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Natural Modulators of Large-Conductance Calcium-Activated Potassium Channels

Abstract

Large-conductance calcium-activated potassium channels, also known as BK or Maxi-K channels, occur in many types of cell, including neurons and myocytes, where they play an essential role in the regulation of cell excitability and function. These properties open a possible role for BK-activators (also called BK-openers) and/or BK-blockers as effective therapeutic agents for different neurological, urological, respiratory and cardiovascular diseases. The synthetic benzimidazolone derivatives NS004 and NS1619 are the pioneer BK-activators and have represented the reference models which led to the design of several novel and heterogeneous synthetic BK-openers, while very few synthetic BK-blockers have been reported. Even today, the research towards identifying new BK-modulating agents is proceeding with great impetus and is giving an ever-increasing number of new molecules. Among these, also a handsome number of natural BK-modulator compounds, belonging to different structural classes, has appeared in the literature. The goal of this paper is to provide a possible simple classification of the broad structural heterogeneity of the natural BK-activating agents (terpenes, phenols, flavonoids) and blockers (alkaloids and peptides), and a concise overview of their chemical and pharmacological properties as well as potential therapeutic applications.

Key words
Natural products · potassium channels · large-conductance calcium-activated BK channels · BK-activators · BK-blockers

Introduction

Among the different factors exerting an influence on the activity of the different classes of potassium (K^+) channels, a rise in the intracellular concentration of free calcium ions causes the activation of a family of channels, known as calcium-activated channels. Calcium-activated K^+ channels are further classified into three principal subtypes on the basis of their biophysical single channel conductance: probably, the most studied subtype is represented by the large-conductance calcium-activated K^+ channels, also known as BK, BK_{Ca} or Maxi-K channels. Besides their calcium-dependent activation, BK channels possess also a voltage-operated mechanism, and this peculiarity accounts for the highly effective role of feed-back regulation against the rise of intracellular Ca^{2+} and membrane depolarisation, promoting a massive outward flow of K^+ ions and leading to a membrane hyperpolarisation, i.e., to a stabilisation of the cell [1].

As far as molecular structure is concerned, the BK channel is a tetrameric protein complex formed by four α subunits, shaping the channel pore. β Accessory subunits can exert a significant influence on the sensitivity towards certain activating factors and on the binding properties to exogenous and endogenous ligands [2], [3].

Furthermore, BK channels, almost ubiquitously distributed among tissues, are expressed in both non-excitable and excitable cells, where they are involved in the control of a number of cell func-

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tions. The key roles of BK channels in non-excitable cells, such as secretory epithelial cells, liver cells and endothelial cells, have been recently reported [4], [5]. In particular, with regard to the expression and function of BK channels in endothelial cells, which are not directly involved in vasomotor responses but play a fundamental role in the regulation of the vascular smooth muscle activity through the biosynthesis and the release of vasoactive endogenous modulators, it has been documented that BK channels in the endothelium are composed of α subunits without association to any β subunit [6] and that their activation in the arterioles of several districts is obligatory for the transduction of the signal triggered by changes in intraluminal flow/shear stress, leading to the release of endothelium-derived factors evoking vasodilation [7]. With regard to the physiological role of BK channels in excitable cells, this has been especially studied in the nervous system [8], [9], [10], where they are key regulators of neuronal excitability and of neurotransmitter release, and in smooth muscle [11], [12], [13], [14], [15], where they are crucial in modulating the tone of vascular, broncho-tracheal, urethral, uterine or gastro-intestinal musculature. Given these implications with the dense distribution and the large conductance of BK channels, small natural or synthetic agents with BK-opener properties, named BK-openers or BK-activators, could have a potentially powerful effectiveness in the modulation and control of numerous upshots of muscular and neuronal hyperexcitability [1], [8], [16], [17], [18], [19], [20], [21], [22], such as asthma, urinary incontinence and bladder spasm, gastroenteric hypermotility, hypertension, coronary vasospasm, psychoses, convulsions and anxiety. Hence, the very large and fast escalation of the pharmaceutical interest on BK channels, which is providing a wider series of natural and synthetic BK-openers, connected with the rapid advances of molecular biology techniques, biochemical studies and screening procedures, are contributing to turning out more and more numerous and diverse potentially therapeutic opportunities, including post-stroke neuroprotection [23], erectile dysfunctions [24], some cardiac diseases [1] etc.

