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Molecular Determinants for the Activating/Blocking Actions of the 2*H*-1,4-Benzoxazine Derivatives, a Class of Potassium Channel Modulators Targeting the Skeletal Muscle K_{ATP} Channels

Domenico Tricarico, Antonietta Mele, Giulia Maria Camerino, Antonio Laghezza, Giuseppe Carbonara, Giuseppe Fracchiolla, Paolo Tortorella, Fulvio Loiodice, and Diana Conte Camerino

Departments of Pharmacobiology (D.T., A.M., G.M.C., D.C.C.) and Medicinal Chemistry (A.L., G.C., G.F., P.T., F.L.), Faculty of Pharmacy, University of Bari, Bari, Italy

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ABSTRACT

The 2*H*-1,4-benzoxazine derivatives are modulators of the skeletal muscle ATP-sensitive-K^+ channels (K_{ATP}), activating it in the presence of ATP but inhibiting it in the absence of nucleotide. To investigate the molecular determinants for the activating/blocking actions of these compounds, novel molecules with different alkyl or aryl-alkyl substitutes at position 2 of the 1,4-benzoxazine ring were prepared. The effects of the lengthening of the alkyl chain and of branched substitutes, as well as of the introduction of aliphatic/aromatic rings on the activity of the molecules, were investigated on the skeletal muscle K_{ATP} channels of the rat, in excised-patch experiments, in the presence or absence of internal ATP (10^{-4} M). In the presence of ATP, the 2-*n*-hexyl analog was the most potent activator ($DE_{50} = 1.08 \times 10^{-10}$ M), whereas the 2-phenylethyl was not effective. The rank order of efficacy of the openers was 2-*n*-hexyl ≥2-cyclohexylmethyl >2-isopropyl = 2-*n*-butyl ≥ 2-phenyl ≥ 2-benzyl = 2-isobutyl analogs. In the absence of ATP, the 2-phenyl analog was the most potent inhibitor ($IC_{50} = 2.5 \times 10^{-11}$ M); the rank order of efficacy of the blockers was 2-phenyl ≥ 2-*n*-hexyl > 2-*n*-butyl > 2-cyclohexylmethyl, whereas the 2-phenylethyl, 2-benzyl, and 2-isobutyl 1,4-benzoxazine analogs were not effective; the 2-isopropyl analog activated the K_{ATP} channel even in the absence of nucleotide. Therefore, distinct molecular determinants for the activating or blocking actions for these compounds can be found. For example, the replacement of the linear with the branched alkyl substitutes at the position 2 of the 1,4-benzoxazine nucleus determines the molecular switch from blockers to openers. These compounds were 100-fold more potent and effective as openers than other KCO against the muscle K_{ATP} channels.

Potassium channel openers (KCO) are chemically diverse compounds that belong to a number of structural classes, including benzopyrans (cromakalim, bimakalim), benzothiadiazines (diazoxide), cyanoguanidines (pinacidil), cyclobutenediones (WAY-151616), nicotinamides (nicorandil), pyrimidines (minoxidil), tertiary carbinoles (ZD-6169), thioformamides (aprikalim), and dihydropyridine-like structures (ZM-244085) (Mannhold, 2004; Jahangir and Terzic, 2005). Several derivatives of these compounds have been synthesized and tested against the ATP sensitive K^+-channel (K_{ATP}) subunits expressed in cell lines, which is the primary target for KCO action.

