human hBK_{Ca}^{low} and hBK_{Ca}^{high} BC cells. Live-cell K⁺ imaging was performed prior and after bath-application of (A) endoxifen, (B) 4-OH-TAM, (A and C) PAX+endoxifen and (D) PAX+4-OH-TAM. C, PAX+endoxifen and (D) PAX+4-OH-TAM treated hBK_{Ca}^{low} and hBK_{Ca}^{high} BC cells were compared with 1 μ M 4-OH-TAM and 1 μ M endoxifen (Fig. 2, C and D) respectively. Data represents average \pm SD. n(isolated tumors) = (A and B) 4–6 and n(independent experiments) = (C) 3–5, (D) 4–6 with * p \leq 0.05 and *** p \leq 0.001. A, one-way ANOVA followed by Tukey's multiple comparisons test. (B) Unpaired t test. C and D two-way ANOVA followed by Sidak's multiple comparisons test. Statistics are described in detail in Table S3.

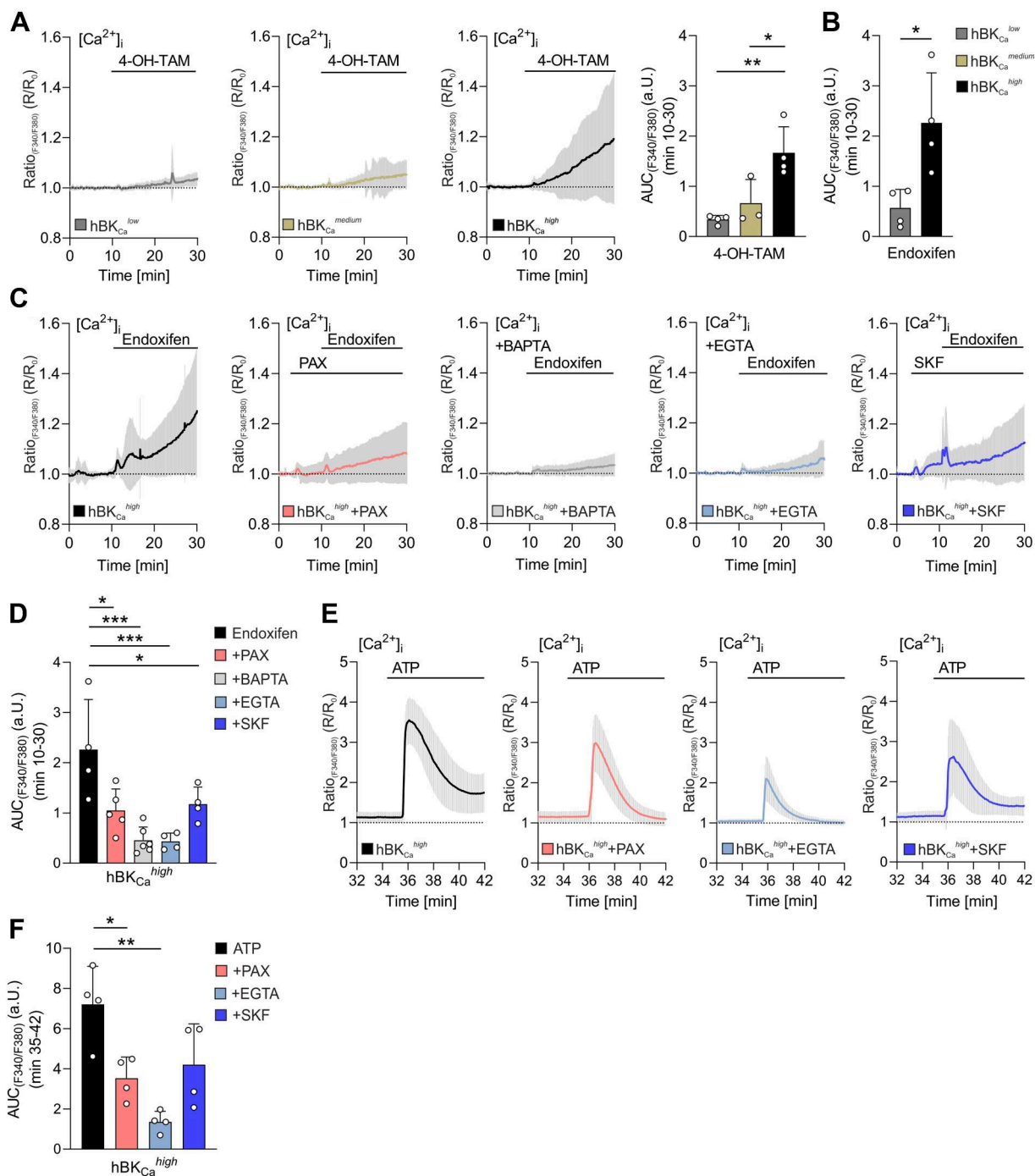


Figure 4. TAM+M-induced BK_{Ca} activation elevates [Ca²⁺]_i. Fluorescence emission ratio signals over time and area under the curve (AUC) statistics of hBK_{Ca}^{low}, hBK_{Ca}^{medium}, and hBK_{Ca}^{high} BC cells loaded with the ratiometric Ca²⁺ dye Fura-2 in presence of (A) 4-OH-TAM or (B) endoxifen. Fluorescence emission ratio traces (F₃₄₀/F₃₈₀) of hBK_{Ca}^{low} (Fig. S3A) were compared with hBK_{Ca}^{high} (Fig. 4C). F₃₄₀/F₃₈₀ nm emission ratio for Fura-2 loaded (C) hBK_{Ca}^{high} BC cells in response to endoxifen in addition with further treatment by (from left to right) BK_{Ca}²⁺ inhibitor PAX, BAPTA AM (BAPTA) for intracellular Ca²⁺ depletion, EGTA-buffered extracellular Ca²⁺-free solution, and TRP/C, STIM/ORAI1 channel blocker SKF-96365 (SKF). D, analysis of the area under the curve (AUC) of Fura-2 loaded hBK_{Ca}^{high} BC cells under recording conditions specified in (C). E, fura-2 fluorescence emission ratio signals (F₃₄₀/F₃₈₀ nm) in response to Ca²⁺ changes over time and statistics obtained from monitoring hBK_{Ca}^{high} BC cells after pre-treatment with endoxifen, PAX+endoxifen, EGTA+endoxifen, SKF+endoxifen (from left to right) followed by extracellular ATP application to provoke emptying of intracellular Ca²⁺ stores. F, analysis of the AUC of Fura-2 loaded hBK_{Ca}^{high} BC cells under recording conditions specified in (E) i.e. after endoxifen pre-treatment (for 20 min followed by a 5 min washout) in addition to the treatments as specified in the panels. Data represents average ± SD. n(independent experiments) = (A, B) 3-4, (C, D) 4-6, (E, F) 4, with *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001. A, D, F, one-way ANOVA followed by Tukey's multiple comparisons test. B, unpaired t test. Statistical values are described in detail in Table S4.

