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The isolated human umbilical vein as a bioassay for kinin-generating proteases: an *in vitro* model for therapeutic angioedema agents†

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Abstract

Aims: The isolated human umbilical vein is a robust contractile bioassay for ligands of the bradykinin (BK) B₂ receptor (B₂R), also extendable to B₁ receptor (B₁R) pharmacology. We hypothesized that, as a freshly isolated vessel, it also contains traces of plasma proteins that may confer responses to exogenous proteases via the formation of kinins.

Main methods: Rings of human umbilical veins were mounted in organ baths containing Krebs buffer maintained at 37°C and purified proteases were introduced in the bathing fluid along with additional drugs/proteins that permit mechanistic analysis of effects.

Key findings: The previously described contractile response to human recombinant tissue kallikrein (KLK-1, 1-10 nM) is not influenced by metabolic inhibitors, suggesting its dependence on a preexisting reservoir of low molecular weight-kininogen (LK). Active plasma kallikrein (apK, ≤5 nM) was inactive in fresh tissues, unless high molecular weight-kininogen (HK, 39-197 nM) replenishment was applied. The effects of KLK-1 and HK+apK are abolished by pretreating tissues with icatibant, but not with tranexamic acid. C1-esterase inhibitor inhibited only HK+apK. Purified plasmin and neutrophil proteinase-3 produced small contractions in the presence of HK only, and tissue plasminogen activator, none. B₁R stimulation was pharmacologically evidenced in response to KLK-1 if LK was supplied.

Significance: The pharmacology of KLK-1 and HK+apK in the human isolated umbilical vein is essentially based on the activity of locally generated kinins and this assay models the inhibitory action of some therapeutic agents active in angioedema states. Proteases that indirectly generate kinins have little activity in the system.

Keywords: tissue kallikrein, plasma kallikrein, plasmin, bradykinin B₂ receptor.

1. Introduction

The human isolated umbilical vein is a robust contractile bioassay for agonist and antagonist ligands of the bradykinin (BK) B₂ receptor (B₂R), extendable to the inducible B₁ receptor (B₁R) [1-4]. In this system with low intrinsic sensitivity to endothelium-dependent vasorelaxation and possessing ≥ 20 smooth muscle cell layers, BK or Lys-BK essentially induce B₂R-mediated contractions. The kinin B₁R is preferentially stimulated by kinin metabolites generated by ubiquitous arginine-carboxypeptidases. The umbilical vein preparation, consistent with the pharmacologic profile of the human B₁R [5], is ~100-fold more sensitive to Lys-des-Arg⁹-BK than to des-Arg⁹-BK [6].

We recently analyzed the effect of a pharmaceutically refined form of human recombinant tissue kallikrein (KLK-1) on umbilical vein rings maintained in Krebs buffer [7]. KLK-1 induced contractions that were highly tachyphylactic, dependent on the B₂R (as shown by the effect of a non-peptide B₂R antagonist) and on the catalytic effect of the protease (inhibited by aprotinin). The tachyphylaxis was reversed if tissues were replenished with low-molecular weight kininogen (LK), the preferential substrate of KLK-1. Thus, the freshly isolated vein contains traces of plasma proteins that may confer an effect to exogenous proteases via the formation of kinins.

The present therapeutic showcase of the kallikrein-kinin system is hereditary angioedema (HAE); in this autosomal dominant disease, most patients exhibit a mutated *SERPING1* gene that codes for a defective or non-expressed C1-esterase inhibitor (C1-inh) protein

[8]. Other patients have a constitutively active form of Factor XII. All these molecular alterations point to a hyperactive contact system, with active plasma kallikrein (apK) generating kinins during attacks. In addition to the replenishment of C1-inh, a B₂R antagonist, icatibant, as well the pharmacological inhibition of plasma kallikrein are effective to abort attacks of HAE angioedema [8]. The acquired angioedema occasionally associated with the pharmacological blockade of angiotensin converting enzyme (a major kinin-destroying peptidase in the extracellular compartment) is also responsive to icatibant [9]. Various other forms of angioedema, many idiopathic, are clinically observed and may be associated with the use of other drugs, malignancies or autoimmune disease [10]. The place of plasmin(ogen) inhibitors in the therapy of angioedema is debated: tranexamic acid is reportedly effective to prevent attacks in a fraction of HAE patients and in certain atypical angioedema cases [8]. Kinin generation may explain acquired angioedema associated with tissue plasminogen activator (tPA) treatment in patients with arterial thrombosis [11, 12]. Other proteases reported to release vasoactive kinins from HK include neutrophil proteinase-3 (PR3), the lectin pathway complement component MSAP-1, and pancreatic trypsin [13-16].

We have exploited the umbilical vein assay to investigate the effect of additional proteases, human purified apK, plasmin and tPA, that may also generate kinins. Further, therapeutic agents used in the treatment of HAE were tested against the effect of the active proteases, in a “reverse engineering” effort to model the *in vitro* effect of currently used drugs vs. the putative pathways of vasoactive kinin generation. The assay

theoretically allows the detection of possible non-conventional effects of proteases, such as the direct activation of the B₂R (as previously proposed [17]).

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2. Methods

2.1. Drugs

Human recombinant tissue kallikrein (KLK-1; DM199) was provided as a catalytically active and pharmaceutically refined form (average molecular weight of 38.5 kDa) by DiaMedica, Inc. (Minneapolis, MN) [18]. Human active plasma kallikrein (apK) purified from plasma ($\geq 95\%$; ≥ 15 units/mg protein) was from EMD Millipore. Human plasmin purified from plasma (≥ 2 units/mg protein) was obtained from Sigma-Aldrich (St. Louis, MO) under a lyophilized powder form. Recombinant human tPA (ateplase, Cathflow, Roche) was reconstituted as recommended by the manufacturer. Proteinase 3 (PR3), purified from human neutrophils ($>95\%$), was from Athens Research & Technology (Athens, GA).

Purified single chain high molecular weight kininogen ($>95\%$, 120 kDa) was purchased from Enzyme Research Laboratories (South Bend, IN), icatibant (Hoe 140; D-Arg[Hyp³, Thi⁵, D-Tic⁷, Oic⁸]BK), from Phoenix Pharmaceuticals (Burlingame, CA), and tranexamic acid and pefabloc SC (4-(2-aminoethyl)benzenesulfonyl fluoride hydrochloride), from Sigma-Aldrich. C1-esterase inhibitor (C1-inh) was under the form of Berinert (CSL Behring Canada, Ottawa, ON), reconstituted as recommended by the manufacturer. Compound 11 (2-((2R)-1-[(3,4-dichlorophenyl)sulfonyl]-3-oxo-1,2,3,4-tetrahydroquinoxalin-2-yl)-N-{2-[4-(4,5-dihydro-1H-imidazol-2-yl)phenyl]ethyl}acetamide) is a powerful antagonist at the human and rabbit B₁ receptor [19] (gift from Dr. D. J. Pettibone, Merck Research Laboratories, West Point, PA).

Bradykinin and histamine were from Sigma-Aldrich and Sar-[D-Phe⁸]des-Arg⁹-bradykinin, a selective B₁R agonist resistant to peptidases [3], was purchased from Phoenix Pharmaceuticals (Burlingame, CA).