On the other hand, the possible therapeutic applications of BK-blockers seem to be confined in the field of a few neurological disorders [8], [18] such as depression and memory impairment, or in the symptomatic treatment of some cognitive disorders, such as Alzheimer-type diseases.

Natural BK-Activators

The synthetic benzimidazolone derivatives NS004 (**1**) and NS1619 (**2**) (Fig. **1**) are the pioneer BK-activators [25], [26] and have represented the reference models which led to the design of several novel and heterogeneous synthetic BK-openers [1]. Also some natural BK-activator compounds, belonging to different structural classes, have appeared in the literature and, among these, are some constituents of *Desmodium adscendes* (Papilionaceae), a folk medicinal herb used in Ghana as a remedy against asthma and other diseases associated with dysfunctions of smooth muscle contractile responses. The powerful modulation on muscular hyperexcitability of extracts of this herb and the study of the involvement of BK channels in this action are well documented. These effects are promoted by three glycosylated triterpenes called dehydrosoyasaponin-I (DHS-I) (**3**) (Fig. **2**),

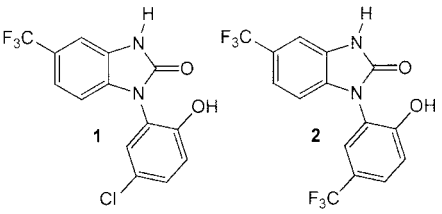

Fig. **1** Synthetic BK-activators NS004 (**1**) and NS1619 (**2**).

soyasaponins I and III and, among them, DHS-I has been demonstrated to be the most effective. Nevertheless, its BK-activating properties, first studied in bovine tracheal smooth muscle cells [27], are clear only when applied at the intracellular side of the channel and, consequently, given its poor membrane permeability, it has been difficult to study its pharmacological activity on smooth muscle function *in vitro*. *In vivo*, instead, DHS-I is probably metabolised into other active molecules that penetrate cells more easily. Interestingly, because DHS-I increases open channel probability only when the α and β subunits are co-expressed, its site of action is associated or allosterically coupled to the β subunit [27], [28]. In contrast, maxikdiol (**4**) (Fig. **2**), a 1,5-dihydroxy-isoprimane diterpenoid obtained by a fermentation process of an unidentified Coelomycete, besides its DHS-I-like limited membrane permeability and ability to activate BK channels of bovine aortic myocytes when applied to the cytoplasmatic side of the channel, was also demonstrated to activate the α subunit alone, without requiring the obligatory co-expression of the β subunit [29].

Very recently, the BK-opener properties of pimaric acid (PiMA) (**5**) (Fig. **2**), a diterpene compound structurally related to maxikdiol and widely present in the resin of the genus *Pinus*, and other related pimarane derivatives (such as isopimaric acid, sandaracoisopimaric acid, dihydropimaric acid, dihydroisopimaric acid, and dihydroisopimarinol) have been demonstrated on human BK channels, expressed in human embryonic kidney cells [30]. Interestingly, PiMA, maxikdiol likewise, seems to act only on the α-subunit, but, differently, acts from either side of the cell membrane. Despite the modest differences in the chemical structures, the BK-opener pharmacodynamic pattern has not been shown by other closely related terpenes, such as abietic acid, sclareol, and methyl pimarate, giving some useful indications for a study on the possible structure-activity relationships [30].

Other terpene derivatives, structurally not related with the above discussed compounds, isolated from the fungus *Trichoderma virens*, have been proposed as BK-activators. Among these, the carotane sesquiterpene CAF-603 (**6**) (Fig. **2**) showed a potent ability to displace radiolabelled ChTX in aortic sarcolemmal membranes, but it had no consistent effects on the BK-currents [31]. Instead, the introduction of an ester tail in position 8, to give the oleic acid ester L-735,334 (**7**) (Fig. **2**), isolated from *Thrichoderma virens* too, determines also the increasing of the open channel probability, but only when applied to the intracellular surface of the channel [32]. Another interesting natural chemotype of BK-activators is represented by some (poly)phenol compounds, such as *trans*-resveratrol (**8**) (3,4′,5-trihydroxy-*trans*-stilbene) (Fig. **3**), a phytoalexin produced by plants, whose medicinal interest is well established. In fact, this compound and its glucoside derivatives are believed to be the active agents of

