Vascular smooth muscle contractility assays for inflammatory and immunological mediators

François Marceau[a], Denis deBlois[b], Eric Petitclerc[c], Luc Levesque[d], Guy Drapeau[a], Ritchie Audet[e], Denis Godin[f], Jean-François Larrivée[a], Steeve Houle[a], Thierry Sabourin[a], Jean-Philippe Fortin[a], Guillaume Morissette[a], Lajos Gera[g], Marie-Thérèse Bawolak[a], Gérémy Abdull Koumbadinga[a], Johanne Bouthillier[a]

[a]Centre de recherche en rhumatologie et immunologie, Centre Hospitalier Universitaire de Québec, Québec, QC, Canada G1V 4G2;

[b]Faculté de pharmacie, Université de Montréal, Montréal, QC, Canada H3C 3J7;

[c]Ingénérie cellulaire, Héma-Québec, Québec, QC, Canada G1V 5C3;

[d]Department of Medical Oncology, McGill University, Jewish General Hospital, Lady Davis Institute, Montreal, QC, Canada H3T 1E2;

[e]Département de biologie, Faculté des sciences et de génie, Université Laval, Québec, QC, Canada G1V 0A6;

[f]Natural Sciences and Engineering Research Council of Canada, Ottawa, ON, Canada, K1A 1H5;

[g]Department of Biochemistry, University of Colorado Denver, Aurora, CO, 80045, U.S.A.

Corresponding author: Dr. F. Marceau, Centre Hospitalier Universitaire de Québec, Centre de Recherche en Rhumatologie et Immunologie, T1-49, 2705 Laurier Blvd., Québec QC, Canada G1V 4G2. Tel. +1-418-656-4141 ext. 46155 ; fax : +1-418-654-2765. E-mail: francois.marceau@crchul.ulaval.ca

Abstract

The blood vessels are one of the important target tissues for the mediators of inflammation and allergy; futher cytokines affect them in a number of ways. We review the use of the isolated blood vessel mounted in organ baths as important source of pharmacological information. While its use in the bioassay of vasoactive substances tends to be replaced with modern analytical techniques, contractility assays are effective to evaluate novel synthetic drugs, generating robust potency and selectivity data about agonists, partial agonists and competitive or insurmountable antagonists. For instance, the human umbilical vein has been used extensively to characterize ligands of the bradykinin B_2 receptors. Isolated vascular segments are live tissues that are intensely reactive, notably with the regulated expression of gene products relevant for inflammation (e.g., the kinin B_1 receptor, inducible nitric oxide synthase). Further, isolated vessels can be adapted as assays of unconventional proteins (cytokines such as interleukin-1, proteases of physiopathological importance, complement-derived anaphylatoxins, recombinant hemoglobin) and to the gene knockout technology. The well known cross-talks between different cell types, e.g., endothelium-muscle, nerve terminal-muscle, can be extended (smooth muscle cell interaction with resident or infiltrating leukocytes, tumor cells). Drug metabolism and distribution problems can be modeled in a useful manner using the organ bath technology, which, for all these reasons, opens a window on an intermediate level of complexity relative to cellular and molecular pharmacology on one hand, and in vivo studies on the other.

Keywords Vascular smooth muscle contractility; isolated organs; rabbit aorta; bradykinin; anaphylatoxin C5a; interleukin-1; histamine.

Contents

1. Foreword

The Nobelist John R. Vane was a virtuoso of smooth muscle bioassays which he used notably to show the inhibitory effect of aspirin-like drugs on the biosynthesis of prostaglandins; in his vivid career recollection, he recognized the influence of former physiologists and pharmacologists such as Henry Dale [1]. Vane exploited a battery of smooth muscle assays, some of which from vascular origin, to detect and measure such substances as prostacyclin and thromboxane in the effluent of perfused organs [1]. A seminal discovery based on vascular smooth muscle bioassays was that of endothelium-dependent vasorelaxation [2] that eventually lead to the discovery of nitric oxide (NO) roles in biology and opened other research fronts, such as the cross-talk between different vascular cell types. In this paper, we would like to illustrate known and novel concepts and approaches that have kept the isolated vascular smooth muscle assay useful and interesting to this day with application to inflammatory and immunological mediators. Indeed, blood vessels are among prime targets for inflammatory changes associated with tissue injury (hyperemia, increased permeability, thrombosis, interaction with leukocytes). Inflammatory microvascular changes that are controlled by soluble mediators include vasodilation, increased permeability, blood flow stasis, and exudative movement of plasma fluid and proteins into the inflamed tissue. Isolated vessels support a reductionist approach of certain relevant problems as the hierarchy of soluble mediators (e.g., a cytokine releasing prostanoids or NO or inducing the expression of a receptor for another mediator). This review will be biased in an autobiographical manner, stressing results obtained by F. Marceau's collaborators and himself over the 25 years of existence of his laboratory. The reader may forgive this self-centered exercise if he/she finds inspiration in a fresh look at this technology.

2. The contractility assay and its variants

What is measured using this technique is the tone of smooth muscle (fig. 1). An isolated macroscopic vessel, such as the rabbit aorta, has often little basal tonus, but may be kept alive for several hours in a tissue bath and intense contractile responses can be recorded by injecting known concentrations of drugs into the bathing fluid. In a modern installation, thermostat-controlled warmed water flows around the inner ("jacketed") chamber containing a physiological fluid made only of salts, glucose and water, like Krebs' solution. A vascular ring or helical strip is fixed both at the bottom of the bath and to a thread transmitting the muscle tone to a force transducer (in the isometric model). A gas mixture, typically 95% O_2 + 5% CO_2, is bubbling in the organ bath to deliver oxygen to the tissue in the absence of hemoglobin, to reproduce the bicarbonate buffer of blood plasma in equilibrium with the CO_2 present in the pulmonary alveolar air and to mix the bathing fluid. Drugs that may affect smooth muscle tone are simply injected via the top of the bath and disposed of by amply washing the tissues with fresh buffer. Unlike smooth muscle of other origin (gastrointestinal, reproductive…), vascular muscle often maintains excellent contraction plateaus as a function of time, a physiological necessity for the uninterrupted regulation of blood flow. This is an advantage for constructing cumulative concentration-effect curves and to study relaxing agents in precontracted preparations. As we shall see, isolated tissues are surprisingly apt to respond to stimulation conditions, and to isolation itself, by gene regulation. This is an interesting fact considering that the bathing fluid is not a complete culture medium, and that the whole observation period is probably confined to the pre-apoptotic time window initiated by tissue isolation and incubation in a nutrient-poor medium.

The isolated vascular smooth muscle preparations have been used in quasi-analytical modes, e.g., in the blood bathed organ cascade and other superfusion systems [1] or to assess the inactivation or conversion of synthetic agents in biological milieus. An example of the latter application is the conversion of bradykinin into des-Arg^9-bradykinin in blood plasma, respectively agonists of the kinin B_2 and B_1 receptors that stimulate the contraction of the rabbit jugular and mesenteric vein, respectively [3]. However, these uses tend to be replaced by physicochemical or immunological analytical techniques of high sensitivity and specificity. A perfectly valid use of the isolated vascular smooth muscle preparations is to characterize novel synthetic drugs related to a receptor represented in a given tissue. Classical quantitative modeling of antagonist potency on the logarithmic pA_2 scale based on Schild's regression [4] is readily applicable to smooth muscle contractility assays, and we will repeatedly cite such results.