2.2. Contractility assay involving the human umbilical vein

The institutional research ethics board (CHU de Québec) approved the anonymous use of human umbilical cord segments obtained after elective cesarean section deliveries. Informed consent was obtained from mothers. The experimental procedures have been recently reported [7]. Briefly, most experiments were based on a 3 hr-equilibration period in Krebs' buffer followed by stimulation with a protease and other agents. Experiments dealing with B₁Rs had an extended equilibration period and a cytokine mixture was present (interleukin-1 β and tumor necrosis factor- α), as reported in Results, due to the time- and stimulus-dependent induction of this pharmacological entity in vascular smooth muscle cells [2, 3, 20].

2.3. Radioligand binding competition assays

The construction of a myc-tagged human B₂R is reported elsewhere, as well as the techniques applied in a competition assay for the binding of [³H]BK to the recombinant receptor transiently expressed in HEK 293a cells [7]. This assay is performed at 0°C to identify unlabeled ligands of the B₂R by their displacement of the specific binding of 3 nM [³H]BK. Similarly designed experiments were the basis of a binding competition

assay involving the displacement of 1 nM [³H]Lys-des-Arg⁹-BK from recombinant FLAG-tagged human B₁Rs transiently expressed in HEK 293a cells [21].

2.4. Data analysis

Numerical results are presented as mean ± S.E.M. Considering the non-normal distributions observed in several experimental groups, sets of numerical data were compared by the non-parametric ANOVA, the Kruskal–Wallis test, and Dunn’s multiple comparison test was applied to compare pairs of values. Pairs of values were likewise compared with the non-parametric Mann-Whitney test. All computations were performed using the InStat3.05 computer program, GraphPad Software (SanDiego, CA). Data from the radioligand competition assays were fitted by nonlinear regression to a one-site competition equation (Prism 4.0, GraphPad Software Inc.).

3. Results

3.1. Contractility studies of the human umbilical vein preparation

The direct application of apK (up to 5 nM) to rings of human umbilical artery did not contract the human umbilical vein preparation maintained in Krebs buffer (Fig. 1A, B). However, HK replenishment 30 min prior to testing (39 or 197 nM; physiological plasma concentration ~600 nM) revealed that apK can slowly contract the preparation in a manner dependent on the HK concentration (Fig. 1C, D). The tissues remained responsive to BK (10 nM) under all circumstances.

Therapeutic agents active in the therapy of HAE were tested against responses induced by the combination of HK + apK (99 nM and 5 nM, respectively), BK (10 nM) or an irrelevant contractile agent (histamine 10 μ M, which is a H₁ receptor agonist in this preparation) [22] (Fig. 2A, sample control tracing). The peptide B₂R antagonist icatibant (100 nM) effectively prevented the effect of apK and BK, but not that of histamine (Fig. 2B). C1-inh and tranexamic acid, both used a clinically relevant plasma concentrations, were tested against the 3 contractile agents. Only C1-inh showed an inhibitory effect against HK + apK.

Minor or ineffective contractile proteases were identified in the contractility assay (Fig. 3). Neutrophil PR3 (up to 31 nM) had a negligible direct effect, but protracted contractile responses were observed if the HK replenishment scheme was applied (Fig. 3A-C). The

effect of PR3 was not dependent on the concentration of HK. The latencies are reported in the Fig. 3 legend and this phenomenon is specific for PR3, because all other contractile proteases produce immediate responses upon application. For instance, immediate contractions were induced by apK (5 nM) in the presence of HK (Fig. 3A). Similarly, plasmin (10 nM) had no direct contractile effect on the umbilical vein preparation, unless a HK replenishment protocol was applied (Fig. 3D, E). The effects did not seem concentration-related vs. HK, and the amplitude of plasmin effects was small and inconsistent from one preparation to the other. Recombinant tPA (ateplase) had no contractile effect on the venous preparation, whether or not HK replenishment was applied (Fig. 3F, G).

The lack of direct effect of apK on the umbilical vein preparation contrasted with the reproducible, but tachyphylactic effect of KLK-1 [7]. A possible explanation for this is that their respective preferential substrates, HK and LK respectively, have a differential expression in the isolated tissue. To test whether LK presence is due to its post-isolation *de novo* formation (as reported in cultured endothelial cells derived from this vein) [23], we have treated venous rings continuously with metabolic inhibitors from the time of organ bath mounting (Fig. 4A). Blockade of protein synthesis with cycloheximide, of RNA synthesis with actinomycin D and of the endoplasmic reticulum-Golgi transition with brefeldin A failed to modify the initial contractile effect of KLK-1 or the one later recorded in response to BK. These results suggest that a certain LK reservoir derived from fetal blood plasma persists in the freshly isolated vein. In this set of experiments, the serine protease inhibitor pefabloc SC (100 μ M) inhibited by 79% the effect of KLK-1

vs. responses recorded in paired control tissues (Fig. 4B). However, it had a cumulative toxicity on tissues, depressing the late effect of BK by 42%, showing that both responses are reactive to this particular metabolic inhibitor, if not always for the expected reason.

The previously described tachyphylactic effect of KLK-1 on the venous preparation was re-examined in relationship with drugs active against HEA (Fig. 5, control tracing).

Icatibant abated the effects of both KLK-1 and BK; this was previously observed with the alternate B₂R antagonist anatibant [7]. In contrast with the susceptibility of apK, KLK-1-induced contractions were not abated in the presence of C1-inh. Tranexamic acid also failed to inhibit KLK-1-induced responses (Fig. 5).

Since KLK-1 reportedly releases Lys-BK from LK, it is possible that widely distributed arginine-carboxypeptidases generate in situ the high affinity of the human B₁R, Lys-des-Arg⁹-BK, from it. We applied special conditions to upregulate the expression of the B₁R in the venous assay and we controlled its presence in experiments reported in Fig. 6.

Thus, umbilical vein rings were stimulated for the first 3 hrs of incubation with inflammatory cytokines; the contractile response to the selective B₁R agonist Sar-[D-Phe⁸]des-Arg⁹-BK recorded 1 hr later proved the time- and protein synthesis-dependent expression of this entity [2, 3]. Then, B₂Rs were blocked with a low concentration of icatibant (20 nM) and the KLK-1 stimulation (10 nM) took place. Very small or no contractions were recorded in response to the protease (Fig. 6A, B). However, if LK replenishment (15.2 nM) was applied before KLK-1 stimulation, a contractile response

was recorded in icatibant-treated tissues; it was attributed to the B₁Rs because addition of the non-peptide B₁R antagonist, compound 11, significantly reduced the response to KLK-1 in separate tissues (Fig. 6B).

The same reasoning was applied in tissues stimulated with HK+aPK (Fig. 6C). In tissues sensitized to the B₁R agonist and in the presence of icatibant, HK+aPK had no reliable effect, consistent with the low affinity of des-Arg⁹-BK in the system.

3.2. Ancillary experiments

The binding of some agents to human recombinant B₂R was examined using a [³H]BK binding competition assay. While BK, Lys-BK and their antagonist icatibant were approximately equipotent competitors of this binding with nanomolar potencies (Fig. 7A), KLK-1 failed to displace [³H]BK in the same experimental system [7], failing to support a direct effect of KLK-1 on the receptor. We have verified that tranexamic acid has no affinity for either human recombinant B₂R or B₁R using radioligand binding competition assays (no displacement of the cognate tritiated agonists from the receptors, Fig. 7A, B).