TAM-BK_{Ca} axis in BC cells

Ca²⁺ homeostasis using Fura-2, a ratiometric (F₃₄₀/F₃₈₀) Ca²⁺ indicator. 4-OH-TAM administration elevated [Ca²⁺]_i levels in a BK_{Ca}-dependent manner. Accordingly, the rise in Fura-2 ratio was significantly lower in hBK_{Ca}^{low} and hBK_{Ca}^{medium} cells compared to hBK_{Ca}^{high} cells (Fig. 4A). Application of the alternative TAM+M endoxifen confirmed these results as the largest increase in [Ca²⁺]_i was induced in hBK_{Ca}^{high} (Fig. 4B). We also treated hBK_{Ca}^{low} (ER⁻) cells with endoxifen and observed a similar increase in the Ca²⁺ signal compared to hBK_{Ca}^{low} (ER⁺) cells (Fig. S3A), indicating TAM+M act on Ca²⁺, independent from ER-mediated genomic events.

Next, we monitored [Ca²⁺]_i in hBK_{Ca}^{high} cells exposed to endoxifen and PAX for pharmacological BK_{Ca} inhibition. Under these co-treatment conditions, the endoxifen-induced increase in Fura-2 ratio was significantly diminished, suggesting that TAM+M require functional BK_{Ca} channels to affect [Ca²⁺]_i (Fig. 4, C and D). To examine the Ca²⁺ accumulation in response to TAM+M in more detail, we depleted intra- and extracellular Ca²⁺ stores by administration of BAPTA AM (BAPTA) and EGTA, a cell-permeant and -impermeant chelator of Ca²⁺, respectively, in Ca²⁺-free buffer. Under both conditions Fura-2 ratio signals were significantly decreased during TAM+M treatment (Fig. 4, C and D), suggesting that Ca²⁺ mobilization across the PM from extracellular sources and from intracellular stores contributes to the observed increase in [Ca²⁺]_i. In BC cells, Ca²⁺ release from the endoplasmic reticulum is recognized by the Ca²⁺ store sensor protein STIM and results in SOCE, which is an important mechanism to raise [Ca²⁺]_i. Ultimately, SOCE is triggering a Ca²⁺ entry pathway involving PM Ca²⁺ channels (f. ex. ORAI) to restore [Ca²⁺]_i (49). High log₂ transcript expression counts of *STIM1*, *ORAI1* and *ORAI3* were identified by gene expression analysis of human BC biopsies (Fig. S3B). In subsequent Fura-2 experiments, we used SKF-96365 (SKF), an inhibitor of SOCE (50) and TRP/C channels (51). We recognized an initial increase in [Ca²⁺]_i levels (Fig. 4C) which was also previously observed to be induced in nasopharyngeal carcinoma cells by SKF-mediated release of Ca²⁺ in the endoplasmic reticulum (52) or, by directly affecting Ca²⁺ entry in human leukemic HL-60 and MDCK cells (53, 54) and/or by enhancing Na⁺/Ca²⁺ exchanger function in glioblastoma cells (55). After Fura-2 fluorescence emission ratio signals stabilized in response to SKF, endoxifen treatment was still effective in increasing [Ca²⁺]_i, albeit not to the same extent as in the absence of SKF (Fig. 4, C and D). We conclude from the Fura-2 measurements that STIM-ORAI complexes, as well as PM Ca²⁺ channels (TRP/C), contribute to the BK_{Ca}-dependent cellular [K⁺]_i loss in response to endoxifen.

To investigate the influence of TAM+M on the mobilization of Ca²⁺ from intracellular stores, hBK_{Ca}^{high} were stimulated with adenosin-5'-triphosphate (ATP) that acts through purinergic receptors to subsequently trigger the release of Ca²⁺ from endoplasmic reticulum *via* inositol-triphosphate (IP₃) (56–58). ATP was applied following pre-treatment of the cells with endoxifen under basal conditions or in the presence of PAX, EGTA or SKF (Fig. 4E from left to right). Statistical analyses revealed significantly lower levels of ATP-

induced release of Ca²⁺ to accumulate as [Ca²⁺]_i under PAX and EGTA conditions (Fig. 4F). These results indicate TAM+M-induced BK_{Ca}-dependent K⁺ dynamics increase the driving force for Ca²⁺ uptake from both extracellular and intracellular stores. Because PAX-mediated inhibition of BK_{Ca} decreased the ATP-induced rise in [Ca²⁺]_i, we conclude that Ca²⁺ released from intracellular stores is functionally linked to Ca²⁺-activated BK_{Ca} channel activity and thus part of a feed-forward mechanism.

Using Fura-2 as Ca²⁺ sensitive probe highlights that TAM+M, through BK_{Ca} activation, causes a K⁺ loss across the PM, which enhances the driving force for Ca²⁺ influx *via* PM localized store- and non-store- operated Ca²⁺ channels. It additionally appears that Ca²⁺ release from intracellular stores, *i.e.* the endoplasmic reticulum, contributes to the observed rise in [Ca²⁺]_i. hBK_{Ca}^{low} BC cells or pharmacological channel blockade reduced all TAM+M-induced changes in [Ca²⁺]_i, proving that BK_{Ca} activity is essential to functionally connect TAM+M to Ca²⁺ signaling.

TAM+M-induced BK_{Ca}-activity mediates voltage-and Ca²⁺ alterations which sustain BK_{Ca} stimulation

As TAM+M affects BK_{Ca}-dependent K⁺ and Ca²⁺ dynamics, we next monitored [K⁺]_i, again employing the FRET-based K⁺ probe Ic-LysM GEPII1.0 while (i) chelating intracellular Ca²⁺ using BAPTA AM, (ii) under Ca²⁺-free conditions with extracellular EGTA, and after blockade of PM TRP/C and STIM/ORAI using SKF. Interestingly, none of these experimental conditions prevented induction of the TAM+M-mediated drop in [K⁺]_i (Fig. 5A). Importantly, the initial drop in [K⁺]_i induced by SKF did not lead to continuous BK_{Ca} activation in absence of endoxifen (Fig. S3C).

Hence, the systematic elimination of intra- and extracellular Ca²⁺ sources did not interfere with the TAM+M-induced BK_{Ca}-dependent K⁺ dynamics in hBK_{Ca}^{high}, indicating that the interaction of TAM+M with BK_{Ca} precedes any subsequent events on [Ca²⁺]_i (Fig. 5B). To confirm these K⁺ live-cell imaging results, whole-cell patch-clamp experiments were performed under EGTA-buffered, Ca²⁺-free conditions to record current density-voltage relationships of hBK_{Ca}^{high} cells. In the absence of [Ca²⁺]_{ex}, we again identified significantly elevated K⁺ outward currents in response to endoxifen (Fig. 5C), while the patch success rate decreased under EGTA-buffered conditions (Figs. S1C, 50%) compared to physiological [Ca²⁺]_{ex}-buffered bath solution (Fig. 1I and S1C, 61.54%).