There are methodological improvements of the vascular smooth muscle contractility assays that extend their usefulness in specific experimental situations and allow to examine specific scientific questions, of which we will give only a few examples:

- The oil immersion technique [5] consists of replacing the bathing fluid of vascular strips with prewarmed mineral oil, thus reducing the diffusion of hydrophilic agonists from the bathing fluid to the tissue. This allowed the evaluation and analysis of drug disposition within the tissue, as the contractile response faded more or less rapidly as a result of agonist concentration decrease at the vicinity of receptors. An application of this technique is further reviewed in section 8.
- Mulvany and Halpern [6] have adapted the contractility assay to small resistance arterial vessels of 150-250 µm of diameter from the rat mesenteric vascular bed. These preparations, for which force transducers and microscopic observation were exploited

simultaneously, responded to high K^+ (depolarization) by contraction, allowed addressing the effect of genetic hypertension on arteriolar structure and function and this technology had an important influence on subsequent hypertension research. As for inflammatory changes in the microcirculation, streptozotocin-induced diabetes in rats determined a loss of relaxing response of isolated resistance arterioles to acetylcholine [7]. This change was largely reversed by the expression of human tissue kallirein (KLK1) in transgenic rats [7], suggesting a protective effect of endogenous bradykinin on the endothelial function.

- Trains of electrical transmural stimulation with electrodes at certain potential amplitude and frequencies (e.g., 50 V, 8 Hz) allow to depolarize with selectivity the nerve terminals contained in the preparation (fig. 1A), which are usually sympathetic varicosities in arteries such as the rabbit aorta [8]. Adventitial nerve terminals release norepinephrine which determines the contraction of the smooth muscle via α-adrenoceptors.
- A special organ chamber adapted to optical measurements allowed the simultaneous measurement of cytosolic calcium concentration (based on the Fura2 fluorescent probe) and vascular contraction upon agonist stimulation of the rat isolated aorta [9]. The raise in cytosolic calcium concentration always preceded contraction induced by norepinephrine and KCl, however the α-adrenoceptor agonist also sensitized the preparation to calcium as its contractile response was greater than expected based on strictly the intracellular calcium.
- Rabbit aorta and pulmonary artery preparations were mounted in organ baths and their plasma membrane were dissolved with the detergent β-escin. A special bathing fluid based on intracellular composition and containing ATP, creatinine phosphate and calmodulin, allowed to record contractile responses to second messengers such as calcium

(500 nM) and inositol triphosphate (100 µM) as a function of time [10], an analysis of contractile signaling that exploits the "real time" tone measurement of the organ bath methodology.

- Organs used for contractility studies can be isolated from animals, usually mice, in which a selected gene has been deleted by homologous recombination (gene knockout). Among many examples, knockout of the AT_{1B} subtype receptor gene reduced to a major extent the contractile response of the mouse isolated abdominal isolated aorta to angiotensin II without altering its response to a thromboxane mimetic or to high KCl [11]. In another application relevant to inflammation, the aortas of male mice was exploited [12]. Estrogen receptor-α protected tissues from wild type mice from lipopolysaccharide (LPS)-induced loss of response to NO, released either from the endothelium by acetylcholine or by a nitrovasodilator agent, as shown by the worse outcome in tissues isolated from estrogen receptor-α knockout treated with LPS [12].
- In principle, freshly isolated vascular tissue should lend itself to other molecular biology approaches that permit modulating the expression of a single gene, such as gene knockdown (siRNA) or adenoviral gene transfer. The latter was illustrated in the rabbit carotid arteries where virally encoded murine inducible NO synthase (iNOS) was expressed 20-24 h after infection of arterial rings [13]. The tissues were mounted after culture and displayed the expected NO-mediated nonspecific inhibition of contractile responses.

3. Versatile bioassay for synthetic drugs: the example of the bradykinin B_2 receptor

The human umbilical vessels have a special sensitivity to various agents that may be generated in stagnant blood, like platelet-derived 5-hydroxytryptamine and thromboxane A_2 and peptides derived from plasma proteolytic cascades like bradykinin [14] and complement-derived anaphylatoxins [15]. This sensitivity serves a probable role in the post-partum closure of the lumen of these vessels. This physiological knowledge has been exploited for the quantitative assay of novel bradykinin B_2 receptor ligands using rings of freshly isolated umbilical veins [16]. Fig. 2 shows the structure of most agents discussed below. We found that the peptide antagonists icatibant (D-Arg[Hyp^3, Thi^5, D-Tic^7, Oic^8]-bradykinin) and the first nonpeptide antagonist of this receptor, WIN64338, were competitive bradykinin antagonists with pA_2 values of 8.2 and 6.0, respectively [16]. Other laboratories have seen the potential of the human umbilical vein as a bioassay for novel drugs binding to the human form of the B_2 receptor [17-20]. This approach complements nicely the modern drug development efforts, often based on radioligand binding competition assays and cellular systems of signal transduction, as it allowed to identify the competitive or insurmountable nature of antagonists [21], their specificity and special properties of ligands. Thus, the nonpeptide antagonist LF 16-0687 was inactive against 5-hydroxytryptamine-induced contraction of the vein but potent against bradykinin, pA_2 8.3 [22]. Partial agonist effects were recorded in response to a nonpeptide stimulant of the B_2 receptors, compound 47a, structurally related to antagonists [23]. A special response to the bradykinin analog B-9972 (= D-Arg-[Hyp^3, Igl^5, Oic^7, Igl^8]-bradykinin) and to compound 47a is the slow relaxation upon tissue washing of contractions induced by these B_2 receptor stimulants that are resistant to enzymatic breakdown [23, 24]. Further, in a series of nonpeptide B_2 receptor antagonists, a certain lack of correlation between potency at a binding assay to recombinant human receptor and at antagonizing bradykinin in the umbilical vein has been observed (the "binding paradox" [19]), suggesting that the vascular bioassay has the potential to eliminate drug

candidates deficient on other accounts than receptor affinity (perhaps related to the ability of specific drugs to maintain a concentration in the extracellular space, see below Section 8). Vascular smooth muscle preparations confirmed species-specific behavior of B_2 receptor antagonists: structurally constrained peptides related to icatibant and B-9430 are insurmountable and quasi-irreversible (nonequilibrium) antagonist of bradykinin in the rabbit jugular vein, but surmountable and reversible in the human umbilical vein [16, 25]. The nonpeptide antagonist bradyzide has high potency at the B_2 receptors from rodent species and the rabbit and low potency at the human receptor, and vice versa for its analog compound 19c (pA_2 7.53 at the human umbilical vein [21, 26]). Finally, the human umbilical vein has been used to confirm the competitive antagonist status of a fluorescent B_2 receptor ligand, B-10380 (pA_2 6.83), that is an N-terminally extended version of the conventional antagonist B-9430 (pA_2 7.70) [27]. B-10380 labels the plasma membrane of cells that express recombinant B_2 receptors at high densities (epifluorescence) [27].

The only bradykinin B_2 receptor ligand in clinical use is presently icatibant, indicated for aborting episodes of hereditary angioedema, but successfully used "off label" in alternative acquired forms of angioedema, those associated to therapy with angiotensin converting enzyme inhibitors or estrogens [28-31]. The benefits of icatibant in angioedemas stem from antagonizing the inflammatory and vascular effects of bradykinin.