4. Discussion

The human umbilical vein preparation is suitable to examine the pharmacology of B₂R ligands, as well as that of B₁R ligands if special experimental conditions are applied. The present study extends the finding that KLK-1 contracts the preparation in a tachyphylactic manner by enzymatically releasing a kinin from a substrate, probably LK, present in limited quantity in the blood free system [7]. Consistent with the probable consumption of the contact system in the veins collected post-partum, apK contracts the preparation only if HK replenishment is applied. The umbilical vein system also suggests that multistep enzymatic pathways leading to kinin formation are not favorable stimuli. Thus, as one introduces proteases with more distal actions relative to apK, i.e. plasmin and atelase (tPA; Fig. 8), the contractile effects progressively decreases. Both tPA and plasmin generate BK when added to human blood, parallel to HK consumption [11]. However, this may be indirect, rather mediated by an effect of plasmin on Factor XII [25], and the present experimental tissues seem to contain a trace of the latter component of the contact system, accounting for the small effect of plasmin. Neutrophil PR3, that reportedly releases Met-Lys-BK-Ser-Ser from HK [13], generates a delayed contractile effect on the venous system in the presence of HK (Fig. 3A-C). We have recently shown that Met-Lys-BK-Ser-Ser has virtually no affinity for the B₂R, but that it is paradoxically activated by angiotensin converting enzyme (ACE) present in vascular tissues, including the human umbilical vein [22] (Fig. 8). Thus, this obligatory additional reaction may make the neutrophil PR3 enzyme a less effective contractile agent than apK, the latter releasing BK from HK.

There is no animal model of the HAE attack, as mice with a complete knockout of the gene (*SERPING1*) corresponding to the C1-inh protein have no phenotype, except for an asymptomatic increase in microvascular permeability mediated by B₂R_s [26]. Mental and physical stressors, including infection, surgery, trauma and chronic inflammation, are statistically associated with the frequency of HEA and ACE inhibitor-induced attacks [27, 28]. A certain fraction of these factors may be parallel to the expression of the cytokine-controlled B₁R. Also, local circulatory condition such as fibrinolysis, KLK-1 generation, neutrophil leukocyte activation, digestive enzyme spillover... may either trigger the attack or amplify it. Therefore, a comparison of the proposed BK-generating proteases (Fig. 8) may help to unravel their role in angioedema states, as well as the spectrum of effect of drugs currently used or proposed in the therapy of such states. As far as effects of kinins on B₂R are concerned, icatibant may be the universal inhibitor of the vascular effects of these proteases. C1-inh, as expected, inhibited the contractile effect of apK+HK, but not that of KLK-1, itself previously shown to be sensitive to aprotinin [7] and to pefabloc SC in present experiments (Fig. 4B). This spectrum of susceptibility to inhibitors is well known for KLK-1 [24], however the cell impermeant peptide was not toxic to tissues, as opposed to pefabloc SC. Tranexamic acid at the used concentration inhibits both tPA and plasmin [29], proteases that possess “kringle” domains and that are upstream of the contact system vs. the generation of BK (Fig. 8). The lack of effect of this drug on the contractile effects of either type of kallikrein is consistent with this idea. We verified that tranexamic acid has no unexpected effect on

kinin receptors using radioligand competition assays; this cyclic amine is not dissimilar to some non-peptide antagonists of either B₁ or B₂Rs [30].

The only subnanomolar affinity agonist of the human B₁R is Lys-des-Arg⁹-BK [5, 6].

The formation of this peptide may be limited to the KLK-1/LK pathway, and a moderate LK supplementation that overcomes the “multistep reaction” limitation has indeed led to the pharmacological stimulation of the B₁Rs in the venous preparation (Fig. 6A, B).

Speculations about a role of the inducible B₁R in HAE attacks [31] may be criticized on the account that the apK/HK pathway theoretically only produces some des-Arg⁹-BK, a low affinity B₁R agonist. Accordingly and within the limitations of the present experimental system, the HK+apK combination failed to stimulate B₁Rs (Fig. 6C).

However, it is not excluded that KLK-1 activity is secreted and/or upregulated during hereditary or other forms of angioedema attacks, and participates in a C1-inh-resistant manner to symptoms via B₁ and B₂Rs.

Conclusion

The pharmacology of KLK-1 and HK+apK in the human isolated umbilical vein is essentially based on the activity of locally generated kinins and this assay models the inhibitory action of some therapeutic agents active in angioedema states. Proteases that initiate the formation of pharmacologically active kinin via multiple enzymatic steps exhibit little or no activity in the system.

Conflict of interest

None declared.

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References

- [1] F. Marceau, L. Levesque, G. Drapeau, F. Rioux, J.M. Salvino, H.R. Wolfe, P.R. Seoane, D.G. Sawutz. Effects of peptide and nonpeptide antagonists of bradykinin B₂ receptors on the vasoconstrictor action of bradykinin. *J. Pharmacol. Exp. Ther.* 269 (1994) 1136-1143.
- [2] S.P. Sardi, F.M. Daray, A.E. Errasti, F.G. Pelorosso, V.A. Pujol-Lereis, V. Rey-Ares, M.P. Rogines-Velo, R.P. Rothlin. Further pharmacological characterization of bradykinin B₁ receptor up-regulation in human umbilical vein. *J. Pharmacol. Exp. Ther.* 290 (1999) 1019-1025.
- [3] S. Houle, M. Landry, R. Audet, J. Bouthillier, D.R. Bachvarov, F. Marceau. Effect of allelic polymorphism of the B₁ and B₂ receptor genes on the contractile responses of the human umbilical vein to kinins. *J. Pharmacol. Exp. Ther.* 294 (2000) 45-51.
- [4] F. Marceau, D. deBlois, E. Petitclerc, L. Levesque, G. Drapeau, R. Audet, D. Godin, J.F. Larrivée, S. Houle, T. Sabourin, J.P. Fortin, G. Morissette, L. Gera, M.T. Bawolak, G.A. Koumbadinga, J. Bouthillier. Vascular smooth muscle contractility assays for inflammatory and immunological mediators. *Int. Immunopharmacol.* 10 (2010) 1344-1353.

- [5] L.M. Leeb-Lundberg, F. Marceau, W. Müller-Esterl, D.J. Pettibone, B.L. Zuraw. International Union of Pharmacology. XLV. Classification of the kinin receptor family: from molecular mechanisms to pathophysiological consequences. *Pharmacol. Rev.* 57 (2005) 27-77.
- [6] F. Gobeil, L.H. Pheng, I. Badini, X.K. Nguyen-Le, A. Pizard, A. Rizzi, D. Blouin, D. Regoli. Receptors for kinins in the human isolated umbilical vein. *Br. J. Pharmacol.* 118 (1996) 289–294.
- [7] X. Charest-Morin, A. Raghavan, M.L. Charles, T. Kolodka, J. Bouthillier, M. Jean, M.S. Robbins, F. Marceau. Pharmacological effects of recombinant human tissue kallikrein on bradykinin B₂ receptors. *Pharmacol. Res. Perspect.* 3 (2015) e00119.
- [8] M. Cicardi, W. Aberer, A. Banerji, M. Bas, J.A. Bernstein, K. Bork, T. Caballero, H. Farkas, A. Grumach, A.P. Kaplan, M.A. Riedl, M. Triggiani, A. Zanichelli, B. Zuraw, HAWK under the patronage of EAACI (European Academy of Allergy and Clinical Immunology). Classification, diagnosis, and approach to treatment for angioedema: consensus report from the Hereditary Angioedema International Working Group. *Allergy* 69 (2014) 602-616.