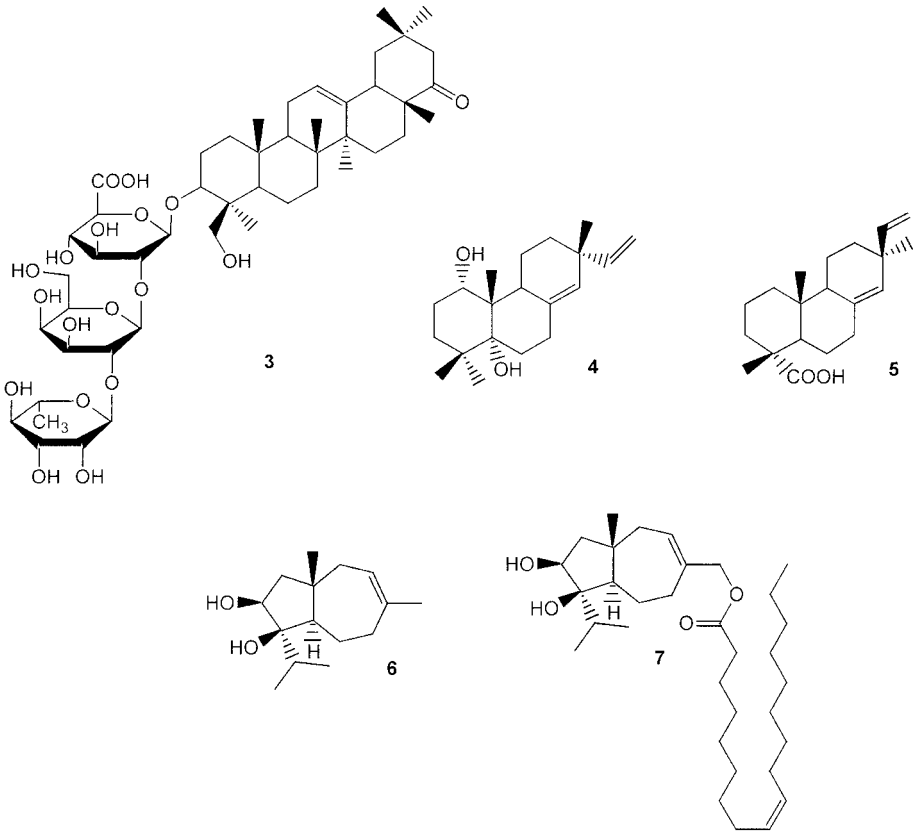

Fig. **2** Chemical structures of the terpene derivatives dehydrosoyasaponin-I (DHS-I) (**3**), maxikdiol (**4**), pimaric acid (PiMA) (**5**), CAF-603 (**6**) and L-735,334 (**7**).

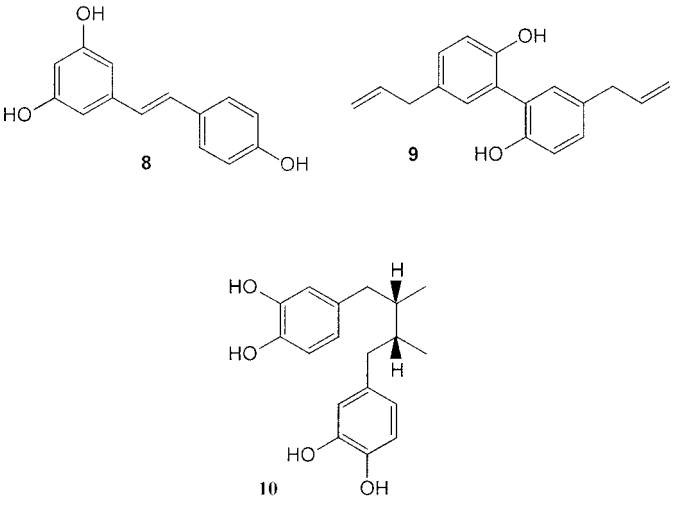

Fig. **3** Chemical structures of the phenolic derivatives *trans*-resveratrol (**8**), magnolol (**9**) and nordihydroguaiaretic acid (NDGA) (**10**).