4. Gene expression in isolated blood vessels: the bradykinin B_1 receptors

It is perhaps surprising that isolated blood vessel preparations respond to the environment, and to the isolation process itself, by differential gene expression, but this has been well documented by

different laboratories. The bradykinin B_1 receptor was initially defined as the receptor subtype that mediates the contractile effect of kinins on the rabbit isolated aorta [32]. In retrospect, this discovery was based on pathologic gene expression because it was later recognized that this and other smooth muscle preparations are totally insensitive to B_1 receptor agonists at the beginning of the in vitro incubation, but gradually become responsive as a function of time via an RNA and protein synthesis-dependent process [33]. Thus, rabbit aortic rings do not respond to the B_1 receptor selective agonist, the fragment des-Arg^9-bradykinin, within the first hour of incubation (fig. 3), while they exhibit an increasing contractile response to this peptide at times 3 or 6-h post isolation. By comparison, the α-adrenoceptor agonist norepinephrine elicited a much more stable response from the beginning (1.5 h) to the end (6.5 h) of the incubation (fig. 3). This kind of experiment has been prolonged up to 10 h, with the maximal response to des-Arg^9-bradykinin continuously increasing [34]. The sensitization behavior is suppressed by protein synthesis inhibitors, such as cycloheximide and anisomycin, and by the RNA synthesis inhibitor actinomycin D introduced in the bathing fluid, a first indication that rabbit vascular preparations maintained in Krebs solution synthesize proteins relevant for signaling. The isolated rabbit aorta maintained in Krebs solution incorporates [^{3}H]leucine and [^{3}H]mannose into proteins and glycoproteins, respectively [35], these assays validating the use of protein synthesis inhibitors and of the glysosylation inhibitor tunicamycin which also abated the time-dependent development of responsiveness to B_1 receptor agonists. The concept that tissue injury and inflammation upregulate the expression of the kinin B_1 receptor with selectivity was derived from experiments initially conducted in isolated tissues and subsequently confirmed in vivo [36]. For instance, treatment of live rabbits with bacterial LPS induces a state of sensitivity to des-Arg^9-bradykinin in all the cardiovascular system, including the aorta that, if isolated from a LPS-treated animal, responds to this peptide during the first hour of isolation (fig. 3). Thus, the early

response of the vascular ring monitors the extent of receptor induction that has developed in vivo before sacrifice and this level can be "frozen" by inhibiting further protein synthesis with cycloheximide (fig. 3). The knowledge of the B_1 receptor structure, a G protein coupled receptor, later allowed to predict its synthesis in the endoplasmic reticulum and transition to the Golgi network before cell surface expression. This transit is blocked by brefeldin A, an agent that does not inhibit protein synthesis per se but nevertheless abolished the development of responsiveness to des-Arg^9-bradykinin in rabbit aortic rings [35].

The rabbit isolated aorta has lent itself to many studies that identified modulators of the B_1 receptor upregulation that, without directly affecting contractility, exerted specific effects on the development of responsiveness to B_1 receptor agonists when added to the bathing fluid in vitro. Among modulators that accelerated the upregulation of B_1 receptors, stimulants of the innate immune systems (LPS, mycobacterial muramyl dipeptide), inflammatory cytokines (interleukin (IL)-1, IL-2) and epidermal growth factor (EGF) were noteworthy [33, 34]; glucocorticoids were powerful negative regulators. Other molecular approaches, including the detection of the B_1 receptor gene mRNA in rabbit aortic rings isolated from LPS-treated rabbits or simply incubated in vitro in serum-free culture medium [37], essentially confirmed that the synthesis of the B_1 receptor is triggered by inflammation and direct tissue injury. Complex transduction events regulating B_1 receptor expression in the rabbit isolated aorta are linked to nuclear factor-κB and MAP kinase signaling [37, 38] and also to autocrine/paracrine EGF, based on the inhibitory effect of a selective receptor tyrosine kinase inhibitor [39].

5. Gene expression in isolated blood vessels: responses to cytokines

Two other inflammatory systems of interest show the de novo synthesis of signaling molecules in isolated vascular preparations. Precontracted rabbit mesenteric arteries maintain a relatively good plateau over a long period, but application of IL-1β or -1α to precontracted tissues induces a slow-developing but intense prostaglandin-dependent relaxation (fig. 4) (prevented or reversed by a cyclooxygenase inhibitor) [40, 41]. This endothelium- and NO-independent response to IL-1 was also prevented or reversed by protein synthesis inhibitors, but not by brefeldin A, indicating that one or more nonsecreted proteins with a short turnover were necessary for this activation of the arachidonate cascade [41]. The other system is linked to the induction of iNOS expression by cytokines in the smooth muscle cells of isolated blood vessels. Rat aortic rings exposed in vitro to IL-1β for several hours exhibit a depressed response to any contractile agent in a NO-, cyclic GMP- and protein synthesis-dependent process [42, 43]. This has been fully reproduced in the rabbit aorta (loss of contractile activity prevented by treatment with protein synthesis inhibitors or a glucocorticoid, acutely reversed by the NOS inhibitor N^G-nitro-L-arginine) [44].

An unusual protein that has found its way into organ baths is a genetically engineered recombinant hemoglobin (rHb1.1) that had been developed for use as a blood substitute [45]. rHb1.1 acutely antagonized or reversed the relaxing actions of NO either released from the endothelium by acetylcholine of from the smooth muscle cells by a prolonged IL-1β treatment that induced iNOS (fig. 5). This was certainly due to the known capability of globins to bind and functionally neutralize NO. Cell free rHb1.1 was much more potent than hemoglobin contained in erythrocytes to antagonize NO [45], which may ultimately explain the termination of this project because the recombinant protein, being designed to be infused directly into the plasma, consistently produced vasoconstriction and hypertension in vivo.

The literature reviewed above suggests that proteins not usually linked with smooth muscle function can nevertheless influence the contractility of vascular smooth muscle yielding robust, if not conventional, bioassays. Thus, the cytokine IL-1 modifies the response of rabbit isolated vessels at least in 3 manners (induction of kinin B_1 receptors, activation of the arachidonic cascade, induction of iNOS) and all 3 readouts were suppressed by the natural IL-1 receptor antagonist (IRA) applied in vitro at nanomolar concentrations [44]. By constructing series of concentration-effect curves for IL-1β-induced relaxing effect in precontracted isolated rabbit mesenteric arteries in the presence of various concentrations of recombinant IRA, a pA_2 value of 8.9 can be estimated (perhaps an objectionable analytical procedure due to the indirect effect of the agonist IL-1 that may not be a system at equilibrium). Similarly, growth factors such as EGF influence smooth muscle preparations in several ways [38, 46, 47]. For instance, the precontracted rabbit mesenteric artery responds to EGF in manner identical to that of IL-1β, by a prostaglandin- and protein synthesis-dependent relaxation [47].