[9] M. Baş, J. Greve, K. Stelter, M. Havel, U. Strassen, N. Rotter, J. Veit, B. Schossow, A. Hapfelmeier, V. Kehl, G. Kojda, T.K. Hoffmann. A randomized trial of icatibant in ACE-inhibitor-induced angioedema. *N. Engl. J. Med.* 372 (2015) 418-425.

[10] J. Levy, G.E. Rivard, E. Wagner, D. Beezhold, N. Berlin, L. Fan, Z. Zhang, G.L. Sussman. Examination of genetic variants involved in generation and biodisposition of kinins in patients with angioedema. *Allergy Asthma Clin. Immunol.* 10 (2014) 60.

[11] G. Molinaro, N. Gervais, A. Adam. Biochemical basis of angioedema associated with recombinant tissue plasminogen activator treatment: an in vitro experimental approach. *Stroke* 33 (2002) 1712–1716.

[12] M.E. Moreau, N. Garbacki, G. Molinaro, N.J. Brown, F. Marceau, A. Adam. The kallikrein-kinin system: current and future pharmacological targets. *J. Pharmacol. Sci.* 99 (2005) 6-38.

[13] R. Kahn, T. Hellmark, L.M. Leeb-Lundberg, N. Akbari, M. Todiras, T. Olofsson, J. Wieslander, A. Christensson, K. Westman, M. Bader, W. Müller-Esterl, D. Karpman. Neutrophil-derived proteinase 3 induces kallikrein-independent release of a novel vasoactive kinin. *J. Immunol.* 182 (2009) 7906-7915.

- [14] L. Gera, C. Roy, M.T. Bawolak, J. Bouthillier, A. Adam, F. Marceau. Met-Lys-bradykinin-Ser-Ser, a peptide produced by the neutrophil from kininogen, is metabolically activated by angiotensin converting enzyme in vascular tissue. *Pharmacol. Res.* 64 (2011) 528-534.
- [15] J. Dobó, B. Major, K.A. Kékesi, I. Szabó, M. Megyeri, K. Hajela, G. Juhász, P. Závodszy, P Gál. Cleavage of kininogen and subsequent bradykinin release by the complement component: mannose-binding lectin-associated serine protease (MASP)-1. *PLoS One* 6 (2011) e20036.
- [16] G.R. Drummond, S. Selemidis, T.M. Cocks. B₂ kinin receptor activation is the predominant mechanism by which trypsin mediates endothelium-dependent relaxation in bovine coronary arteries. *Naunyn Schmiedebergs Arch. Pharmacol.* 378 (2008) 33-41.
- [17] C. Hecquet, F. Tan, D.M. Marcic, E.G. Erdős. Human bradykinin B₂ receptor is activated by kallikrein and other serine proteases. *Mol. Pharmacol.* 58 (2000) 828-836.
- [18] T. Kolodka, M.L. Charles, A. Raghavan, I.A. Radichev, C. Amatya, J. Ellefson, A.Y. Savinov, A. Nag, M.S. Williams, M.S. Robbins. Preclinical characterization of recombinant human tissue kallikrein-1 as a novel treatment for type 2 diabetes mellitus. *PLOS One* 9 (2014) e103981.

[19] G. Morissette, J.P. Fortin, S. Otis, J. Bouthillier, F. Marceau. A novel nonpeptide antagonist of the kinin B₁ receptor: effects at the rabbit receptor. *J. Pharmacol. Exp. Ther.* 311 (2004) 1121-1130.

[20] G.A. Koumbadinga, A. Désormeaux, A. Adam, F. Marceau F. Effect of interferon- γ on inflammatory cytokine-induced bradykinin B₁ receptor expression in human vascular cells. *Eur. J. Pharmacol.* 647 (2010) 117-125.

[21] G. Morissette, J.P. Couture, A. Désormeaux, A. Adam, F. Marceau. Lack of direct interaction between enalaprilat and the kinin B₁ receptors. *Peptides* 29 (2008) 606-612.

[22] L. Gera, C. Roy, X. Charest-Morin, F. Marceau. Vasopeptidase-activated latent ligands of the histamine receptor-1. *Int. Immunopharmacol.* 17 (2013) 677-683.

[23] K. Yayama, N. Kunimatsu, Y. Teranishi, M. Takano, H. Okamoto. Tissue kallikrein is synthesized and secreted by human vascular endothelial cells. *Biochim. Biophys. Acta* 1593 (2003) 231-238.

- [24] P. Goetig, V. Magdolen, H. Brandstetter. Natural and synthetic inhibitors of kallikrein-related peptidases (KLKs). *Biochimie* 92 (2010) 1546-1567.
- [25] S. de Maat, P.G. de Groot, C Maas. Contact system activation on endothelial cells. *Semin. Thromb. Hemost.* 40 (2014) 887–894.
- [26] E.D. Han, R.C. MacFarlane, A.N. Mulligan, J. Scafidi, A.E. Davis AE. Increased vascular permeability in C1 inhibitor-deficient mice mediated by the bradykinin type 2 receptor. *J. Clin. Invest.* 109 (2002) 1057-1063.
- [27] T. Hoover, M. Lippmann, E. Grouzmann, F. Marceau, P. Herscu. Angiotensin converting enzyme inhibitor induced angio-oedema: a review of the pathophysiology and risk factors. *Clin. Exp. Allergy* 40 (2010) 50-61.
- [28] Z. Zotter, D. Csuka, E. Szabó, I. Czaller, Z. Nébenführer, G. Temesszentandrás, G. Fust, L. Varga, H. Farkas. The influence of trigger factors on hereditary angioedema due to C1-inhibitor deficiency. *Orphanet J. Rare Dis.* 9 (2014) 44.
- [29] R.A. Al-Horani, U.R. Desai. Recent advances on plasmin inhibitors for the treatment of fibrinolysis-related disorders. *Med. Res. Rev.* 34 (2014) 1168-1216.

[30] G. Morissette, J. Bouthillier, F. Marceau. Dual antagonists of the bradykinin B₁ and B₂ receptors based on a postulated common pharmacophore from existing non-peptide antagonists. *Biol. Chem.* 387 (2006) 189-194.

[31] Z.L.M. Hofman, A. Relan, C.E. Hack. C-reactive protein levels in hereditary angioedema. *Clin. Exp. Immunol.* 177 (2014) 280-286.

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Figure legends

Figure 1. Effect of purified active plasma kallikrein (apK) on the human isolated umbilical vein. A. Representative tracings of the effect of apK (5 nM) and bradykinin (BK, 10 nM). apK, applied twice at 1-hr interval, had no effect. B. Maximal effects recorded in replicated experiments. C, D. Effect of HK replenishment, applied 30 min before stimulation, on the contractile effect of apK (5 nM; C. representative tracing; D. effect of two concentration levels of HK).

Figure 2. Mechanism of the contraction induced by the HK + apK combination in the human umbilical vein. Tissues were randomly assigned to one of the inhibitory drug treatment, as indicated, from the equilibration time point 2.5 hr; this treatment was maintained for all subsequent recordings. Inhibitory drugs were introduced 30 min before apK stimulation (5 nM), at the same time as HK replenishment (99 nM), and maintained thereafter in the bathing fluid. Kruskal-Wallis test showed that the response to HK+apK and BK stimulation significantly differed across treatments with inhibitory drugs (HK+apK: $P = 0.0004$; BK: $P = 0.0011$). The effect of each drug vs. control responses was further tested using Dunn's multiple comparison test. * $P < 0.05$; ** $P < 0.01$.