The observed changes in ion homeostasis and in particular the activation of BK_{Ca} should affect the membrane potential of BC cells. The fluorescent and slow-response voltage-sensitive probe DiBAC₄(3) (59) was therefore used to detect changes in Ψ_{PM} (ΔΨ_{PM}) in hBK_{Ca}^{high} and hBK_{Ca}^{low} BC cells in response to TAM+M. Endoxifen significantly increased DiBAC₄(3) fluorescence intensity in hBK_{Ca}^{high}, but not in hBK_{Ca}^{low} BC cells, suggesting Ψ_{PM} was more depolarized by TAM+M administration if BK_{Ca} was expressed (Fig. 5, D and F). Further, Ψ_{PM} was recorded during pharmacological inhibition of BK_{Ca} with PAX, intracellular depletion of Ca²⁺ by

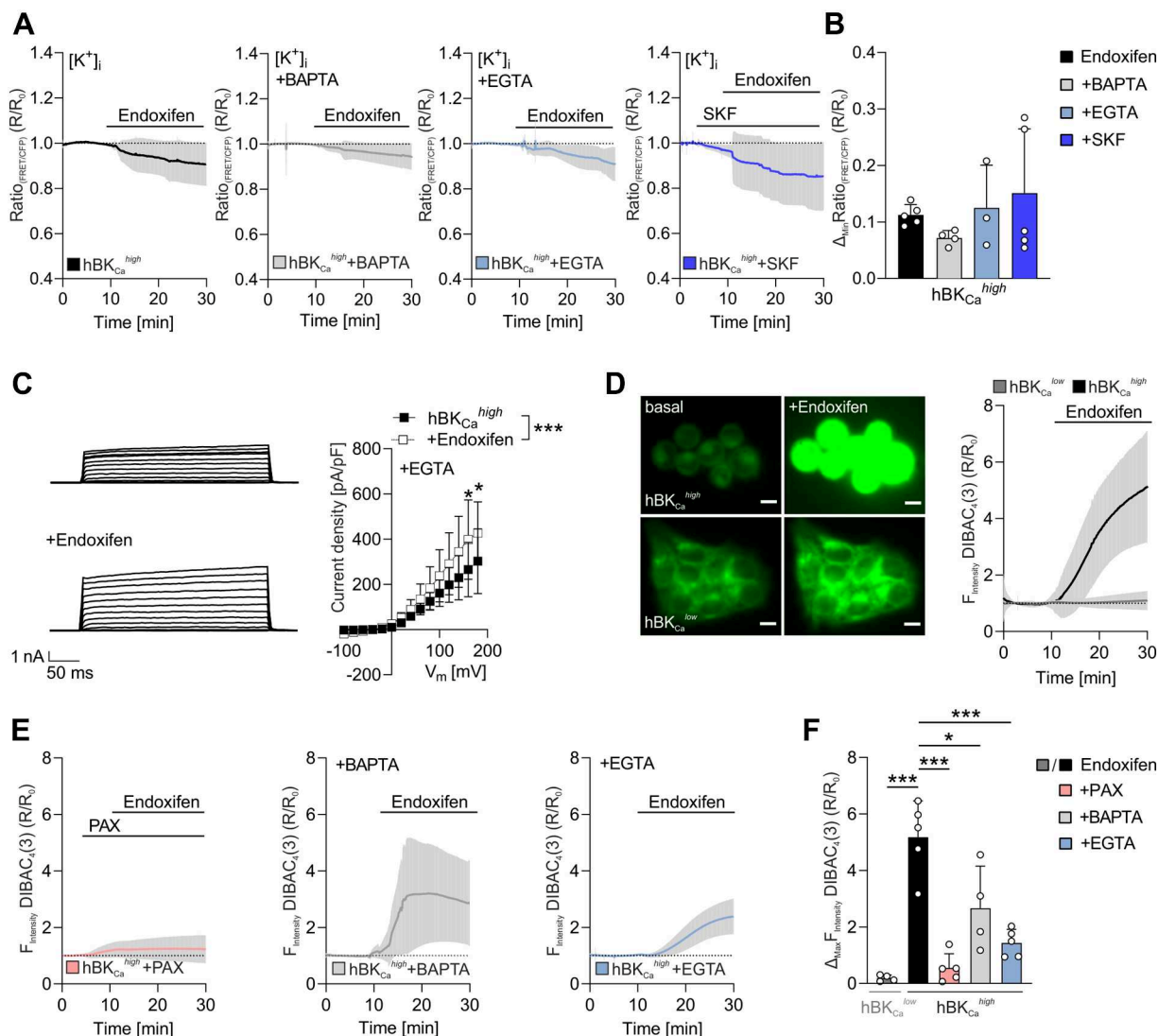


Figure 5. The TAM+M-BK_{Ca} channel axis, by modulating [K⁺]_i, initiates intracellular Ca²⁺ signals and a depolarized Ψ_{PM} in BC cells. *A*, FRET ratio signals of cytosolic K⁺ changes over time and *B*) statistical presentation of minimal (R) to basal (R₀) ratio (Δ_{Min}) monitored in hBK_{Ca}^{high} BC cells in presence of endoxifen (Fig. 2D, 1 μ M endoxifen), depleted intracellular Ca²⁺ by BAPTA AM (BAPTA), Ca²⁺-free, EGTA-buffered extracellular conditions, and TRP/C, STIM/ORAI1 channel block by SKF-96365 (SKF) (from left to right). *C*, representative whole-cell current tracings (left) and current density-voltage curves (right) of hBK_{Ca}^{high} BC cells in presence of EGTA before and after endoxifen administration. *D*, representative fluorescence images (basal (left), endoxifen (middle)) and fluorescence intensity signals recorded over time (right) from hBK_{Ca}^{low} and hBK_{Ca}^{high} BC cells loaded with the membrane potential (Ψ_{PM})-sensitive dye DiBAC₄(3) and administration of endoxifen. Scale bar = 10 μ m. *E* fluorescence intensity signals of hBK_{Ca}^{high} BC cells loaded with Ψ_{PM} -sensitive dye DiBAC₄(3) in the presence of PAX+endoxifen, BAPTA AM+endoxifen and EGTA+endoxifen. *F*, quantification of endoxifen-induced maximal change (Δ_{Max}) in DiBAC₄(3) fluorescence intensity in hBK_{Ca}^{low} and hBK_{Ca}^{high} BC cells under recording conditions that are specified in *D*, *E*. Data represents average \pm SD. *n*(independent experiments) = (*A*, *B*) 3-5, (*C*) 7, (*D*, *E*, *F*) 4-5 with **p* < 0.05, ***p* < 0.01, ****p* < 0.001. *B*, one-way ANOVA followed by Tukey's multiple comparisons test. *C*, two-way ANOVA followed by Sidák's multiple comparisons test. *F*, one-way ANOVA followed by Dunnett's multiple comparisons test. Statistical values are described in detail in Table S5.

BAPTA AM and [Ca²⁺]_{ex}-free conditions with EGTA (Fig. 5, *E* and *F*). Again, increased fluorescence intensity indicated a more depolarized Ψ_{PM} , but this TAM+M-induced effect in hBK_{Ca}^{high} cells was largely abrogated by pharmacological BK_{Ca} inhibition and considerably reduced in the presence of the Ca²⁺-chelators (Fig. 5*F*).

Combined, TAM+M influences [K⁺]_i dynamics by BK_{Ca} activation, leading to depolarized $\Delta\Psi_{PM}$ and mobilization of intracellular and extracellular Ca²⁺ sources to maintain BK_{Ca} stimulation.