These compounds show a broad spectrum of therapeutic applications, including asthma, urinary incontinence, hypertension, angina, hypoglycemia, neuromuscular disorders, and some forms of epilepsy (Andersson, 1992; Longman and Hamilton, 1992). KCO drugs exert their effects on pancreatic β cells, neurons, and vascular/nonvascular smooth muscle and cardiac muscle by opening K_{ATP} channels, thus shifting the membrane potential toward the reversal potential for potassium and reducing cellular electrical activity. In skeletal muscle, nicorandil is effective in restoring the depressed

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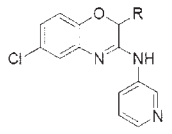
ABBREVIATIONS: KCO, potassium channel openers; WAY-151616, 3-(2,4-dichloro-6-methylbenzylamino)-4-(1,1-dimethyl-propylamino)cyclobut-3-ene-1,2-dione; ZD-6169, (*s*)-*N*-(4-benzoylphenyl)-3,3,3-trifluoro-2-hydroxy-2-methyl-propionamide; ZM-244085, 9-(3-cyanophenyl-)hexahydro-1,8-acridinedione; DE_{50}, drug concentration needed to enhance the current by 50%; IC_{50}, drug concentration needed to inhibit the current by 50%; hypoPP, hypokalemic periodic paralyses; MOPS, 3-(*N*-morpholino)propane-sulfonic acid; DMSO, dimethyl sulfoxide.

contractility function in humans after neuromuscular blocker-induced paralysis (Saitoh, 2005). Pinacidil is capable of reducing the severity and frequency of paralysis in patients affected by hypokalemic periodic paralyses (hypoPP); however, the patients suffer severe hypotension, which limits its use in this muscle disorder (Links et al., 1992). Cromakalim is able to repolarize the insulin-depolarized muscle fibers of patients with hypoPP "in vitro" as well as of K^+-depleted rats, an animal model of hypoPP, by opening the skeletal muscle K_{ATP} channels (Tricarico et al., 1998, 1999). In some myotonic patients, it is also able to suppress "in vitro" the abnormal hyperexcitability of the fibers; however, this compound is not available for clinical use (Quasthoff et al., 1990). Blockers of the skeletal muscle K_{ATP} channels may instead be useful in those conditions associated with insulin resistance and neuromuscular symptoms (Koster et al., 2005; Flechtner et al., 2006). Sulfonylureas used for the treatment of diabetes type II also block the skeletal muscle types. Unfortunately, the currently available openers and blockers of the K_{ATP} channels were not developed against the skeletal muscle channel types and are therefore not indicated for neuromuscular disorders (Camerino et al., 2007).

Recent data show differences in the biophysical properties, subunit expression, and drug responses between muscles types and phenotypes, which corroborates the idea that molecular composition of the skeletal muscle K_{ATP} channels is complex (Tricarico et al., 2006). In native skeletal muscle fibers, the K_{ATP} channel is indeed an hybrid assembly of Kir6.2/SUR2A and Kir6.2/SUR1 subunits organized as homomeric complexes with the possible contribution of SUR2B to the functional channels. However, the cromakalim-sensitive K_{ATP} channels are the main complexes found in the different muscle types and phenotypes, thereby representing a valuable drug target in this tissue (Tricarico et al., 2006).

We have shown previously that the 2-*n*-propyl-1,4-benzoxazine derivative **1** (Fig. 1), a novel modulator of the muscular K_{ATP} channels, in the presence of internal ATP leads to 54% activation of the fast-twitching muscle K_{ATP} channels at 10^{-7} M concentration (Tricarico et al., 2003). A downturn in response is observed with this compound when a certain dose is exceeded, suggesting that its effectiveness is reduced at higher concentrations. Furthermore, the same compound in the absence of internal ATP, caused a 41% inhibition of the K_{ATP} channels at 10^{-4} M concentration, which seems to be mediated by interaction with the Kir6.2 subunit (Tricarico et al., 2003; Rolland et al., 2006). Dual hypotheses have been proposed to explain this peculiar behavior: first, the drug action is dependent on the ability to bind two distinct sites of skeletal muscle K_{ATP} channel complex modulating opposite actions; second, the drug binds to a single site whose affinity and/or effect are modulated by ATP or generally by tissue metabolism (Tricarico et al., 2003).