Figure 3. Effect of minor or ineffective contractile proteases. A. Representative tracing of the protracted effect of purified neutrophil proteinase 3 (PR3, 31 nM) in the presence of HK. This was followed by stimulations with BK (10 nM) and apK (5 nM) in the presence

of HK to put the effect of PR3 in perspective. B. Purified neutrophil proteinase 3 (3.1 or 31 nM), applied twice at 1-hr interval, had negligible effect on the umbilical vein. C. When HK replenishment was applied 30 min before stimulation, protracted contractile effects were recorded (latency at the 39 nM HK concentration: 9.8 ± 1.8 min; at the 197 HK concentration: 7.7 ± 1.2 min). D. Purified plasmin (10 nM), applied twice at 1-hr interval, had no effect. E. When HK replenishment was applied 30 min before stimulation, small and inconsistent contractile effects were recorded. F, G. Recombinant tissue plasminogen activator (169 nM) has no effect, whether or not HK replenishment was applied.

Figure 4. Effect of metabolic inhibitors applied in a continuous manner on the contractions induced by human recombinant tissue kallikrein (KLK-1) or BK (10 nM of each). A. Inhibitors of intracellular processes. ANOVA indicated that the responses to either KLK-1 or BK were homogeneous between groups ($P > 0.05$). B. Inhibitor of serine proteases, pefabloc SC. * $P < 0.05$, ** $P < 0.01$, Student's t test vs. control response to each stimulus.

Figure 5. Mechanism of KLK-1-induced contraction in the human umbilical vein. A. Representative tracing of a control tissue: effect of continuous drug treatments on the contractile response to KLK-1 (10 nM, recorded at the 3-hr equilibration time point), BK (10 nM, 4 hr) and histamine (100 μ M, 4.5 hr). B. Tissues were randomly assigned to one of the inhibitory drug treatment, as indicated, from the equilibration time point 2.5 hr;

this treatment was maintained for all subsequent recordings. Kruskal-Wallis test showed that the response to KLK-1 and BK stimulation significantly differed across treatments with inhibitory drugs (KLK-1: $P = 0.0024$; BK: $P = 0.0012$). The effect of each drug vs. control responses was further tested using Dunn's multiple comparison test. * $P < 0.01$.

Figure 6. Investigation of the generation of a B_1R stimulant by kallikreins. A. Isolated umbilical vein preparations were treated to optimize the expression of B_1R s, with successive cytokine pretreatment during a long in vitro incubation, testing of the B_1R presence using the selective agonist Sar-[D-Phe⁸]des-Arg⁹-BK, B_2R blockade with icatibant (20 nM) and optional kininogen replenishment, as outlined in the sample tracings (LK and KLK-1 tested). B. Effect of KLK-1 (10 nM) during B_2R blockade with or without LK supplementation. The effect of Sar-[D-Phe⁸]des-Arg⁹-BK was recorded in each tissue prior to B_2R blockade. In the presence of LK and icatibant, adding compound 11 had a significant effect only for KLK-1 (* $P < 0.05$, Mann-Whitney test). C. Inconsistent effect of apK (5 nM) during B_2R blockade with HK supplementation in tissues expressing the B_1R s. Responses recorded in the presence or absence of compound 11 did not significantly differ (Mann-Whitney test).

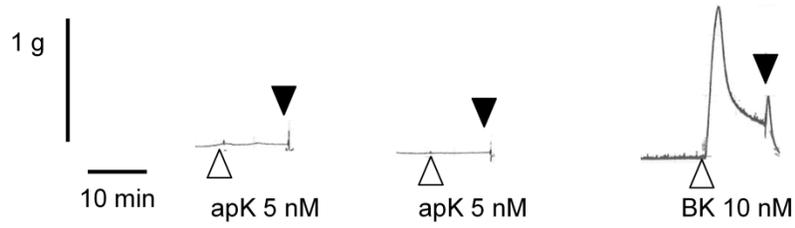
Figure 7. A. Competition of [³H]BK binding to human recombinant B_2R s stably expressed in HEK 293a cells by a panel of unlabeled peptides/drugs. BK, Lys-BK and icatibant are approximately equipotent competitors of nanomolar potency. KLK-1 (10^{-10} - 10^{-6} M) or tranexamic acid do not compete for [³H]BK in this assay (Charest-Morin et al., 2015; present results). B. Tranexamic acid fails to displace [³H]Lys-des-Arg⁹-BK from

either human recombinant B₁Rs. The unlabeled form of the radioligand is used as a positive control.

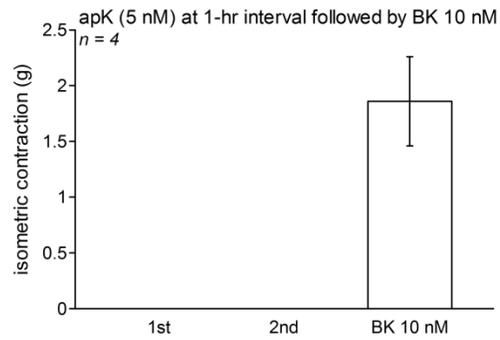
Figure 8. Schematic representation of the kinin-mediated responses to proteases in the isolated human umbilical vein preparation. Reactions difficult to evidence in the system are represented in progressively shaded areas. A reservoir of LK and preformed BK B₂Rs are present in freshly isolated veins, explaining the tachyphylactic response to KLK-1; however the system is depleted of HK. The contractile effect of apK is revealed by HK replenishment. Fibrinolysis is postulated to potentiate kinin generation upstream of the contact system. The inducible B₁Rs may be shown to mediate KLK-1 effects under specific experimental conditions. The effects of some inhibitors are indicated, that of tranexamic acid postulated. Some of the peptidases that metabolize kinin are abbreviated as ap, aminopeptidase; cp, carboxypeptidase; ace, angiotensin converting enzyme.

A. Active plasma kallikrein (apK) 5 nM at 1-hr intervals

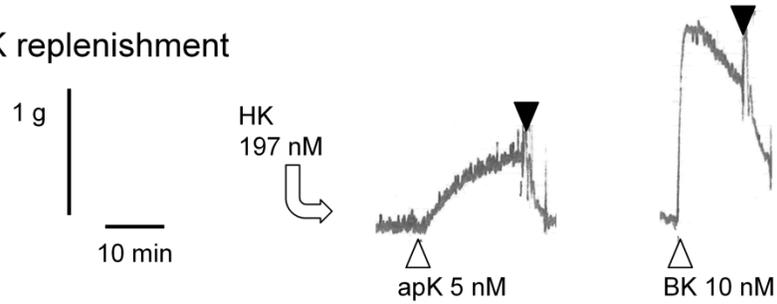
Fig. 1



B.



C. HK replenishment



D.