some herbal remedies, such as the weed *Polygonum cuspidatum*, whose roots, dried and infused, are used in Japanese and Chinese folk medicine to treat diseases of the heart and blood vessels [33]. Recently, it has been demonstrated that *trans*-resveratrol can stimulate BK-channels in vascular endothelial cells, as measured using the patch-clamp technique in whole cell recordings [34]. These electrophysiological measurements, recorded in cultured smooth muscle cells of the human trachea, were used by the same authors to unmask the BK-activator profile of magnolol (**9**) (5,5′-di-2-propenyl-1,1′-biphenyl-2,2′-diol) (Fig. **3**) [35], a neolignan secondary metabolite isolated from the cortex of *Magnolia officinalis*. Magnolol has been considered as one of the major compounds responsible for the therapeutic effects of Saiboku-to, a mixture of ten different herbal extracts (including *Magnolia officinalis*, *Glycyrrhiza glabra*, *Scutellaria baicalensis*, etc.), used in Asian countries as a remedy against asthma. Nevertheless, it has not yet been demonstrated that the modulator effects of magnolol on BK channels is the primary mechanism of the anti-asthmatic action of magnolol and of Saiboku-to. In this chemotype, again, a natural lignan can be also included, nordihydroguaiaretic acid (NDGA) (**10**) (Fig. **3**), present in high amounts in the leaves and twigs of *Larrea tridentata* Cav, a plant belonging to the family Zygophyllaceae, commonly known as chaparral or creosote bush or greasewood and whose tea was described in the American Pharmacopeia as a treatment for tuberculosis, arthritis and cancer [36]. This catechol, a well-known inhibitor of lipoxygenase (that represents a key biological target for many diseases such as asthma, atherosclerosis and cancer), besides its wide range of pharmacological activities, such as antioxidant capability, also showed a BK-activator profile in single smooth muscle cells of porcine coronary artery, as investigated by some Japanese authors using the patch-clamp technique [37]. Some of the same researchers, more recently, have published two studies about its mechanism and site of action on BK channels, both of which seem to be independent of the inhibition of lipoxygenase and of antioxidant activity [38], and to directly involve the α subunit [39], respectively.

Another structural class of BK-openers is represented by the flavonoid moiety, present in many metabolites of vegetable origin, as shown in a structure-activity hypothesis concerning its phar-

macophoric analogy with the reference compound NS004 [40]. In this study, the effective BK-opener profile of apigenine (**11**) and kaempferol (**12**) (Fig. **4**) was demonstrated in *Xenopus* oocytes injected with *mSlo*. Furthermore, also naringenine (**13**) [41], as well as hesperetin (**14**), luteolin (**15**), and 5-hydroxyflavone (**16**) (Fig. **4**), showed tetraethylammonium- or IbTX-sensitive vasorelaxant effects on rat aortic smooth muscle, while other related flavonoids, such as 5-methoxyflavone, 7-hydroxyflavone, and 4′-hydroxyflavanone, induced IbTX-insensitive (and thus, BK-independent) vasorelaxing responses, indicating the presence of a hydroxy group in position 5 of the flavonoid moiety as an important structural requirement for the activation of BK channels [42]. In this chemical class, finally, the dihydrochalcone flavonoid phloretin (**17**) (Fig. **4**), possessing BK-activating properties too, as established in amphibian myelinated nerve fibres by patch clamping [43], can be also included.

Finally, worthy of mention is vinpocetine (**18**) (Fig. **5**), also known as ethyl apovincaminate, whose well-established neuroprotective, anticonvulsant and potent cerebral vasodilator properties have been clinically widely used for decades in various cerebrovascular syndromes, even if it has not been approved by any regulatory body [44]. This alkaloid, characterised by an eburnamenine skeleton, was synthesised from vincamine [45], obtained from the leaves of *Vinca minor*. Interestingly, vinpocetine has been reported to have direct BK-channel stimulating properties, as shown in rat pituitary GH_3 cells [46], even though it remains to be studied if this BK-action could contribute to its beneficial effects.