TAM+M increases single-channel currents in a Ca²⁺-independent manner

As our data suggests that the TAM+M-BK_{Ca} axis includes mobilization of Ca²⁺ from multiple stores, we applied inside-out patch-clamp recordings to study TAM+M-mediated BK_{Ca} single channel behavior in the presence and absence of Ca²⁺. Accordingly, we used hBK_{Ca}^{high} BC cells to study the influence of endoxifen on channel open probability under Ca²⁺-free conditions or at physiological Ca²⁺ levels at either negative (-50 mV) or positive (+50 mV) voltages (Fig. 6, *A* and

TAM-BK_{Ca} axis in BC cells

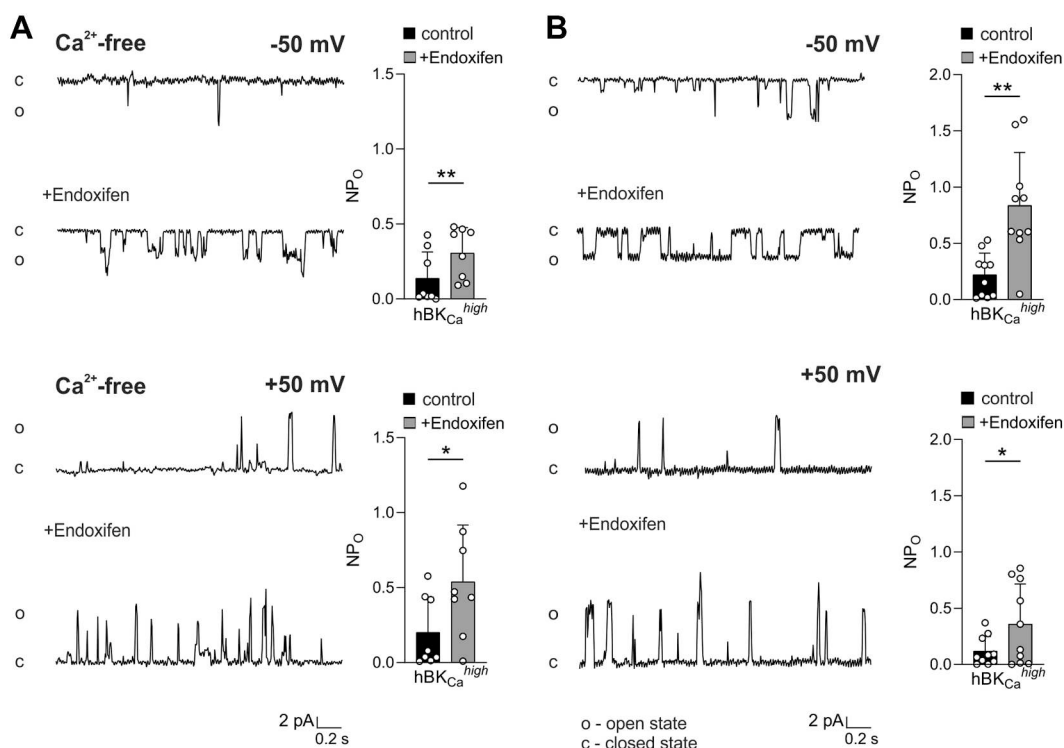


Figure 6. TAM+M enhances single channel open probabilities independent of Ca²⁺ levels. A and B, representative single channel recordings (left) and corresponding bar charts (right) of hBK_{Ca}^{high} BC cells at negative and positive voltages (-50 and +50 mV) using a symmetric isotonic KCl solution containing either (A) Ca²⁺-free or (B) physiological Ca²⁺ levels. 1 μM endoxifen significantly increased BK_{Ca} open probability (NP₀) under all at investigated conditions. Data represents average ± SD. n (cells) = (A) 8 and (B) 10 with **p* ≤ 0.05 and ***p* ≤ 0.01. (A) Wilcoxon test. (B) Paired *t* test. Statistical values are described in detail in Table S6.

B). At both voltages and Ca²⁺ levels, endoxifen increased the number of channel openings, resulting in a significantly increased open probability (NP₀). Single channel conductance yielded values of approximately 200 pS (data not shown), which is in the expected range of canonical BK_{Ca} channels. As the effect of endoxifen on NP₀ was independent of Ca²⁺, we conclude that TAM+M modulates BK_{Ca} function through a direct interaction and not indirectly by influencing Ca²⁺ handling mechanisms like transporters, channels or stores. Interestingly, the highest NP₀ in response to endoxifen was recorded at physiological Ca²⁺ levels and negative potentials (Fig. 6B), supporting our initial hypothesis that TAM+M-mediated BK_{Ca} activity results in a feed-forward mechanism, including Ca²⁺-dependent sustained BK_{Ca} channel activation.

In sum, these single-channel recordings confirmed TAM+M-promoted BK_{Ca} channel activity in a voltage- and Ca²⁺-independent manner. This suggests a direct interaction between TAM+M and BK_{Ca} channels, which results in an increased channel open probability.

Molecular modelling of TAM+M interaction with hBK_{Ca}

Docking of TAM+M against entire hBK_{Ca} protein structures (PDB Codes 6V38, 6V22 (60)), suggest two putative extracellular binding sites, one located close to the N-terminus (termed “WDF”, based on the amino acids forming major interactions with TAM+M) (Fig. 7, A and B and Fig. S3, D and

E) and one posed between subunits close to the pore (termed “TSYF” based on the same principle) (Fig. 7, C and D and Fig. S3, D and E). Both pockets show high docking scores for 4-OH-TAM and endoxifen with virtually the same binding modes, indicating similar effects. The binding modes place the stilbene scaffolds in the hydrophobic part of the transmembrane region. They show π-π interactions with W22 and F252 for the “WDF” site, as well as with F131 and Y274 for the “TSYF” site. In the “WDF” site, the positively charged secondary or tertiary amines of endoxifen and 4-OH-TAM form a salt bridge to D261. Interestingly, in the “TSYF” site, the protonated tertiary amine of 4-OH-TAM appears to preferentially interact with the hydroxyl function of the side chain of S134, while the protonated secondary amine of endoxifen can, in principle, form two hydrogen bonds targeting the side chain hydroxyl group of S134 and the backbone carbonyl of F131. The phenolic hydroxyl functions of both metabolites are located in close proximity to the backbone of W23 (“WDF” site) or the backbone of T298 (“TSYF” site). In addition to the high docking scores in 6V38 (Fig. S3D), a homology model (Fig. S3F) showed the “WDF” binding site close to the recently described binding site of E2 in the structure of BK_{Ca} with β1-subunit in smooth muscle cells (21).

In sum our results reveal that TAM+M activates BK_{Ca} by potential binding sites in the α-subunit of BK_{Ca} to trigger changes in the [K⁺]_i vs [K⁺]_{ex} balance that in turn allows