6. Assays for proteases of physiopathological importance

Proteolytic enzymes may also act as nonconventional stimuli of vascular preparations. α-Thrombin is a key enzyme involved in blood coagulation, exerting its activity on soluble substrates in the circulation, but also exhibiting in many cell types hormone-like activities that are mediated by cleavable receptors. Indeed, the molecular explanation for thrombin signaling hinged on the discovery of a G protein coupled receptor activated by the limited proteolysis of the N-terminal extracellular domain in which a thrombin binding/cleavage site exists [48]. A G protein coupled receptor subclass, the protease activated receptors (PARs), is presently represented by this PAR_1 and 3 other members [49]. These receptors can be activated by synthetic peptides, as

short as five or six amino acids, such as Ser-Phe-Leu-Leu-Arg-Asn-NH_2 (NAT_6-NH_2) for PAR_1, corresponding to the newly generated amino terminus (NAT) sequence acting as a "tethered ligand" following proteolytic cleavage of the receptor. Thrombin also cleaves PAR_3 and PAR_4, corresponding to other NAT sequences, and PAR_2 is cleaved by mast cell-derived tryptase, interestingly [49]. Thrombin is a modulator of vessel tone that can act by either endothelium-dependent or -independent mechanisms and the relaxation or contraction induced by thrombin can be reproduced by synthetic agonists of the cleavable receptors. In the intact rat aorta, PAR_1 peptide agonists are relaxing agents [50]; however, contractile responses are observed when the endothelium is removed in this preparation and in the canine coronary arteries [51, 52]. In the rabbit aorta, thrombin and NAT_6-NH_2 produce a dose-dependent contraction suggesting that the cleavable PAR_1 is involved in this activity [53, 54].

Another example of protease-induced vasomotor activity is that elicited by tissue kallikrein (KLK1) in the rabbit jugular vein [55]: this tissue is contracted via endogenous bradykinin B_2 receptors, based on the preventive effect of the specific antagonist LF 16-0687, but in a tachyphylactic manner, meaning that the contraction cannot be obtained twice. By contrast, bradykinin itself is not tachyphylactic. These and other results suggest that kallikrein activates the release of bradykinin from blood-derived kininogen(s) that have remained associated with the blood vessel after the isolation process.

7. Cross-talk between different vascular cell types

Furchgott and Zawadzki [2] exploited the precontracted rabbit isolated aorta to show that the relaxant effect of acetylcholine was due to the presence of endothelial cells that could be removed

by either scrubbing them from the intimal surface or detaching them enzymatically. Further, a diffusible but instable substance, endothelium-derived relaxing factor (EDRF), was shown to be the intermediate between the endothelial and the smooth muscle cells. EDRF was later identified as the NO gas, itself formed from a special metabolic pathway catalyzed by the endothelial NO synthase from the L-arginine substrate [56, 57]. This exemplary demonstration of a cross-talk between different cell types present in an isolated vessel has had a considerable impact, notably leading the way to other forms of endothelium-dependent vasomotricity. One may add that the rabbit isolated aorta has surprising metabolic resources, relying on its own arginine supply to produce sustained and nontachyphylactic endothelium-dependent responses.

Other forms of interactions between cell types present in the vascular structures may be considered, and the resident or infiltrating leukocytes may have been largely overlooked (fig. 1A). Histologic techniques show that resident leukocytes are abundantly found in the connective tissue lining blood vessels of all sizes, and preatherosclerotic lesions in the subendothelial layer is another location rich in macrophages [58]. There is no uniformly effective method to remove these cells from vascular preparation, as it exists for the fragile and accessible endothelial cells, but accumulating circumstantial evidence support the vasomotor roles of resident or infiltrating leukocytes in vascular preparations [58]. First, agonists or receptors believed to activate phagocytic leukocytes with selectivity, such as the formylated peptide f-Met-Leu-Phe and the complement-derived anaphylatoxin C5a, exert contractile or, more rarely, relaxant effects largely mediated by prostanoids on isolated blood vessels in the absence of blood. Although receptors for C5a may be found in nonleukocyte cell types as the endothelium [59], the calcium signaling necessary for eicosanoid production is not produced by C5a in these cells. In some isolated vessels, such as the guinea pig portal vein and pulmonary artery, C5a has a contractile effect

based at least in part on resident mast cells, based on the large histaminic component of the response [60]. In the human umbilical artery, devoid of mast cells, the binding sites for C5a and f-Met-Leu-Phe or C5a receptor immunoreactivity are restrained to a rare cell population located more densely in the periphery and corresponding to histiocytes [15, 61]. The human umbilical artery contractility has been the basis of an assay for synthetic analogs of C5a, culminating in the characterization of C5a antagonists [62].

Organ culture has been attempted for regenerative medicine approaches and the pharmacological responses of such macroscopic "vascular equivalent" have been studied in organ baths [63]. Thus, vascular equivalents made only of pure cultured smooth muscle cells cultured from the umbilical artery and subsequently mounted in organ baths do not respond to C5a, f-Met-Leu-Phe or an analog of the later, unlike the fresh arterial tissue, although direct smooth muscle stimulants like the thromboxane mimetic U-46619 were effective in both types of preparations (fig. 6) [58]. It is of interest that the rabbit isolated aorta is also essentially unresponsive to C5a or f-Met-Leu-Phe, unless the animals were submitted to pathologies that determine phagocyte infiltration in the vascular wall, like a cholesterol enriched diet or serum sickness [64]. In these cases, prostanoid-dependent contraction was recorded in response to the chemotactic peptides and it may be speculated that thrombogenic actions may also arise in such systems from the release of thromboxane-like lipids. These experimental systems where the leukocyte density varies also support leukocyte-derived vasomotion.

Little is known about the interaction of tumor cells with host vascular smooth muscle cells. In reconstitution experiments, tumorigenic cell lines (including the rat hepatocarcinoma Morris 7777 and human melanoma M-21) were cultured for 17 hr in the presence of rat aortic rings,

subsequently evaluated in contractility assays (response to phenylephrine and KCl) [65]. An agonist-independent loss of contractility was observed in rings pre-incubated with either tumorigenic cell lines or their conditioned medium. The depressing effect of Morris cells depends largely on iNOS expression, whose immunoreactivity was found in some muscular vessels at the periphery of tumors formed by the Morris cell line in rats. The Morris cells release low molecular weight mediator(s) that activate nuclear factor-κB and iNOS synthesis in smooth muscle cells. M-21 melanoma cell coculture rather produces apoptosis in rat aortic rings, accounting for the irreversible loss of contractility. Either mechanism may contribute to the maximal, unregulated blood flow observed in tumors.

8. Distribution, gradients and pharmacologic distortion

Kenakin [66] has provided a theoretical approach to situations of drug removal from the receptor compartment. Thus, the sequestration (e.g., in acidic intracellular organelles, pathway 3 in fig. 7A) or breakdown (e.g., hydrolysis by an ectopeptidase, pathway 2, fig. 7A) of some drugs in the tissue extracellular fluid in a manner that is not completely compensated by diffusion from the bathing fluid (pathway 1) alters the available agent concentration at the vicinity of receptors (pathway 4, fig. 7A). The compact structure of the contractile tissue, like the rabbit aorta which contains about 25 layers of smooth muscle cells along with elastic laminae and other conjunctive tissue (fig. 7, top right) [67], is the physical basis of diffusion barriers that will support the lack of equilibrium between the drug concentrations in the bathing fluid and that at the receptor level, given that a sink mechanism is active within the tissue. The result of these situations is an underestimation of drug potency that may be important, relative to "true" receptor affinity. The actions of neurotransmitters are prone to quantitative distortions of potency in isolated organs

where, for instance, norepinephrine uptake by presynaptic transporters or acetylcholine hydrolysis by cholinesterases occur at the vicinity of receptors [66]. Can the inactivation mechanisms within the tissue (pathways 2, 3) be isolated from the diffusion rate from the bathing fluid (pathway 1, fig. 7A) ? Yes, the oil immersion technique [5] acutely interrupts pathway 1. When this was applied to rabbit aortic rings precontracted with the PAR_1 agonist peptide NAT_6-NH_2, the tissue relaxed rapidly under the influence of aminopeptidase N present in the tissue [52]; this and other results lead to the rational design of an aminopeptidase-resistant analog of the peptide. The inactivation mechanisms themselves can be inhibited with an appropriate drug (e.g., the aminopeptidase inhibitor amastatin) or with the gene knockout technology, as for the deletion of the ectonucleotidase CD39 gene which allowed to record important contractile effects of ATP and UTP mediated by purinergic receptors ($P2Y_6$) present in the mouse aorta [68], these nucleotides exhibiting little activity in the aorta of wild type mice.