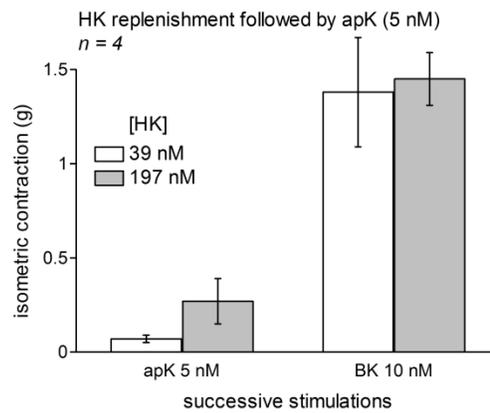
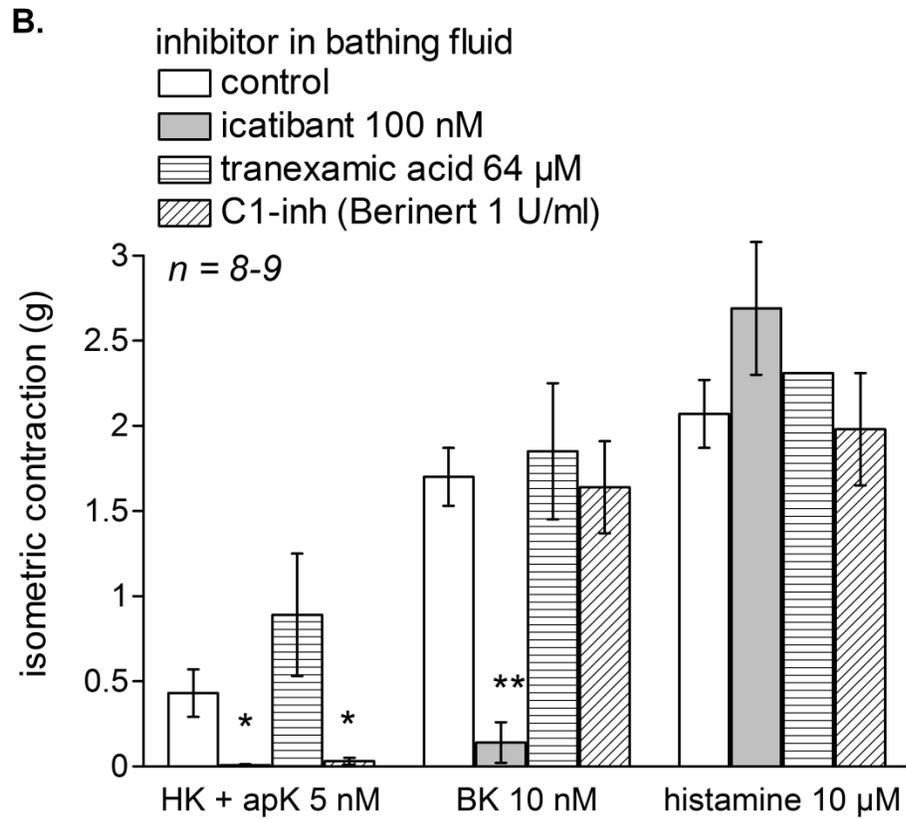
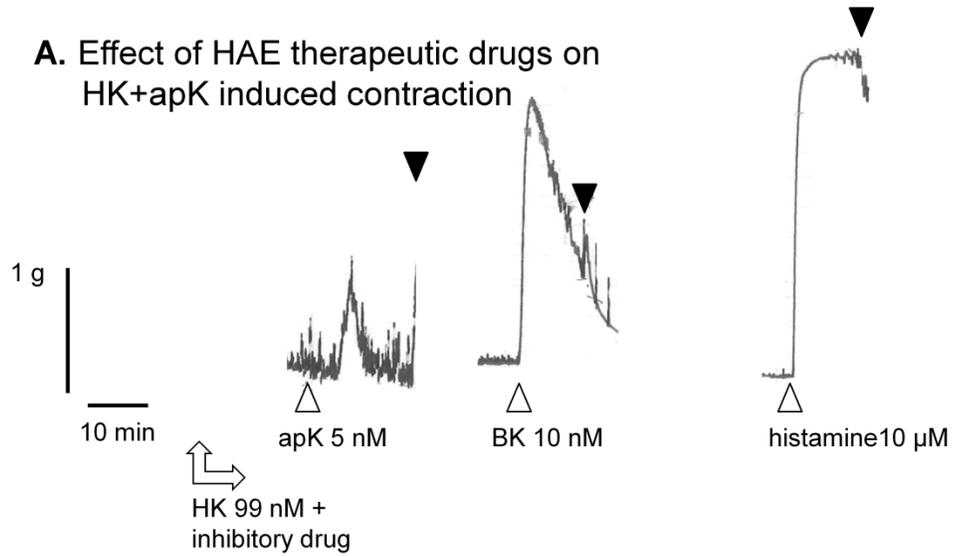