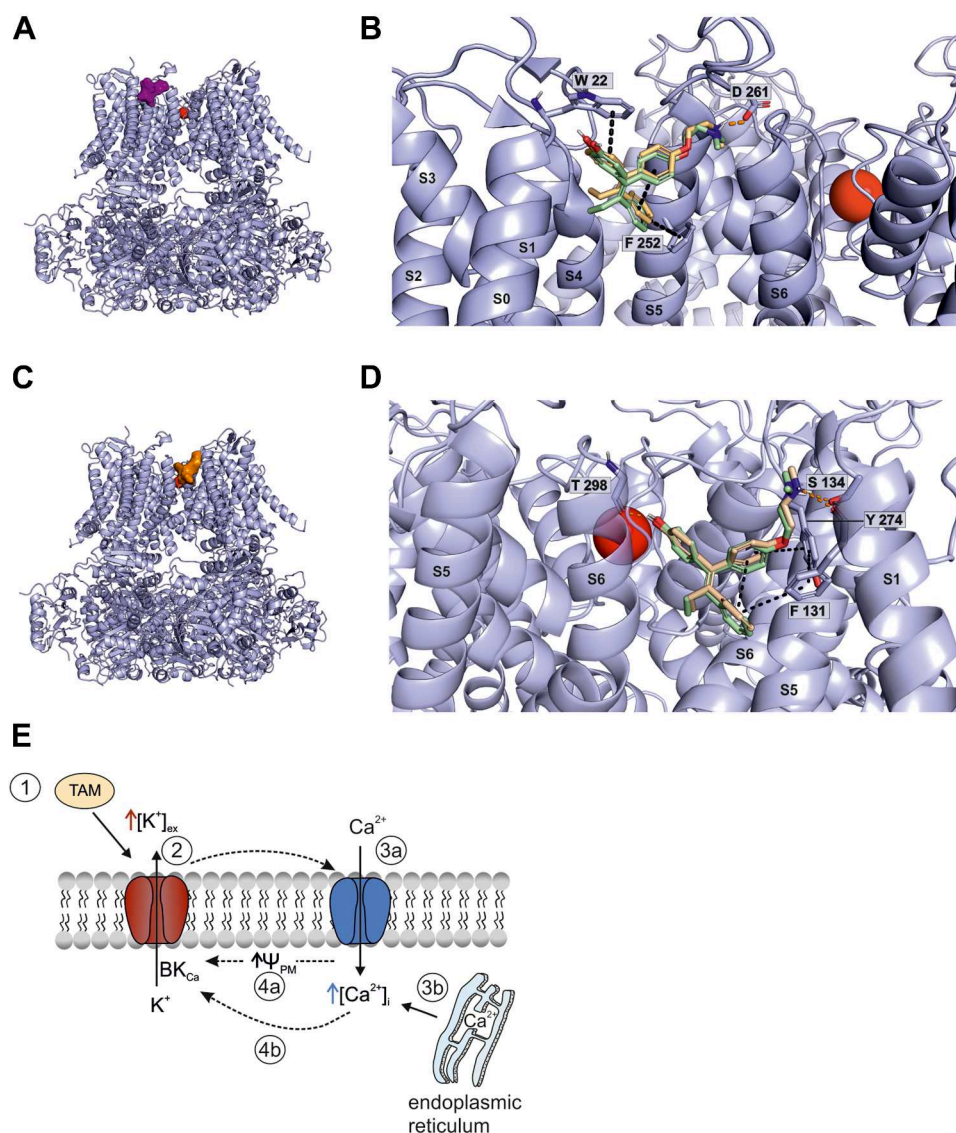


Figure 7. Molecular modelling of TAM+M interaction with hBK_{Ca}. Results of docking simulation of TAM+M into (A, B) extracellular binding pocket “WDF” and (C, D) pore-interacting binding pocket “TSYF” in (A, C) tetrameric arrangement of α -subunits and (B, D) magnified regions of putative binding modes in cryo-EM structures of Ca²⁺-bound BK_{Ca} (PDB 6V38) after induced fit docking. The high docking scores indicate strong hydrophobic, polar and π - π interactions at (A, B) segments S0-S5 with the amino acids W22, D261 and F252 and (C, D) S1, S5-S6 with T298, S134, Y274, F131. Color code: Segments, light blue; K⁺, red sphere; Endoxifen, yellow; 4-OH-TAM, green. E, summarizing scheme of BK_{Ca}-mediated alterations in K⁺ and Ca²⁺ homeostasis due to TAM+M stimulating BK_{Ca} channel activation. (1) TAM+M provoke BK_{Ca} channel activity, (2) BK_{Ca}-dependent K⁺ dynamics lead to an increased extracellular K⁺ ($[K^+]_{ex}$) and a lower $[K^+]_i$; Ca²⁺ entry through (3a) TRP/C-like Ca²⁺ channels in the PM and (3b) by its release from intracellular stores such as the endoplasmic reticulum results in a cytosolic Ca²⁺ accumulation ($[Ca^{2+}]_i$) in BC cells. This increase in $[Ca^{2+}]_i$ in turn causes (4a) voltage- and (4b) Ca²⁺-dependent activation of BK_{Ca}, which is thus part of a feed-forward amplification mechanism in TAM+M exposed BC cells.

elevated $[Ca^{2+}]_i$ by facilitating Ca²⁺ influx across the PM and by Ca²⁺ release from the endoplasmic reticulum. Ca²⁺ accumulation and depolarized Ψ_{PM} subsequently maintain BK_{Ca} activity in terms of a feed-forward mechanism (Fig. 7E).

Discussion

We combined live-cell imaging with electrophysiological and pharmacological approaches in human and murine BC cell systems and obtained novel insights into the interaction of clinically relevant TAM+M with the “oncochannel” BK_{Ca}, a K⁺ channel that is suspected to confer a negative influence on endocrine treatment in BC patients (9).

Supported by molecular docking calculations, our analyses of TAM+M-mediated effects in human and murine MMTV-PyMT-based BC cell models expressing either no, low, medium or high levels of BK_{Ca} show a close correlation between the cells’ BK_{Ca} status and their response to endocrine agents.

We suggest that the TAM+M-BK_{Ca} axis in BC cells directly controls the intracellular K⁺ and Ca²⁺ balance, as the observed effects of endocrine agents on ion homeostasis occurred within seconds or minutes and were limited to BK_{Ca}-proficient cells, and were independent from genomic actions of the ER. In fact, BK_{Ca} but not ER status of the BC cells had an influence on whether TAM+M exposure caused a K⁺ drop in the cells and consequently a Ca²⁺ accumulation.

TAM-BK_{Ca} axis in BC cells

Our observed TAM+M-mediated activation of BK_{Ca} in BC is consistent with our previous findings that low concentrations of TAM+M promote rather than inhibit BC cell growth in the presence of functional BK_{Ca} channels (9). Accordingly, high *KCNMA1* expression levels resulted in lower recurrence-free survival rates in ER⁺ BC patients (9). By quantifying K⁺ channel transcripts from human ER⁺ BC biopsies and human BC cells, we confirmed *KCNMA1* expression (7) is aberrantly increased in bulk tumor samples and selected BC cell lines.

When we validated the functional expression of BK_{Ca} channels in our BC cell models, we observed a significant PAX-sensitive K⁺ current component in hBK_{Ca}^{medium} and hBK_{Ca}^{high} cells that was not present in hBK_{Ca}^{low} BC cells, thereby confirming previous reports of BK_{Ca} expression in hBK_{Ca}^{low/high} cell models (7, 9). In contrast, pharmacological modulation of BK_{Ca} by the channel opener NS11021 led to an increase in current densities in whole-cell patch-clamp analysis and a drop in [K⁺]_i, from which we conclude that human BC cells with varying BK_{Ca} channel levels represent a suitable model to study the putative effects of TAM+M on BK_{Ca} channel activity and ion homeostasis.

In this current work, we further established a correlation between the BK_{Ca} expression level, the reduction of [K⁺]_i and the elevation in [Ca²⁺]_i in response to TAM+M. Our BK_{Ca}^{rescue} experiments in hBK_{Ca}^{low} BC cells confirmed TAM+M-mediated BK_{Ca} activity by restoring [K⁺]_i dynamics. Additionally, we demonstrated that the “pore death” mutant BK_{Ca}^{G354S} expressed in hBK_{Ca}^{low} BC cells did not mediate TAM+M-provoked [K⁺]_i dynamics. These defined amendments in BK_{Ca} expression and activity that we implemented either as BK_{Ca}-RFP^{rescue} or BK_{Ca}^{G354S} confirmed a direct interaction of TAM+M with the pore-forming α -subunit. Importantly, ER-independent actions of TAM are frequently reported, especially in the context of cancer cell proliferation (61–63), however, these have not been examined at the level of K⁺ and Ca²⁺ homeostasis.