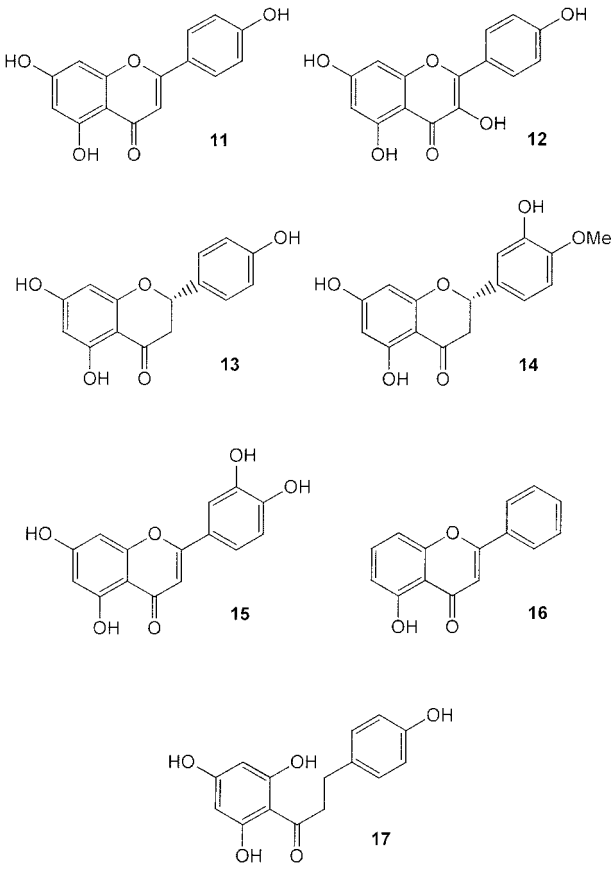

Fig. **4** Chemical structures of the flavonoids apigenine (**11**), kaempferol (**12**), naringenine (**13**), hesperetin (**14**), luteolin (**15**), 5-hydroxyflavone (**16**) and phloretin (**17**).

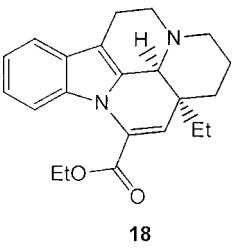

Fig. **5** The alkaloid vinpocetine (**18**), a *cis*-(3*S*,16*S*)-derivative of vincamine.

In spite of the great and ever-increasing number of natural and synthetic BK-openers, little is known about their molecular site/s of interaction/s and only for synthetic openers, are a basic pharmacophoric model and some essential structural features for the BK-opening action now are emerging [47], [48]. To date, the BK-openers of first choice as tools for studying BK channels are generally represented by synthetic compounds, such as NS1619 [26] (which activates BK currents at 10 – 30 μM in vascular and non-vascular smooth muscle [47]), NS1608 [49] (which significantly activates bovine aortic smooth muscle cells at 1 μM [18]) or CGS7184 [50] (whose BK-activating concentration in some smooth muscle cells is 20 – 50 fold lower than that of NS1619 [47]). Nevertheless, also some natural compounds, which are today easily available in commerce, are often quoted in the literature for experimental aims, such as DHS-I [27] (10 nM to the inside face reversibly increases the open probability of single BK channel recordings [28]), phloretin (which at concentrations from 10 – 200 μM determines the increase of BK channel probability due to a shift of the membrane potential for half-maximal activation [43]) or maxikdiol (that in inside-out configuration shows a significant effect at 3 – 10 μM [29]).

Natural BK-Blockers

Peptide toxins

Scorpion venom has proved to be a good source of peptide toxins with ion channel-blocker properties. In particular, scorpion peptide toxins which block potassium channels are short peptides of about 30 – 40 amino acid residues cross-linked by three or four disulphide bridges [51]. Among these, the 37-amino acid peptide charybdotoxin (ChTX), isolated from the venom of *Leiurus quinquestriatus* (var. *hebraeus*) [52], was the first potent and specific peptide BK-blocker able to occlude the external mouth of the pore and to block the conduction pathway. Unfortunately, ChTX possesses blocking properties also towards small-conductance calcium-activated K^+ channels (SK channels) and other subtypes of voltage-operated K^+ channels, and this can represent a limiting experimental condition [53]. Iberiotoxin (IbTX), a 37-amino acid peptide isolated from the venom of the scorpion *Buthus tamulus* and having a block mechanism analogous to that of ChTX [54], is the first example of a high affinity inhibitor of BK channels [55] and is probably the most used BK-blocker for experimental purposes (half maximal concentration: 10 nM [56]). Nevertheless, as of today, given the pharmaceutical disadvantages as a result of their peptide nature (not orally active, poor blood-brain permeability, disadvantages about synthesis and so on), these and other related peptide toxins, such as limbatustoxin (LbTx) [57], do not represent potential therapeutic agents, but they are considered as powerful tools to delineate