Fig. 2



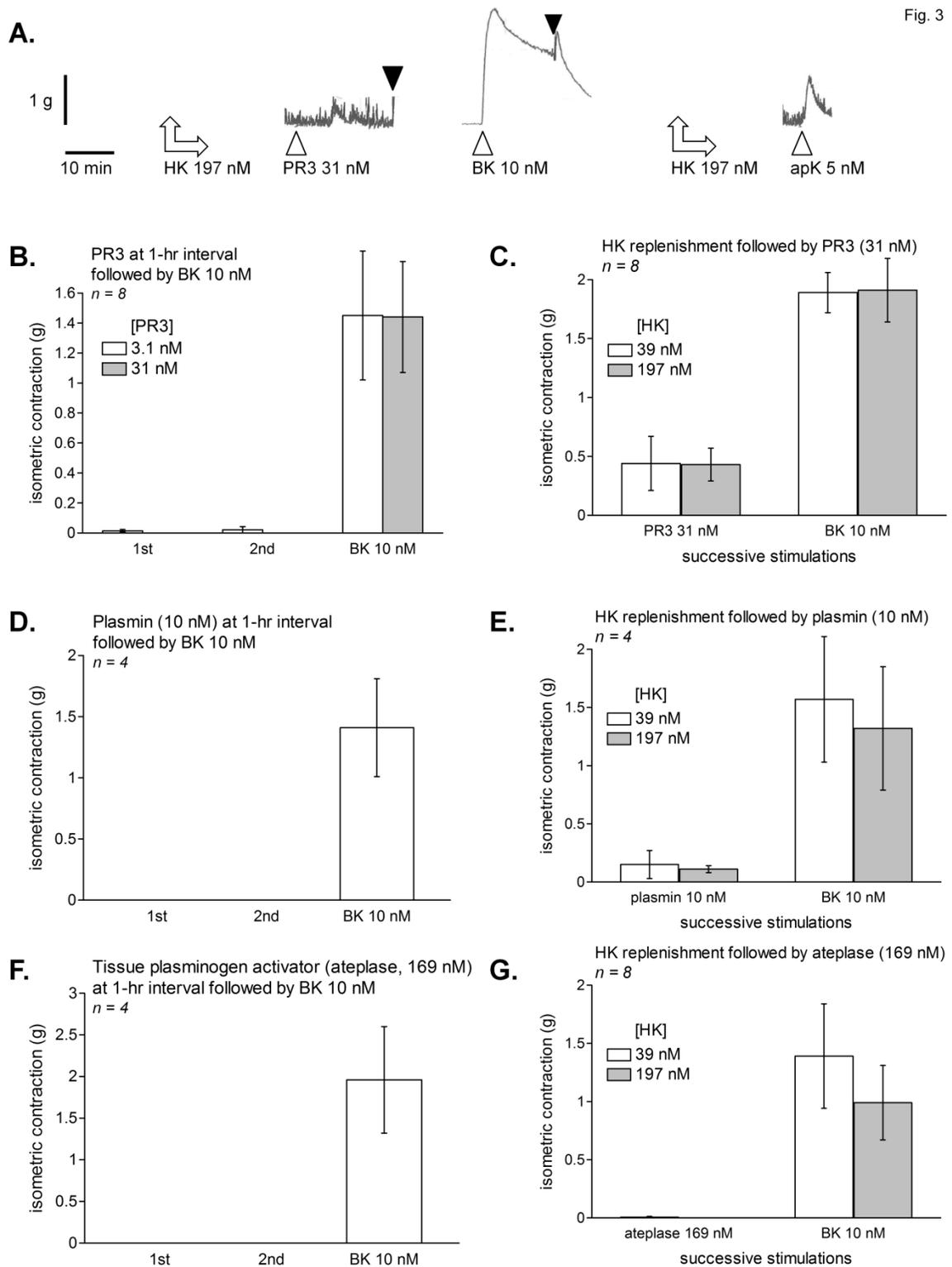


Fig. 4

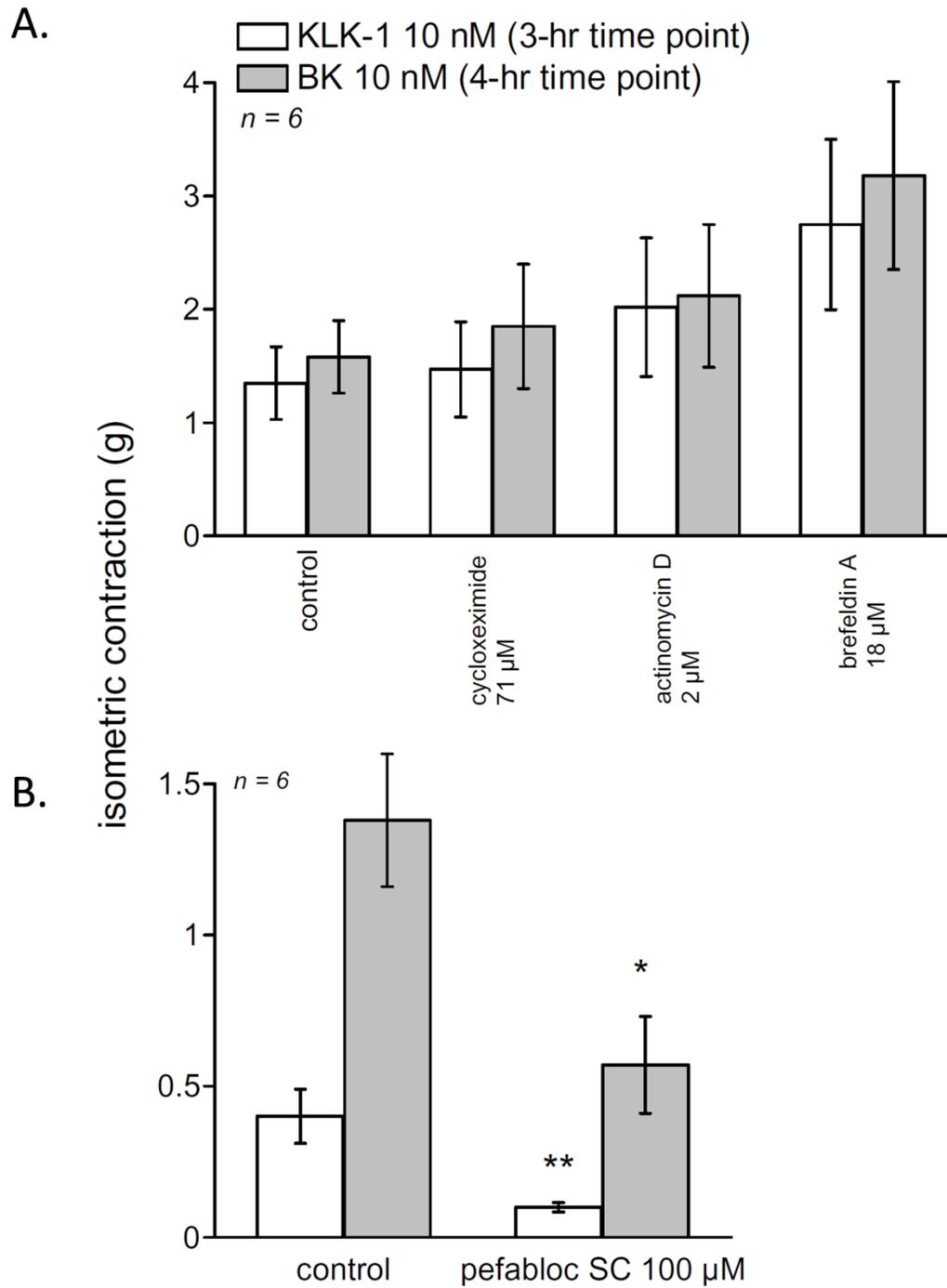


Fig. 5

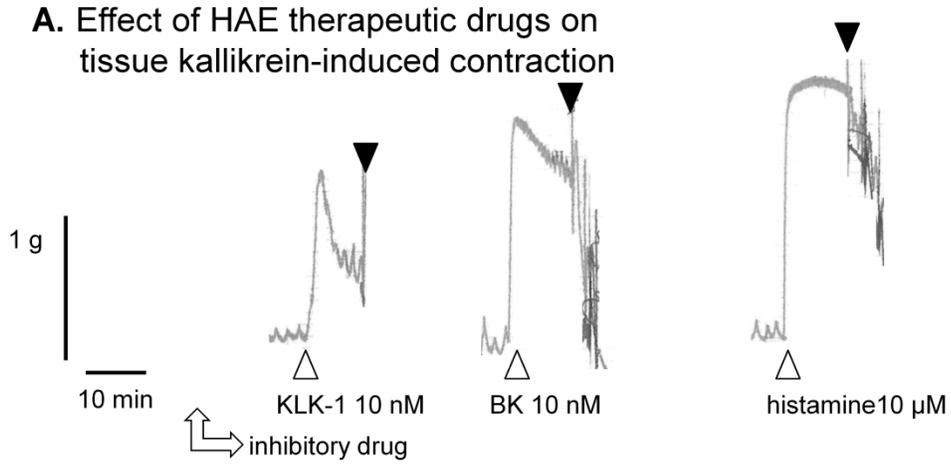
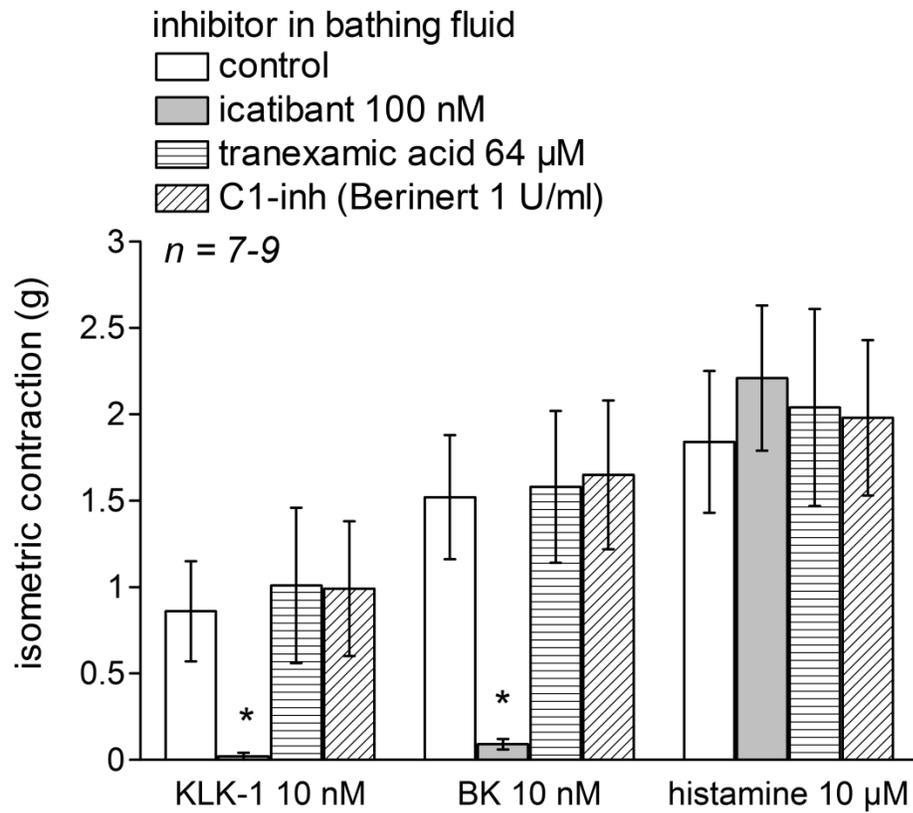
**B.**