2H-1,4 benzoxazine nucleus

Compound	R
1	*n*-propyl
2	*n*-butyl
3	*n*-hexyl
4	*i*-propyl
5	*i*-butyl
6	cyclohexylmethyl
7	benzyl
8	2-(phenyl)ethyl
9	phenyl

Fig. 1. Molecular structures of 2*H*-1,4-benzoxazine derivatives. R indicates the substitutes at position 2 of the 2*H*-1,4-benzoxazine nucleus.

To investigate on the molecular determinants responsible for the opening/blocking actions of these compounds, several new molecules were prepared and tested on the muscular K_{ATP} channels. The influence of the lengthening of the alkyl chain and of the branched alkyl chain substitutes on the activity of the molecules against the K_{ATP} channels was therefore investigated by preparing the 2-*n*-hexyl and 2-*n*-butyl-1,4-benzoxazine derivatives, and the 2-isopropyl and 2-isobutyl-1,4-benzoxazine derivatives, respectively. Whereas the influence of the introduction of aliphatic/aromatic rings on the activity of the molecules was investigated by preparing the 2-cyclohexylmethyl, 2-phenyl, 2-benzyl and 2-phenylethyl-1,4-benzoxazine derivatives. The drug experiments were performed "in vitro" in excised-patches on K_{ATP} channels, cromakalim-glibenclamide-sensitive and tolbutamide-insensitive, of native fibers of the rat, in the presence or absence of internal ATP (10^{-4} M).

Materials and Methods

Muscle Preparations and Single Fiber Isolation. The flexor digitorum brevis muscles were dissected from male Wistar rats under urethane anesthesia (1.2 g/kg). After dissection, the animals were rapidly killed with an overdose of urethane according to the *Guide for the Care and Use of Laboratory Animals* prepared by the U.S. National Academy of Sciences. Single muscle fibers were prepared by enzymatic dissociation (Tricarico et al., 1998).

Drugs and Solutions. The normal Ringer solution contained 145×10^{-3} M NaCl, 5×10^{-3} M KCl, 10^{-3} M $MgCl_2$, 0.5×10^{-3} M $CaCl_2$, 5×10^{-3} M glucose, and 10^{-2} M MOPS, pH 7.2. The patch pipette solution contained 150×10^{-3} M KCl, 2×10^{-3} M $CaCl_2$, and 10^{-2} M MOPS, pH 7.2. The bath solution contained 150×10^{-3} M KCl, 5×10^{-3} M EGTA, and 10^{-2} M MOPS, pH 7.2. Stock solution of $ATPK_2$ (5×10^{-3} M) was prepared by dissolving the chemical in the bath solution.

Stock solutions of the 2*H*-1,4-benzoxazine derivatives (30×10^{-3} M), cromakalim, tolbutamide and glibenclamide (20×10^{-3} M) (SIGMA, Mi) were prepared by dissolving the compounds in dimethyl sulfoxide (DMSO). Microliter amounts of the stock solutions were then added to the bath solution as needed to obtain concentrations of 2*H*-1,4-benzoxazine derivatives ranging between 10^{-12} and 10^{-4} M (DMSO 0.33%). Because of the low solubility of these compounds in the aqueous solvent, the drugs were tested at concentrations $<10^{-4}$ M. DMSO applied at 0.33% concentration to the excised patches in the presence of ATP (10^{-4} M) did not increase the K_{ATP} channel activity (solvent control). DMSO did not affect K_{ATP} current even in the absence of internal nucleotide. The drugs were tested in the presence or in the absence of internal ATP (10^{-4} M) with no added Mg^{2+} ions to reduce possible ATPase activity in the patches (Russ et al., 2003; Tricarico et al., 2003).