the channel pore structure and the mechanism of ion permeation as well as to study the physiological role of the BK-channel subtype. However, the list of scorpion toxins with BK-blocker properties is destined to grow quickly {as also demonstrated from the recent publication of a new specific BK-blocker, a 37-amino acid toxin, named slotoxin (SloTx) and isolated from *Centruroides noxius* [58], that could even distinguish between α and $\alpha + \beta$ complexes} and to be enriched by peptide toxins isolated from other animal venoms, such as from snake's venom [59], all speeded up by established experimental methodologies developed for scorpion toxins. Then, it can be hoped that, in the next few years, rapid advances in understanding the factors involved in the mechanism of interactions between peptide toxins and their targets will be established, providing a pharmacophoric model for the design of new peptidomimetic small synthetic BK-blockers which, in turn, could be used as new therapeutic agents.

Non-peptide BK-blockers

Very few natural non-peptide chemotypes, possessing BK-channel blocking activity, have been reported. The alkaloids quinine (**19**) and quinidine (**20**) (Fig. **6**), from *Cinchona* species, are fairly selective blockers of BK and IK channels. Then, they can both cause the inactivation of other different K^+ channel subtypes and influence Na^+ and Ca^{2+} currents, so that their experimental use is limited [60], [61], [62]. The first class of potent and selective non-peptide inhibitors was, and still is, a series of indole-diterpenes belonging to a family of tremorgenic mycotoxins isolated from fungi belonging to the genera *Penicillium*, *Aspergillus* and *Claviceps*. This group includes paxilline (**21**), verruculogen (**22**), non-tremorgens paspalicine (**23**), paspalitrem C (**24**), penitrem A (also known as tremortin A) (**25**), aflatrem (**26**) and paspalinine (**27**) (Fig. **7**), identified by their ability to modulate binding of radiolabelled charybdotoxin to BK-channels in bovine aortic smooth muscle sarcolemmal membranes [63]. Probably, among these, the most studied and used for experimental purposes is the potent and selective blocker paxilline, able to block, in electrophysiological experiments, BK-channel currents with a K_i of less than 10 nM [63] and whose binding site is on the α subunit, different from the charybdotoxin receptor but allosterically coupled to it, and accessible from the cytoplasmatic side, as studied in excised inside-out patches [64] and isometric tension recordings [65]. Finally, also tetrandrine (**28**) (Fig. **8**), a bis-benzylisoquinoline alkaloid isolated from the tuberous roots of the *Stephania tetrandra*, a Chinese medicinal herb used to treat hypertension and angina [66], possesses a BK-blocking profile [67], [68], even if conflicting results exist [69].

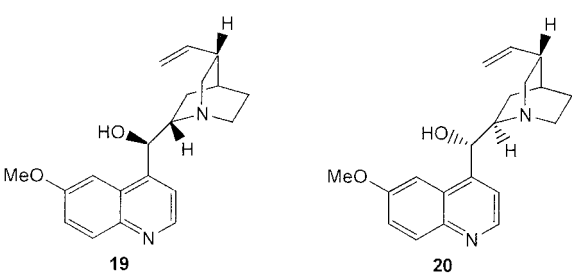

Fig. **6** Chemical structure of quinine (**19**) and quinidine (**20**).

Conclusions

For a long time, the study of BK channels has been confined in the basic electro-physiological disciplines and only in the last decade have pharmacologists and chemists focused their attention on this field, proposing potential therapeutic uses of modulators of these ion channels. Despite this recent interest, the research on new BK-modulating agents has proceeded with great impetus and, to date, a wide number of synthetic BK-activators and of studies on their structure-activity relationships is available. Actually, only the pharmacological profiles of some synthetic BK-activators have been closely investigated in preclinical studies. The indolone derivative BMS-204352 (MaxiPost™) is under a phase III clinical trial, to evaluate its therapeutic application as a neuroprotective agent, in stroke-injured patients [20]. Besides, the investigation of natural compounds, which started with the first paper on DHS-I, has experienced a constant development, well documented by many reports in the scientific literature and by the increase in the number of well-characterised natural BK-openers. Furthermore, it is reasonable to believe that a systematic study on the BK-activating properties of selected classes of natural products (for example, pimarane diterpenes [30] or flavonoids [42]) may give, on one hand, new natural BK-openers and, on the other, useful considerations on structure-activity relationships, in order to define a satisfactory pharmacophoric model which provides a guideline for the synthesis of new drugs.