Fig. 6

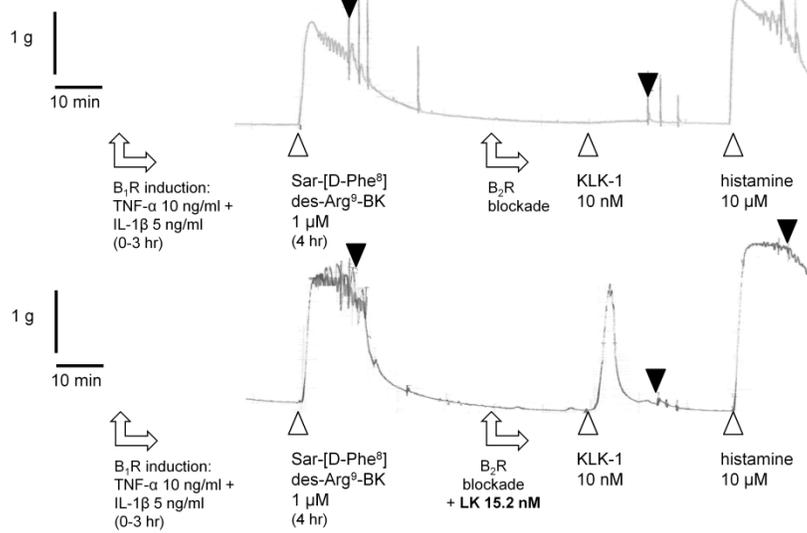
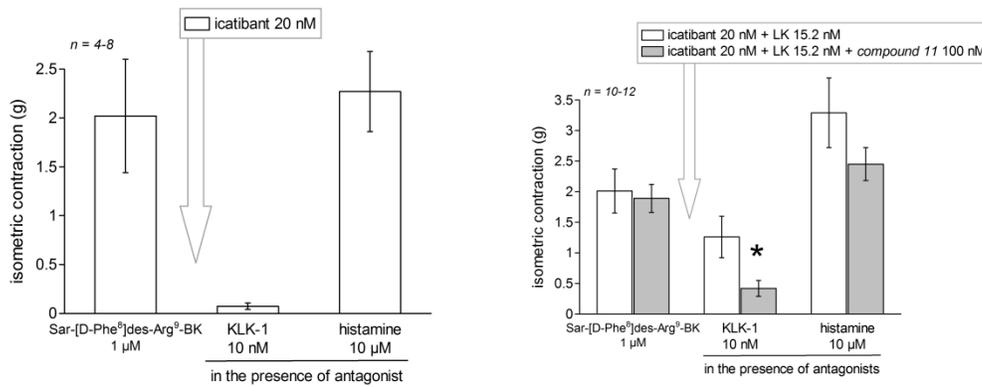
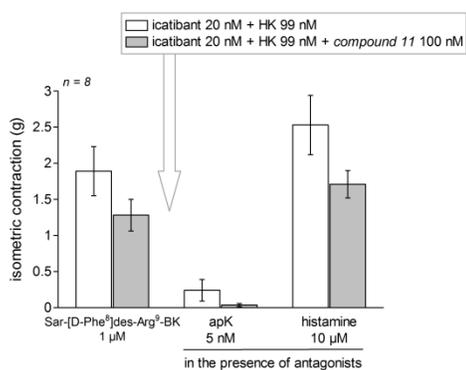
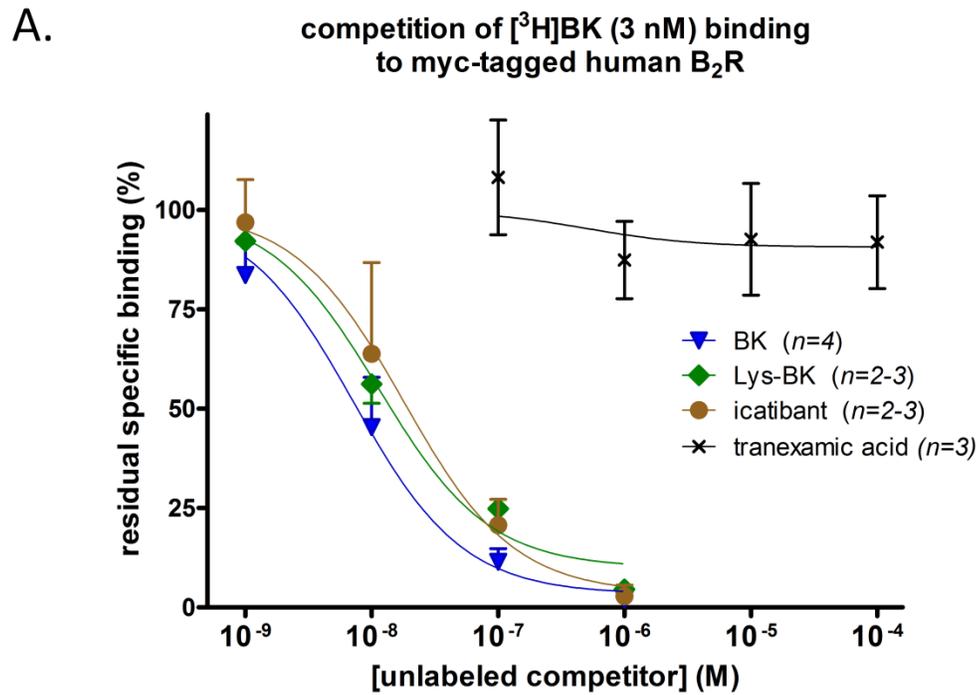
A. Effects of KLK-1 mediated by B₁RsB. Effects of KLK-1 on B₁Rs with or without LK replenishmentC. Effects of apK on B₁Rs

Fig. 7



B. competition of binding of [3 H]Lys-des-Arg⁹BK (1 nM) to hu B₁R-FLAG
 $n = 3-6$