Synthesis of the New 2*H*-1,4-Benzoxazine Derivatives. The key intermediate in the synthetic pathway of all benzoxazine derivatives **2–9** (Fig. 1) was the appropriate 2-substituted-6-chloro-2*H*-1,4-benzoxazine-3-one, which was condensed with 3-aminopyridine either in refluxing dry toluene in the presence of $TiCl_4$ and anisole as previously reported for derivative **1** (compounds **3** and **9**) (Tricarico et al., 2003), or in dry acetonitrile in the presence of triethyl amine and $POCl_3$ (compounds **2** and **4–8**). In addition, the preparation of benzoxazinones followed two different synthetic pathways. The former started from the commercially available α-bromo acids, which were converted to the corresponding acyl chloride with $SOCl_2$ and condensed with 2-amino-4-chloro-phenol in the presence of triethyl benzyl ammonium chloride and $NaHCO_3$ in $CHCl_3$ to give a one-pot cyclization reaction (benzoxazinone intermediate for compounds **2**, **4**, and **9**). The latter started from α-hydroxy ethyl esters whose condensation with 2-nitro-4-chloro-phenol under Mitsunobu conditions followed by reduction and cyclization with 6N HCl and Fe powder in 1,4-dioxane afforded the desired compounds in high yields (benzoxazinone intermediate for compounds **3** and **5-8**). All synthetic details will be reported elsewhere. Microanalyses of all final molecules were within ± 0.4% of theoretical values. For pharmacological experiments, these molecules were used as racemates and free bases. The generic names of the 2*H*-1,4-benzoxazine derivatives (Fig. 1) were: (*R*/*S*)-6-chloro-2-butyl-3- (pyridin-3-yl-amino)-2*H*-1,4-benzoxazine (**2**), (*R*/*S*)-6-chloro-2-hexyl-3- (pyridin-3-yl-amino)-2*H*-1,4-benzoxazine (**3**), (*R*/*S*)-6-chloro-2-isopropyl-3- (pyridin-3-yl-amino)-2*H*-1,4-benzoxazine (**4**), (*R*/*S*)-6-chloro-2-isobutyl-3- (pyridin-3-yl-amino)-2*H*-1,4-benzoxazine (**5**), (*R*/*S*)-6-chloro-2-cyclohexylmethyl-3- (pyridin-3-yl-amino)-2*H*-1,4-benzoxazine (**6**), (*R*/*S*)-6-chloro-2-benzyl-3- (pyridin-3-yl-amino)-2*H*-1,4-benzoxazine (**7**), (*R*/*S*)-6-chloro-2–2- (phenyl)ethyl-3-(pyridin-3-yl-amino)-2*H*-1,4-benzoxazine (**8**), and (*R*/*S*)-6-chloro-2-phenyl-3- (pyridin-3-yl-amino)-2*H*-1,4-benzoxazine (**9**).

Patch Clamp Experiments. Experiments were performed in inside-out configurations using the standard patch-clamp technique. Recordings of channel currents were performed during voltage steps of 10 s, going from 0 mV of holding potential to −60 mV immediately after excision, at 20°C, in the presence of 150×10^{-3} M KCl on both sides of the membrane in the absence (controls) or in the presence of ATP in the bath solution. The macropatch currents with a mean amplitude of −510.5 ± 31 pA (number of macropatches = 211) were recorded at 1-kHz sampling rates (filter = 0.2 kHz) using an Axopatch-1D amplifier equipped with a CV-4 headstage (Molecular Devices, Sunnyvale, CA). Pipettes having a resistance of 1 ± 0.2 MΩ (number of macropatches = 211) measured in KCl on both sides of the membrane patches were used to measure the currents sustained by multiple K_{ATP} channels and their pharmacological properties.

The currents flowing through the macropatches excised from different fibers were digitally averaged and were calculated by subtracting the baseline level of the currents from the open channel level. The baseline level for the K_{ATP} current was measured in the presence of ATP (5×10^{-3} M). Macropatches containing voltage-dependent channels or inward-rectifier K^+ channels were excluded from the analysis. Current amplitude was measured using the Clampfit program (Molecular Devices). No correction for liquid junction potential was made, estimated to be <1.9 mV in our experimental conditions.