In contrast to our previous observations where prolonged E2 exposure increased BC cell proliferation in a BK_{Ca}-dependent manner (9), our current work revealed that E2-mediated effects on K⁺ dynamics were independent of BC BK_{Ca} channels, at least during the few-minute time frame considered here. Thus, follow-up studies are required to address and explain these differences in the mode of action of E2 and TAM+M on BK_{Ca}. For the time being, we suggest that the BK_{Ca}-dependent pro-proliferative effects of E2 may be mediated by an alternative signaling pathway that is independent of any direct interaction between E2 and BK_{Ca}'s α -subunit or ER. This view is in line with findings in non-cancerous cells, where the expression of auxiliary β 1-subunits was essential for the activation of BK_{Ca} by E2 (18, 21, 22). It is also in line with findings in triple negative breast cancer cells (absence of ER, PR and HER2), where E2-dependent activation of G protein-coupled estrogen receptor 1 (GPER) stimulated a non-genomic pathway causing cell proliferation (64, 65). The role of BK_{Ca} in this context, however, was not specifically investigated.

Interestingly, TAM+M-induced [Ca²⁺]_i dynamics were decreased by [Ca²⁺]_{i/ex}-depleted conditions, TRP/C and SOCE inhibition and by BK_{Ca} blockade with PAX, giving rise to the view that BK_{Ca}-mediated K⁺ loss triggers Ca²⁺ elevation. To this end, we showed that the presence of [Ca²⁺]_{i/ex} or intact SOCE is dispensable for the TAM+M-induced response of BK_{Ca}, as FRET recordings demonstrated an unaltered TAM+M-provoked K⁺ efflux, whole-cell patch-clamp experiments showed increased current densities even under Ca²⁺-free EGTA-buffered conditions, and the NP_O of BK_{Ca} in inside-out patches was raised independent of Ca²⁺ stores or transporting mechanisms. It should be emphasized that physiological Ca²⁺ conditions contributed to the highest increase in NP_O, confirming an amplified TAM+M effect on BK_{Ca} activity by Ca²⁺.

To address whether the TAM+M-BK_{Ca} axis plays a role for intracellular Ca²⁺ mobilization, we induced Ca²⁺ mobilization from the endoplasmic reticulum by ATP. The K⁺ loss induced by TAM+M caused an increase in [Ca²⁺]_i under [Ca²⁺]_{ex}-depleted conditions, probably due to an increase in Ca²⁺ driving force.

Hence, BK_{Ca} gating in BC cells is also linked to the homeostasis of this intracellular Ca²⁺ pool, and thus influences cellular signaling processes, as previously demonstrated for a wide range of different cell types (66). This is further corroborated by the PAX-induced BK_{Ca} inhibition, which impaired a significant part of the ATP-elicited [Ca²⁺]_i transients after TAM+M pre-treatment (Fig. 4, E and F). These findings are in line with those previously reported by us, where PAX reduced the basal [Ca²⁺]_i levels in MDA-MB-453 BC cells, while the mean peak amplitude of the [Ca²⁺]_i signals induced by ATP remained unaltered (7).

We also considered that the TAM+M-BK_{Ca} axis would affect the Ψ_{PM} and thus voltage-dependent BK_{Ca} activation in the presence and absence of [Ca²⁺]_{ex/i}. Indeed, we triggered a strong Ψ_{PM} depolarization with TAM+M that depended on the BK_{Ca} status and was sensitive to genetic or pharmacological inhibition of the channel. The measured effects on Ψ_{PM} were less prominent when [Ca²⁺]_i or [Ca²⁺]_{ex} were selectively depleted, indicating that Ψ_{PM} depolarization is a consequence of the (initial) TAM+M-BK_{Ca} interaction. Although this may be counterintuitive at first, as the BK_{Ca}-mediated K⁺ efflux should cause a greater membrane polarization rather than depolarization, we need to be aware of, that DiBAC₄(3) is a slow-acting voltage-sensitive dye (59). We conclude that we have not been able to demonstrate the initial and rapid hyperpolarizing effect of TAM+M, as only the subsequent Ca²⁺ influx leading to membrane depolarization is detected by DiBAC₄(3).

Finally, for the TAM+M binding sites suggested by molecular docking, additional homology modelling of the β 1 subunit to the β 4 subunit (in 6V22) revealed, that the extracellular “WDF” binding site would lie close to the amino acids W163 and F166 of the β 1-subunit, which were found to be critical for the effect of E2 on hBK_{Ca} channel complexes (21). Both binding sites are close to binding sites of the BK_{Ca} channel activator CTIBD (4-(4-(4-chlorophenyl)-3-

(trifluoromethyl)isoxazol-5-yl)benzene-1,3-diol) (67, 68), with CTIBD occupying the same space as TAM+M in the “WDF” binding site. According to our simulations, both binding sites are located in the extracellular region, fitting well to the recently reported activation of BK_{Ca} by a membrane-impermeable form of TAM in smooth muscle cells (69).

In conclusion, we show that TAM+M induces considerable changes in the K⁺ and Ca²⁺ homeostasis in human and murine BC. This is mediated by direct activation of BK_{Ca} oncochannels. TAM+M binding to one or two postulated extracellular binding sites results in BK_{Ca} activation, followed by the elevation of [Ca²⁺]_i and a more depolarized Ψ_{PM}. Both effects consequently lead to a sustained feed-forward mechanism of BK_{Ca} activation. Considering that BK_{Ca} is involved in cell cycle control, proliferation, invasion, migration and malignancy of BC cells, it may be inferred from these changes that in patients with BC they may affect the response to endocrine treatment and promote the development of drug treatment resistance. A future perspective for translation of our results into clinical practice should be the analysis of BK_{Ca} channel expression in tumor biopsies, upfront onset of TAM therapy to predict TAM-modulation based on ER-independent interaction with oncochannels. In addition, we envision that the pharmacological inhibition of BK_{Ca} could be promising as a novel therapeutic in targeted anti-cancer concept, either in patients with endocrine resistance, or in patients with BC having aberrant BK_{Ca} channel expression.

Experimental procedures

Cell culture

Human BC cell lines MDA-MB-453 (hBK_{Ca}^{high} (ER⁻)), MCF-7 (hBK_{Ca}^{low} (ER⁺)), CAMA-1 (hBK_{Ca}^{medium} (ER⁺)), SKBr-3 (hBK_{Ca}^{low} (ER⁻)) were purchased from ATCC: The Global Bioresource Center. Cells were cultivated in Dulbecco's modified eagle's medium (DMEM), 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin (P/S) (Thermo Fisher Scientific). MDA-MB-453 and MCF-7 were additionally supplemented with 1 mM sodium pyruvate (Thermo Fisher Scientific). Murine BC cells were isolated from breast tumor-bearing MMTV-PyMT transgenic FVB/N WT (mBK_{Ca}^{WT}) and BKKO (mBK_{Ca}^{KO}) mice (9). Animal experimental procedures were approved by the local ethics Committee for Animal Research (Regierungspräsidium Tübingen) and in accordance with the German Animal Welfare Act. Animals had access to food and water ad libitum and were kept on a 12 h light/dark-cycle under controlled humidity and temperature. Spontaneous tumor induction and development was monitored at an age of 12 to 14 weeks, and murine BC cells were isolated from n = 5 mBK_{Ca}^{WT} and n = 6 mBK_{Ca}^{KO} mice. Breast cancer cells were isolated from primary tumors as previously described (7, 70) and cultured in modified improved minimal essential medium (IMEM) supplemented with 5% FBS and 1% P/S. Human and murine BC cells were cultivated at 37 °C, 5% CO₂ in a humidified incubator.