Experimental Procedures

In this appendix the authors wish to give a brief and general overview of some techniques most commonly used to study BK-modulators. Naturally, for a detailed description of the various experimental protocols, which does not fit the aims of this review, the authors invite readers to consult the papers quoted in the text and the international literature.

Patch-clamp technique

The patch-clamp technique represents a widely used method for observing the function of individual ion channels in a variety of cell types. The most commonly used method is called the "on cell" or "cell-attached" configuration, because ion channels can be recorded on an intact cell.

This mode is well suited for investigation of ion channels which are activated/inactivated by drugs. Another reliable mode is the "cell-excised" configuration, which is obtained by suddenly removing the patch-pipette from the cell, so that the membrane patch is pulled off the cell. This mode easily allows the exposure of the channel proteins to drugs by changing the bath solution and the recording and analysing of the channel currents.

Various parameters, such as the single channel conductance, open- and closed-times of the channel and the open-state probability and their changes (due to the administration or activating or inactivating agents) can be evaluated.

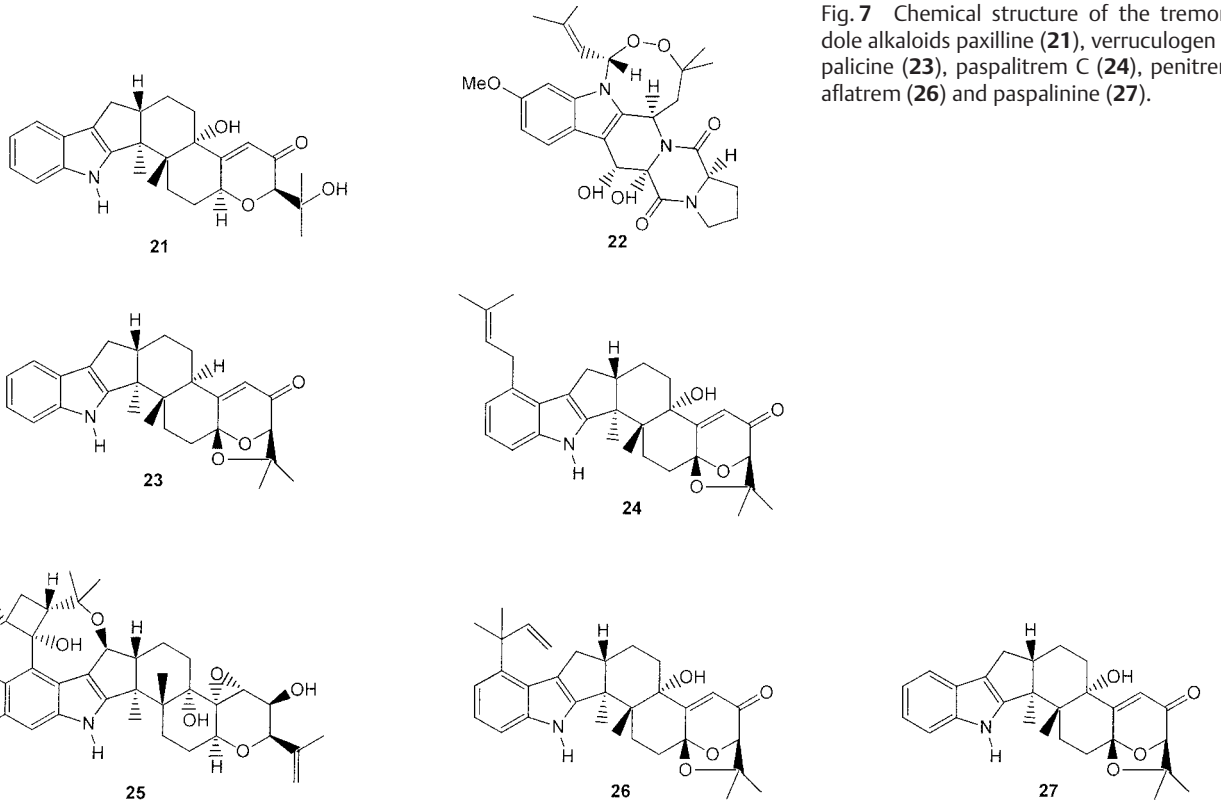

Fig. **7** Chemical structure of the tremorgenic indole alkaloids paxilline (**21**), verruculogen (**22**), paspalicine (**23**), paspalitrem C (**24**), penitrem A (**25**), aflatrem (**26**) and paspalinine (**27**).