We have illustrated the same concept based on pharmacological antagonists of inflammatory/allergic mediators that were inactivated at the vicinity of receptors (fig. 7B-G). In both examples, competitive antagonists were used at several concentrations to generate families of agonist concentration-effect curves shifted to the right and analyzed using the Schild regression, thus yielding potency estimates in the pA_2 scale. The peptide antagonist of the bradykinin B_1 receptor Lys-[Leu^8]des-Arg^9-braykinin is susceptible to aminopeptidase N inactivation in the rabbit isolated aorta because it is considerably more potent in the presence of the aminopeptidase inhibitor amastatin (fig. 7B-D) [67]. This did not apply to the agonist used in this series of experiments, des-Arg^9-bradykinin, but the alternate high affinity agonist Lys-des-Arg^9-bradykinin was also susceptible to potency distortion by the action of aminopeptidase N [67]. Other peptide antagonists protected against aminopeptidases or nonpeptide antagonists of

the B_1 receptors were not potentiated by amastatin [65], indicating a situation closer to equilibrium for these drugs in the aortic tissue.

In a completely different context, while studying the uptake of cationic drugs into acidic intracellular organelles, we have observed that this "pseudotransport" coupled to proton pumping by vacuolar (V)-ATPase alters the potency of the most lipophilic anti-histamines, like astemizole. The antagonist potency of this anti-allergic drug at the H_1 receptor of the rabbit aorta is potentiated by the V-ATPase inhibitor bafilomycin A1, indicating that astemizole is removed from the vicinity of cell surface receptors (Fig. 7E-G) [69]. The efficacy of V-ATPase-mediated sequestration of cationic drugs is critically dependent on their liposolubility and this distortion, also observed with terfenadine, was not observed for more hydrophilic antihistamines pyrilamine and diphenhydramine. The contractile effect of histamine was unaffected by bafilomycin A1 [69].

Metabolic activation can occur in some cases at the vicinity of receptors. Angiotensin I-induced contraction of the rabbit aorta stimulated via smooth muscle cell AT_1 receptors is in fact largely mediated by the shorter peptide angiotensin II produced in situ via the ectopeptidase angiotensin converting enzyme, as shown by the selective potency attenuation of angiotensin I, but not of angiotensin II, by treatments with converting enzyme inhibitors [70]. Similarly, the only kinin receptor subtype present in the rabbit isolated aorta is the B_1 receptor, selectively stimulated by the des-Arg^9 metabolites of native kinins. Thus bradykinin and lysyl-bradykinin contractile effects on this tissue are largely mediated by their in situ conversion into their respective des-Arg^9 metabolites by carboxypeptidase(s) present in the tissue [71].

9. Concluding remarks

The isolated organ technology does not need defense, because it continuously enjoyed popularity for many decades. While models based on macroscopic isolated blood vessels have limitations (low hemodynamic importance in many cases, lack of integration of the in vivo neurohumoral regulation), we have illustrated their versatility as bioassay organs for mediators of inflammation with a "real time" feeling irreplaceable for characterizing nonequilibrium drug responses and the potential to integrate more recent technologies, such as gene deletion. Isolated vascular segments are live tissues that are intensely reactive, notably with the regulated expression of gene products relevant for inflammation. The well known cross-talks between different vascular cell types, e.g. endothelium-muscle, nerve terminal-muscle, can be extended (smooth muscle cell interaction with resident or infiltrating leukocytes, tumor cells). Drug metabolism and distribution problems can be modeled in a useful manner using the organ bath technology, which, for all these reasons, remains a useful window on an intermediate level of complexity between cellular and molecular pharmacology on one hand, and in vivo studies on the other.

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Figure legends

Fig. 1. A. Some of the cell types present in macroscopic blood vessels. B. Schematic view of a vascular ring mounted in an isolated organ bath.

Fig. 2. Structure of peptide (top) and nonpeptide (bottom) ligands of the bradykinin B_2 receptor assayed using the human isolated umbilical vein and other preparations. Abbreviations: BK, bradykinin; [5(6)-CF]: 5(6)-carboxyfluorescein; CpG: α-cyclopentylglycyl; ε-ACA: ε-aminocaproyl; Hyp: *trans*-4-hydroxyproline; Igl: α-(2-indanyl)glycyl; Oic: (3as, 7as)-octahydroindol-2-yl-carbonyl; Thi, β-thienylananyl; Tic: 1,2,3,4-tetrahydroisoquinoline-3-carboxylic acid.

Fig. 3. Contractile effect of des-Arg^9-bradykinin (des-Arg^9-BK, 1.7 μM) and of norepinephrine (NE, 100 nM) on the rabbit aortic strip, as a function of incubation time in vitro. The two strips of the same aorta isolated from a normal rabbit were initially insensitive to des-Arg^9-BK (tracings a and b) whereas a significant response was recorded in paired strips isolated from a rabbit pretreated with lipopolysaccharide (LPS, 50 μg i.v. 5 h before sacrifice; tracings c and d). The subsequent up-regulation of responses to the kinin (tracings a and c) was inhibited in the corresponding paired strip continuously exposed to cycloheximide (71 μM; tracings b and d). Abscissa scale, time; ordinate scale, isometric contraction. Closed symbols refer to the application of agents and open symbols to washout of stimulants. Reprinted from [34] with permission from Elsevier.

Fig. 4. Effect of interleukin-1 (IL-1) of the α and β types on typical preparations of rabbit isolated mesenteric artery. Tissues were precontracted with phenylephrine (PE, 4 μM) before the application of the IL-1. The top tracing illustrates the spontaneous evolution of the PE-induced plateau over the observation period. At the end of the observation period, indomethacin (Indo, 2.8 μM) was injected into the baths without washing the tissues. Abscissa scale: time; ordinate scale: isometric contraction, g. Closed symbols refer to the application of agents and open symbols, to washout of stimulants. From [40] with permission from Wiley-Blackell.

Fig. 5. Effects of recombinant hemoglobin on rabbit aorta rings. A, B. Representative tracings of acetylcholine (ACh)-induced relaxation of rings precontracted with phenylephrine (PE 500 nM) and time courses of reversal of ACh vasorelaxant effects on tissue exposure to human recombinant hemoglobin (rHb1.1, 15 μM). C, D. PE (500 nM)-induced contractions in rings preincubated with vehicle (C) or IL-1β (1.4 nM, 3 h followed by 2 h of cytokine-free incubation, D). After the tissue response to PE has reached a plateau, tissues were challenged with rHb1.1 (15 μM, D). Arrowheads: times of drug or solvent additions to the organ baths; dots: first of a series of stimulant washouts. Reprinted from [45] with permission from Wolters Kluwer/Lippincott, Williams & Wilkins.