Subculturing of cells was performed at 80 to 90% confluency with 0.05% trypsin-EDTA diluted in PBS (Thermo

Fisher Scientific) for 5 min in the incubator. After centrifugation at 1000 rpm for 5 min, cells were seeded into new cell culture flasks or dishes for subsequent experiments. Cell culture materials were purchased from Sarstedt or Corning (Kaiserslautern, Germany). In experiments that aimed to exclude the influence of growth factors and hormones, cells were cultured 72 h in serum-free medium followed by charcoal-stripped serum (CCS) enriched medium.

Electrophysiology

For whole-cell and excised inside-out patch-clamp experiments, 300,000 cells of hBK_{Ca}^{medium/high} and 150,000 cells of hBK_{Ca}^{low} were seeded in 35 mm dishes the day before measurement. For whole-cell patch-clamp experiments, BC cells were washed twice with pre-warmed PBS and extracellular buffer (Bf_{ex}) containing (in mM): 143 NaCl, 2 CaCl₂, 1 MgCl₂, 10 HEPES, 10 D-Glucose, pH 7.4 adjusted with NaOH (all from Carl Roth) was applied. A P-1000 Flaming/Brown micropipette puller (Sutter Instruments) was used to pull micropipettes with 3.5 to 4.5 MΩ from borosilicate glass capillaries (BM150-10P, Science Products GmbH, Hofheim, Germany). Glass micropipettes were polished using an MF-830 Micro Forge (Narishige International Ltd) and filled with intracellular solution (in mM: 130 K-gluconate, 10 KCl, 5 MgCl₂, 0.6 EGTA, 5 HEPES, 0.1 CaCl₂, 0.2 Na₂-GTP, 2.0 Mg-ATP, pH 7.4 adjusted with KOH). Osmolality of intra- and extracellular buffer was adjusted to 300 mOsm/kg. Single-channel recordings in excised inside-out patches were performed in symmetrical isotonic KCl buffer containing (in mM): 10 NaCl, 110 KCl, 2.69 CaCl₂, 5 EGTA, 2 MgCl₂, 10 HEPES, pH 7.4 adjusted with KOH to obtain physiological [Ca²⁺]_i levels (0.1 μM free Ca²⁺) (71) and Ca²⁺-free conditions were achieved by depleting CaCl₂. Micropipettes with 5.0 to 7.0 MΩ were pulled and polished as mentioned above. For both configurations, a Nikon Tc2R microscope equipped with a Nikon S Plan Fluor 40x/0.6 objective (Nikon Instruments Inc.) and a DFK 33U x 174 camera (The Imaging Source Europe GmbH) was used for visual guidance. The recording setup was further comprised of a MP-225 micromanipulator (Sutter Instruments) and an EPC10 amplifier (HEKA Elektronik GmbH). PatchMaster software (HEKA Elektronik GmbH) and nest-o-patch (<http://sourceforge.net/projects/nestopatch>, Dr V. Nesterov) were used for data recording and analysis. In whole-cell mode, currents were recorded in response to 300 ms voltage steps ranging from -100 to +180 mV (hBK_{Ca}^{high}) and -100 to +120 mV (hBK_{Ca}^{low}) in 20 mV increments from a holding potential of -40 mV. Recordings were repeated after addition of PAX (1 min), NS11021 (3 min) and TAM+M (8 min). Whole-cell capacity and access resistance were compensated. Cells for which access resistance changed by more than 20% during the recording were excluded from the analysis (<10%). The mean current amplitude during the last 16.2 ms of the depolarizing pulse was measured and normalized to cell capacity. Single-channel currents were recorded using voltage steps ranging from -80 to +80 mV in increments of 10 mV for 15 s.

TAM-BK_{Ca} axis in BC cells

Recordings were repeated after addition of TAM+M (5 min). For current analysis using Nest-o-Patch, the recordings were filtered at 500 Hz. The open probability from a group of channels (NP_O) in the patches represents channel activity. Representative single-channel recordings and analysis of NP_O are described for -50 and +50 mV at physiological or Ca²⁺-free conditions.

FRET-based K⁺ imaging

BC cells were seeded to a confluency of 70 to 80% on glass coverslips (1.5 H 30 mm, Paul Marienfeld GmbH) in 35 mm dishes (Corning). For FRET-based live-cell K⁺ imaging, cells were transfected 16 h prior to the experiments with PolyJet (SigmaGen Laboratories) according to the manufacturer's instructions. Plasmids used in this study are as follows: FRET-based K⁺ sensor cytosolic Ic-LysM GepIII.0 (40) as a gift from Roland Malli; BK_{Ca} channel overexpression was performed with BK_{Ca}-RFP (7) as well as respective control by the empty RFP plasmid (72). Loss-of-function mutant BK_{Ca}^{G354S} previously reported by (43), was generated by site-directed mutagenesis using: BK_{Ca}-RFP plasmid as template, the primers: BK^{G354S} forward 5'-ACA ATG TCT ACA GTG AGT TAT GG-3' and BK^{G354S} reverse 5'-CAT AAA CGT CCC CAT AAC TCA-3', Q5 High-Fidelity DNA Polymerase (NEB) followed by digestion with *DpnI* (NEB) according to manufacturer's instructions. Plasmid preparation was performed by NucleoBond Xtra Maxi Kit according to the manufacturer's protocol (Macherey-Nagel). The successful mutagenesis was validated by sequencing of the insert (Microsynth SeqLab).

Glass coverslips were transferred to a perfusion chamber (NGFI GmbH, Graz, Austria) and washed twice with Bf_{ex}. To obtain intracellular Ca²⁺-depleted conditions, BC cells were incubated for 30 min prior to the recording with cell-permeant BAPTA-acetoxymethyl ester (BAPTA AM). Extracellular Ca²⁺-free conditions were achieved by the addition of EGTA (Sigma-Aldrich) and omission of CaCl₂. Measurements were performed under the presence of different compounds with stock solutions diluted in DMSO at the following final concentrations: 1 μM endoxifen, 1 μM 4-OH-TAM (Sigma-Aldrich), 10 μM NS11021, 5 μM paxilline, 10 μM SKF-96365 (Tocris). Dilution series were performed with 1 μM endoxifen and 1 μM 4-OH-TAM to perform concentration-dependent experiments. Controls were treated with DMSO.

A Zeiss Axio Observer Z1 (Carl Zeiss AG) microscope was used for FRET-based measurements as previously described (7, 70, 73). The imaging platform contained a BioPrecision2 automatic XY-Table (Ludl Electronic Products, Ltd) and a Plan-Neofluar 40x/1,30 Oil immersion objective (Carl Zeiss AG). An external light source (LED-Hub, Omicron Laserage) equipped with LED at 340 nm, 380 nm, 455 nm, 470 nm and 505 to 600 nm and emission filters: 340x, 380x, 427/10, 473/10 and 575/15 (AHF, Analysentechnik) allowed live-cell imaging by FRET. For FRET measurements, an Optosplit II (Cairn Research) together with dichroic mirrors (459/526/596 nm) and emission filters (475/543/702 nm) was utilized for simultaneous recording of FRET and CFP emissions (all from

AHF Analysentechnik). Images were captured by a pco.panda 4.2 bi sCMOS camera (Excelitas PCO) and VisiView software (Visitron Systems). Addition of buffers and treatments was enabled by a gravity-based perfusion system (NGFI GmbH).