Besides these modes, which enable the recording of single channel currents, it is also possible to measure the current flowing through the entire cell. This "whole-cell mode" is obtained by rupturing the membrane patch in the cell-attached mode. This is achieved by applying suction to the interior of the patch-pipette. The "whole-cell mode" not only allows the recording of the electrical current, but also the measuring of the cell potential. Moreover, the cell interior is dialysed by the electrolyte solution filled into the patch-pipette, allowing also a direct intracellular administration of drugs. Different cell types can be used for a patch-clamp study on BK channels. For example, natural BK-activators have been tested on native BK channels of human cultured endothelial (obtained from the umbilical cord) [34] or trachea [35] cells, as well as of myelinated nerve fibres of *Xenopus* [43], porcine smooth muscle cells [37] or rat pituitary GH3 cells [46]. The experimental study can be carried out on BK channels transfected in different cell types, such as, for example, human embryonic kidney cells [30].

Voltage-sensitive fluorescent dye

Together with the classical electrophysiological tools, also a voltage-sensitive fluorescent dye can be used for the measurement of membrane potential.

Such a compound, $DiBAC_4$, a bis-barbituric acid oxonol dye with maximum excitation at approximately 490 nm, has been used for the evaluation of the pimarane BK-activators, quoted in this article [30]. Hyperpolarisation, due to BK-activation, results in the extrusion of the dye from cells and a subsequent decrease in fluorescence intensity. The decrease in fluorescence intensity by 1% corresponded to approximately 0.5 mV hyperpolarisation in the membrane potential range between –20 and –70 mV.

Binding technique

Usual binding techniques, based on the property of a given molecule to compete with a radiolabelled ligand for a common binding site, can be suitably used to detect a BK-modulating agent. For example, in order to investigate the pharmacology of maxikdiol [29] and CAF-603 [31], their ability to displace radiolabelled [^{125}I]charibdotoxin from native BK channels of bovine aortic sarcolemmal membranes was evaluated. The binding technique is generally considered to be a powerful, easy and rapid method for a preliminary screening, but, unfortunately, it cannot be predictive for the activating or inactivating pharmacodynamic properties of a BK-modulator, needing further experimental tests.

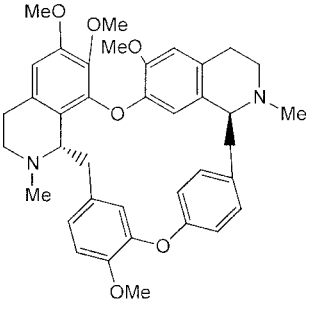

Fig. **8** Chemical structure of (*S*,*S*)-tetrandrine (**28**), a bis-benzylisoquinoline alkaloid.

Functional technique

The property of a BK-activator to induce a myorelaxing response in smooth muscle of different districts makes possible the use of pharmacological functional tests to determine such a mechanism of action. For example, a vasorelaxing effect on vascular smooth muscle preparations (rat thoracic aortic rings are probably the most common ones) can be seen as an indicator of a possible BK-opening activity and this method has been used to evaluate the BK-activator effects of flavonoids mentioned [41], [42]. The vascular preparations should be pre-contracted by a vasoconstricting agonist (for example, norepinephrine) or by a depolarising agent, such as KCl. If the depolarising agent KCl is used, a relatively low concentration (about 20 mM) should be added to the bathing solution, because the effects of a hyperpolarising drug (i. e., a potassium channel opener) are inhibited by high levels of membrane depolarisation (corresponding to higher concentrations of KCl). Really, this last pharmacodynamic characteristic can be usefully investigated in order to individualise a potassium channel opener: the vasorelaxing effect of such a drug, observed in aortic preparations pre-contracted by KCl 20 mM, is dramatically decreased in organs pre-contracted with higher concentrations of KCl (typically, 60 or 80 mM). Of course, a vasorelaxing effect can be evoked by a number of drugs, acting through many mechanisms of action different from a BK-opening effect. Only the antagonism exerted by BK-blockers (typically iberiotoxin 100 – 200 nM or charibdotoxin 100 – 200 nM) against the effects of a vasorelaxing drug represents an acceptable demonstration of the involvement of BK activation in such a functional response.

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