Fig. 6. Effect of chemotactic peptides and other agents on the human isolated umbilical artery. (A) responses of a fresh artery obtained post-partum, prepared as a strip. (B) Response in vascular equivalents made of pure smooth muscle cell cultures. Abscissa scale: time; ordinate scale: isometric contraction. Closed arrowheads refer to the application of agents; open triangles refer to the first of a series of stimulant washouts. From [58] with permission from Elsevier.

Fig. 7. Pharmacodynamic distortion of antagonist drug potencies by inactivating mechanisms in the rabbit isolated aorta. A. Left: schematic representation modified from Kenakin [64]: the concentration of the drug D at the vicinity of receptors (arrow 4) in a dense tissue is inferior to that of the bathing fluid if the diffusion rate of the drug (arrow 1) is inferior to that of inactivating systems within the tissue, like an ectopeptidase (arrow 2) or the sequestration into intracellular acidic organelles (arrow 3). Right: immunohistochemistry for the smooth muscle marker α-actin (brown) in a paraffin section of the rabbit aorta (intimal surface to the left, note compact organization and prominent nonmuscle structure). B-D. Effects of antagonists on des-Arg^9-BK-induced contraction of the rabbit isolated aorta (reprinted from [67] with permission from ASPET). B, C. Lys-[Leu^8]des-Arg^9-BK without or with the aminopeptidase N inhibitor amastatin (3 μM). D. Schild plot analyses derived from B and C data and based on the agonist EC_{50} values from the averaged concentration-effect curves (dose ratio (DR) = EC_{50} in the presence of the antagonists divided by the control EC_{50}). Calculated pA_2 values ± S.E.M. are reported in the figure. E-G. Effects of astemizole on histamine-induced contraction of the rabbit isolated aorta optionally recorded in the presence of the V-ATPase inhibitor bafilomycin A1 (reprinted from [69] with permission from Elsevier). The concentration-effect curves recorded at time 3.5 h are shown and were constructed in the presence of the antagonist or its DMSO vehicle. E, F, astemizole without or with bafilomycin A1 (300 nM), respectively. G, Schild plot analyses derived from E and F data. Values are means ± S.E.M. of the number of determinations indicated by *n*.

Figure 1

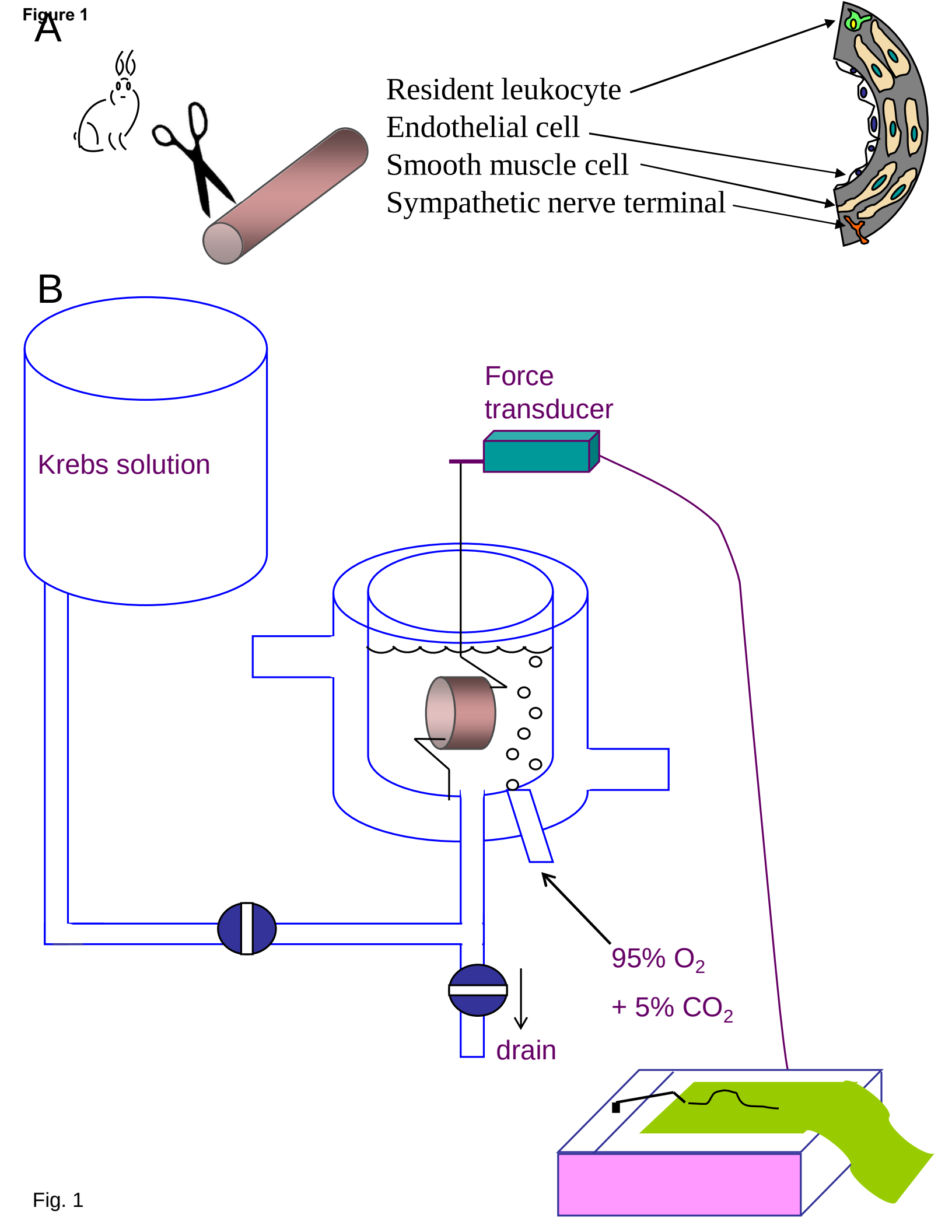

Fig. 1

Figure 2
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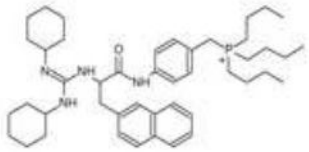
Fig. 2

Peptide B_2 receptor ligands

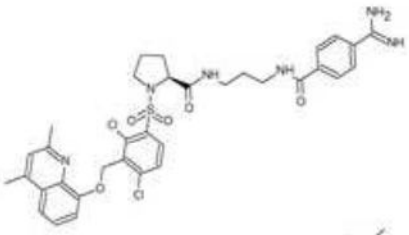
peptide	pharmacological identity	position											
		-2	-1	0	1	2	3	4	5	6	7	8	9
BK	agonist				Arg	Pro	Pro	Gly	Phe	Ser	Pro	Phe	Arg-COOH
B-9972	agonist			D-Arg	Arg	Pro	Hyp	Gly	Igl	Ser	Oic	Igl	Arg-COOH
icatibant	antagonist			D-Arg	Arg	Pro	Hyp	Gly	Thi	Ser	D-Tic	Oic	Arg-COOH
B-9430	antagonist			D-Arg	Arg	Pro	Hyp	Gly	Igl	Ser	D-Igl	Oic	Arg-COOH
B-10380	antagonist	[5(6)-CF]	ε-ACA	D-Arg	Arg	Pro	Hyp	Gly	Igl	Ser	D-Igl	Oic	Arg-COOH