Fura-2-based Ca²⁺ measurement

For the Fura-2 recordings, the BC cells were seeded 24 h before the measurement with a confluence of 50% on glass cover slips in 35 mm dishes. Stocks of 15% Pluronic F-127 (Sigma-Aldrich) and 1 mM Fura-2 AM (Biomol GmbH, Hamburg, Germany) were mixed in a 1:3 dilution (following Fura-2). BC cells were washed with Bf_{ex} and loaded for 20 min at RT with a final concentration of 3.3 μM Fura-2 AM. Subsequently, cells were washed with Bf_{ex} for 10 min at RT. Imaging was performed on a Zeiss Axiovert 200M microscope equipped with a 40x Fluor 1.30 oil immersion objective (Carl Zeiss AG). Further, illumination was provided by a pE-340^{fura} LED (CoolLED), together with excitation filters 340/26 and 380/14 (AHF Analysentechnik). Dichroic mirror 515LP and emission filter 525/15 (AHF Analysentechnik) enabled specific detection of Fura-2 based Ca²⁺ signals. A pco.panda 4.2 sCMOS camera (Excelitas PCO) and VisiView were used for image acquisition. Perfusion of treatments was achieved by a gravity-based system. Next to already mentioned treatments, 100 μM ATP (Carl Roth) was applied.

Plasma membrane potential measurements

Bis-(1,3-dibutylbarbituric acid)Trimethine Oxonol (DiBAC₄(3)) (Thermo Fisher Scientific) was used in a concentration of 0.25 μg/ml to determine ΔΨ_{PM}. BC cells were seeded on 30 mm glass cover slips, washed with Bf_{ex} and incubated with DiBAC₄(3) for 35 min at RT. During the measurement at the microscope Zeiss Axio Observer Z1, DiBAC₄(3) was constantly present in the buffer. Excitation was performed by 470 nm LED and a dichroic mirror (409/493/573/652 nm), and an emission filter (514/605/730 nm) enabled DiBAC₄(3) specific recordings.

qRT-PCR

Gene expression levels of BK_{Ca} subunits in human BC cells were analyzed using total mRNA isolated from hBK_{Ca}^{low}, hBK_{Ca}^{medium} and hBK_{Ca}^{high} BC cells. BC cells were seeded to a confluency of 80% in 100 mm dishes. Isolation, cDNA synthesis and qRT-PCR were performed as previously described (74). RNA was isolated using the NucleoSpin RNA kit (Macherey-Nagel) followed by cDNA synthesis of 0.5 μg RNA using iScript cDNA synthesis kit (Bio-Rad) according to manufacturer's instructions, respectively. SsoAdvanced Universal SYBR Green Supermix (Bio-Rad) and target-specific primer pairs were used to perform qRT-PCR with CFX Connect Real-Time PCR Detection System (Bio-Rad).

Human breast cancer biopsies and nanostring nCounter gene expression analysis

An endocrine treatment study of 1286 BC patients between 2005 and 2011 (German Clinical Trial Register DRKS

00000605, “IKP211” study) was previously described for the analysis of 551 primary tumor biopsies from hormone receptor positive, post-menopausal patients with Nanostring nCounter gene expression analysis (7). In brief, patients were treated by TAM, aromatase inhibitor or switched endocrine therapy in between. Total RNA was isolated from FFPE tissues (Quick-DNA/RNA FFPE, ZymoResearch, Freiburg, Germany) of their breast cancer biopsies. Target-specific capture probes and color-coded reporter sets for ion channel genes and accessory subunits were designed and hybridized with 250 ng RNA in a thermal cycler at 65 °C for 20 h. The Nanostring nCounter system was used to measure fluorescence counts. Data analysis was performed with nSolver 4.0. Normalization to five housekeeping genes (*ABCF1*, *NRDE2*, *POLR2A*, *PUM1*, and *SF3A1*) was performed and cut off of log₂ expression count was set to 5.5. We have previously published some information on the BC patient-related BK_{Ca} channel expression profile in (7).

Molecular docking simulation

TAM+M were docked against two protein cryo-EM structures of BK_{Ca} under Ca²⁺-bound and Ca²⁺-bound in complex with the β4 subunit conditions (PDB Codes 6V38, 6V22, respectively, (60)) using Schrödingers software suite (Schrödinger Release 2024–4). Using the Protein Preparation Wizard (75), bond orders were reassigned, hydrogens replaced and sensible protonation states at pH 7.4 were determined using PROPKA. Each protein was subjected to a restrained minimization using the OPLS4 force field (76) and a 0.3 Å RMSD convergence criterion. Ligands were prepared using LigPrep at pH 7.0. We used SiteMap (77, 78) with shallow settings to identify a multitude of binding sites and split large binding sites using the DBSCAN clustering algorithm. This resulted in 277 and 368 potential binding sites for BK_{Ca} under Ca²⁺-bound and Ca²⁺-bound in complex with β4 conditions, respectively. TAM+M were docked into each binding site using GLIDE (79) with standard precision. After the identification of putative binding sites, the extended induced fit docking protocol (80, 81) with extra precision in the structure of BK_{Ca}-Ca²⁺-bound (6V38) was performed. For the refinement step, an implicit membrane was defined based on the experimental data.

Statistical analysis

Statistical analysis was performed with GraphPad Prism 9 software (GraphPad, Software). All data were tested for outlier. Normal distribution was verified using Shapiro-Wilk or Kolmogorov-Smirnov test. If the variances of the populations were comparable, a two-tailed Unpaired *t* test was used; if F-test was significantly different, Welch’s test was applied for the statistical comparison of normally distributed data. Normally distributed data of paired measurements were evaluated with Paired *t* test, while the Wilcoxon-test was used for non-normally distributed data. For the comparison of >2 groups either One-way ANOVA followed by Dunnett’s or Tukey’s multiple comparisons for normally distributed data

was used, always considering F-test variances, or Two-way ANOVA followed by Dunnett’s, Šidák’s or Tukey’s multiple comparisons test was performed. If data were not normally distributed, a Kruskal-Wallis test followed by Dunn’s multiple comparisons test was performed. Statistical tests are indicated in the Figure legends and described in detail in the Supplementary Tables (Tables S1–S9). *p*-values of ≤ 0.05 were considered as statistically significant with **p* ≤ 0.05, ***p* ≤ 0.01 and ****p* ≤ 0.001.

Data availability

All data are present in the article or the Supporting Information.

Supporting information—This article contains supporting information (7, 9, 21, 44, 82).

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Abbreviations—The abbreviations used are: ATP, adenosin-5′-triphosphate; BC, breast cancer; BK_{Ca}, voltage- and Ca²⁺-activated K⁺ channel of large conductance; CCS, charcoal-stripped serum; ER, estrogen receptor; HER2, human epidermal growth factor receptor; IF, immunofluorescence; IP₃, inositol-triphosphate; MMTV, mouse mammary tumor virus; PM, plasma membrane;

PyMT, polyoma middle T antigen; SOCE, store-operated Ca²⁺ entry; TAM, tamoxifen; TAM+M, tamoxifen and its metabolite; TNBC, triple-negative BC.

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