Nonpeptide B_2 receptor ligands

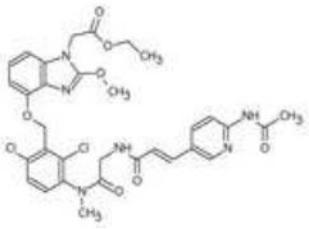
WIN 64338
(antagonist)



LF 16-0687
(anatibant,
antagonist)



compound 47a
(partial agonist)



compound 19c
(antagonist)

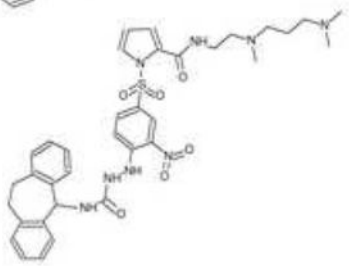


Figure 3

Fig. 3

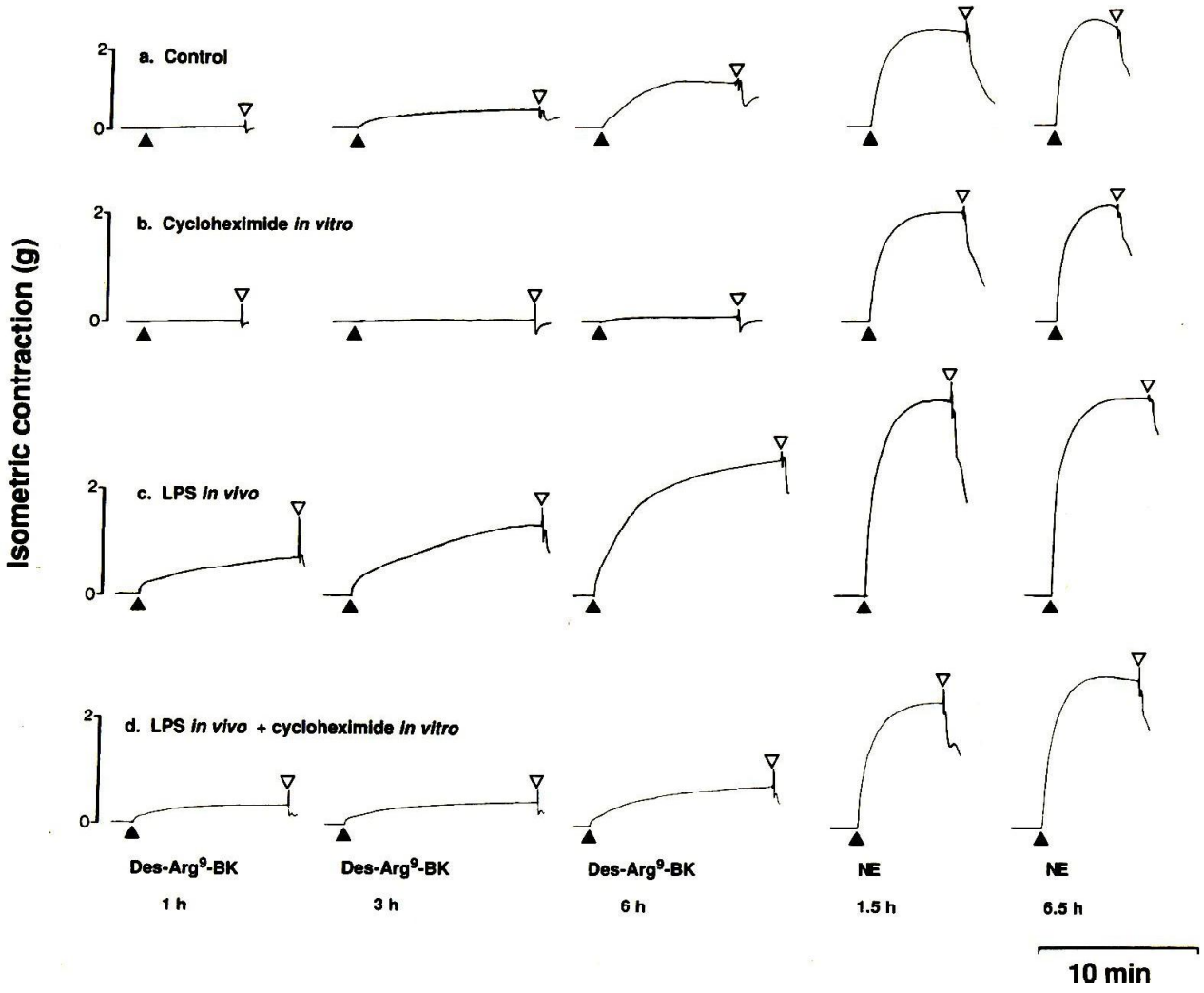


Figure 4

Fig. 4

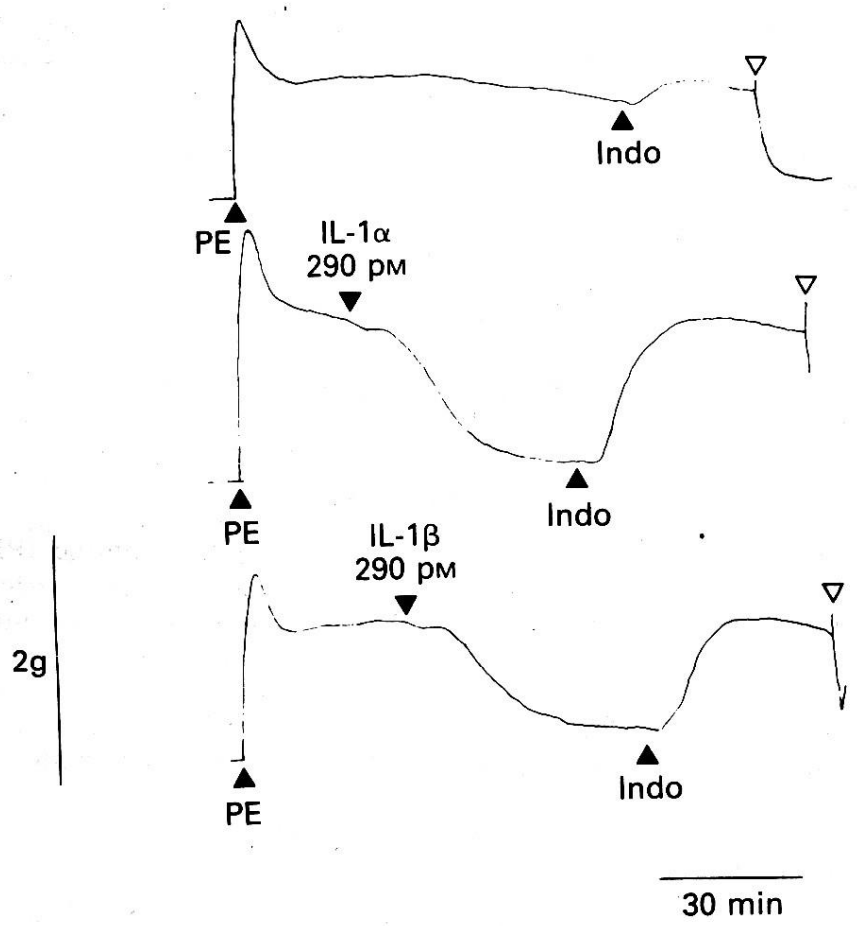


Figure 5

Fig. 5

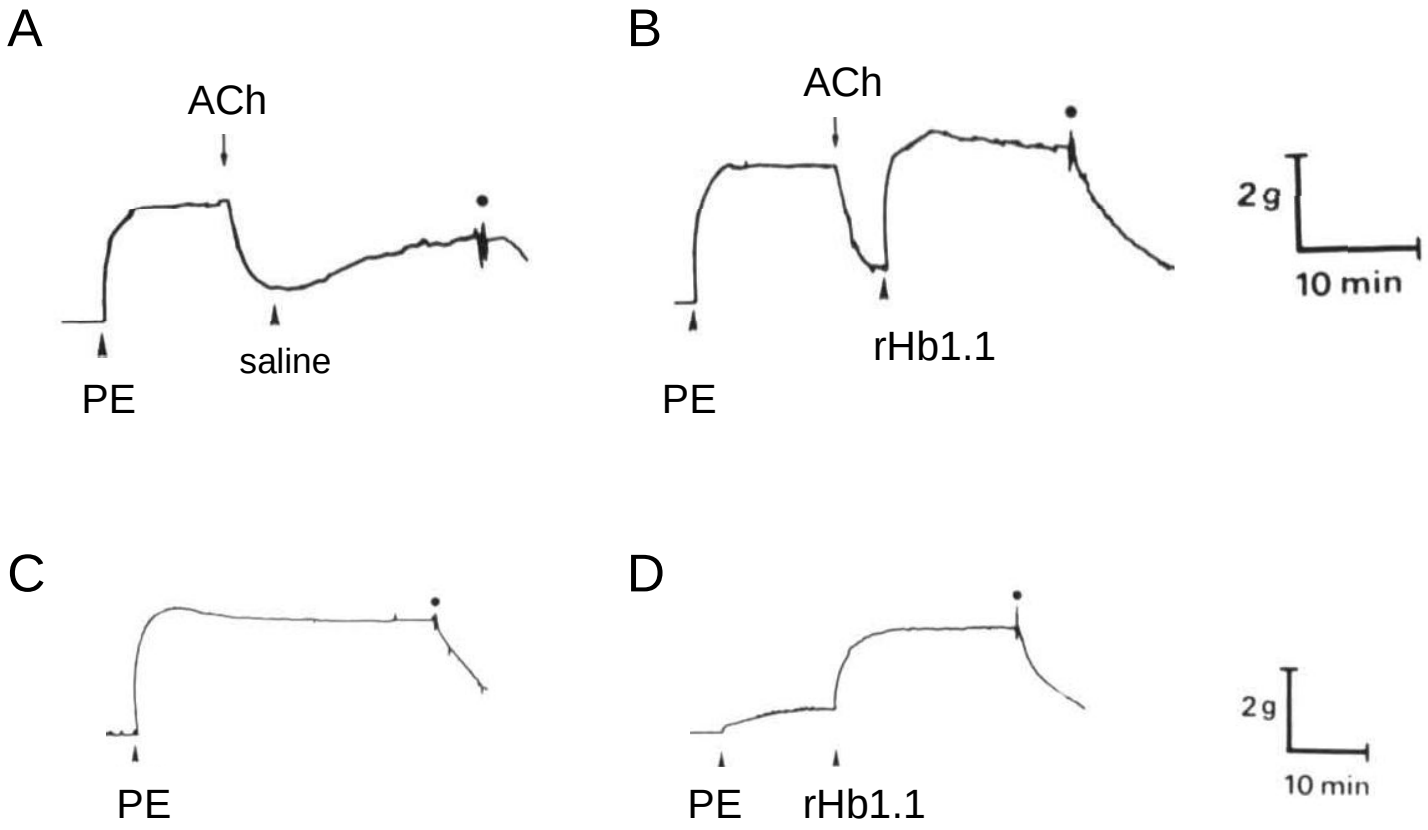


Figure 6

Fig. 6

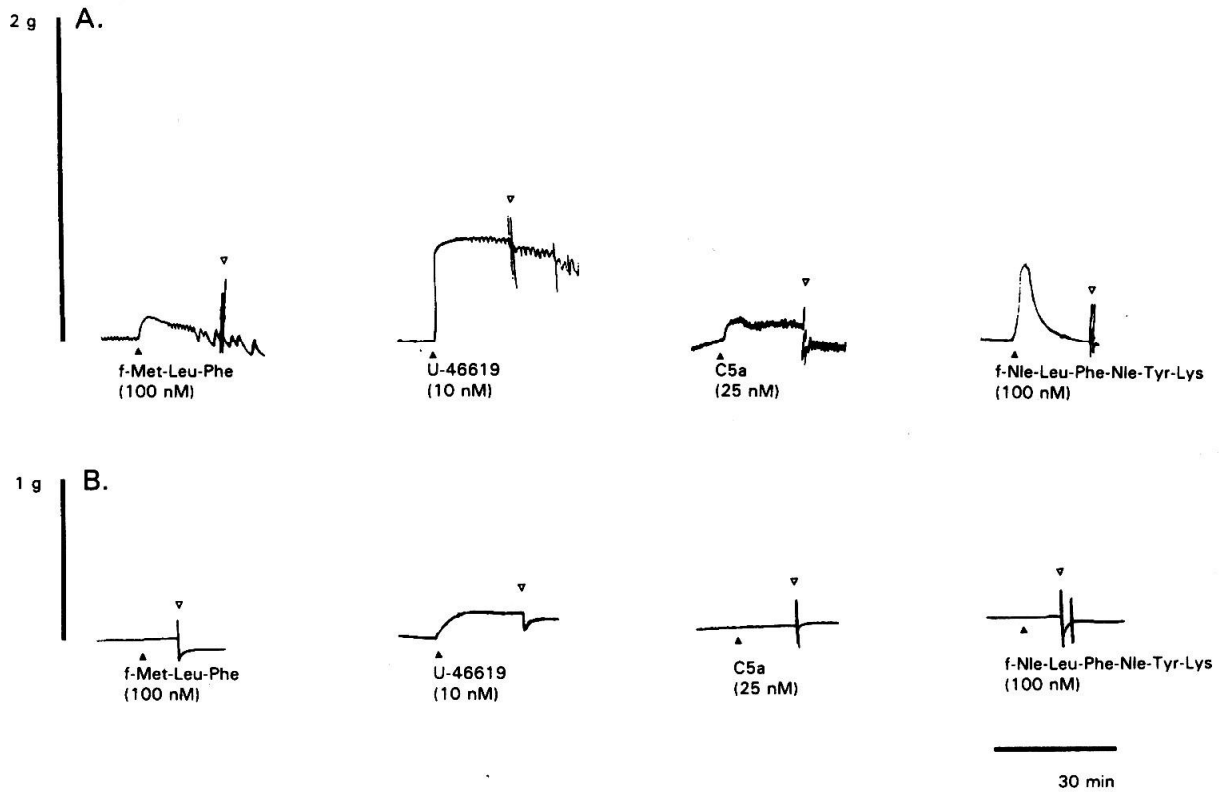


Figure 7
Fig. 7