Concentration-response relationships were constructed as described previously (Tricarico et al., 2006). No more than two different concentrations of the drugs on the same excised macropatch were applied. Washout periods followed the first and second applications of the drug solutions. A solution enriched with the Kir6.2/SUR2A-2B agonist cromakalim (100 nM) followed the second washout period. The cromakalim-sensitive channels were then exposed to solutions enriched with the Kir6.2/SUR1 selective blocker tolbutamide (1.5 mM) and/or to glibenclamide (100 nM), an nonselective channel blocker. Drug solutions were applied to the patches by using the ValveLink8 fast perfusion system (AutoMate Scientific, Berkeley, CA). Patches showing rundown or that did not fully recover during washout after the drug solution applications were excluded from the analysis. Patches containing channels unresponsive to cromakalim but inhibited by glibenclamide and tolbutamide, possibly composed by SUR1 subunit, as well as channels unresponsive to any of these drugs, were excluded from the mean.

Electronic Quantum Chemical Calculations and Conformational Analysis. The 2*H*-1,4-benzoxazine derivatives were first constructed by fragments, and the molecular geometry was optimized to DFT B3LYP/6–31G* level theory; we got the ATP and ADP optimized starting geometry by the Spartan "06 internal database. The optimized structures were submitted for a systematic Merck Molecular Force Field conformational analysis as described previously (Tricarico et al., 2004). We searched the most populated low-energy conformer families within the 3.0 kcal/mol range for each compound and within 7 kcal/mol for nucleotides by a systematic conformational analysis; from among them, some low energy conformers were selected and used for the superimposition. The electrostatic potentials of the selected conformers were also calculated. All calculations were performed by using the SPARTAN '06 software package (Wavefunction Inc. Irvine, CA). Energies were corrected for aqueous solvation by using the Cramer-Truhler SM54 solvation model. Graphical representations and superimpositions were performed by DS Visualizer v1.7 (Accelrys Inc., San Diego, CA) (Tricarico et al., 2004). Two criteria were used for selection and representation of the conformers. First, the threshold of energy levels chosen for selection was 3 kcal/mol, which is generally considered sufficiently low to allow the interconversion between conformers in physiological condition. Second, within the low energy conformers we identified those showing a conformation matching with that adopted by the ATP molecule into the Kir6.2 task (Haider et al., 2007).

Statistics. The concentration – response relationships of the K_{ATP} currents constructed in the presence of internal ATP fits the product of two equations describing the interaction of a ligand with two sites mediating opposite effects, the stimulatory effect or the inhibitory effect (Rovati and Nicosia, 1994; Tricarico et al., 2003). However, the concentration-response relationships of the K_{ATP} currents versus drug concentrations constructed in the absence of ATP are well fitted by one inhibitory term.

The stimulatory component can be described by the term:

$$(I_{drug+ATP} - 1) \times 100 = A_{max}/(1 + (DE_{50}/[Drug])^n) \quad (1)$$

whereas the inhibitory component can be described by the term:

$$(I_{drug} - 1) \times 100 = I_{max}/(1 + ([Drug]/IC_{50})^n) \quad (2)$$

For eq. 1, $I_{drug+ATP}$ is the K_{ATP} current measured in the presence of the molecules under study, in the presence of internal ATP (10^{-4} M) and normalized to that in the absence of drugs, A_{max} is the percentage maximal activation of the K_{ATP} currents produced by the molecules under study, DE_{50} is the concentration of the drug needed to enhance the current by 50%, [Drug] is the concentration of the drug tested, and n is the slope factor of the concentration-response relationships. For eq. 2, I_{drug} is the K_{ATP} current measured in the presence of the molecules under study, in the absence of ATP (controls) and normalized to that in the absence of drugs; I_{max} is the percentage maximal inhibition of the K_{ATP} currents produced by the molecules under study; IC_{50} is the concentration of the drug needed to reduce the current by 50%; and n is the slope factor of the curves calculated in the absence of ATP. The algorithms of the fitting procedures used are based on a Marquardt least-squares fitting routine. Data analysis and plot were performed by using SigmaPlot software (Systat Software, Inc., San Jose, CA). The data are expressed as mean ± S.E. unless otherwise specified. The unpaired t test was used to compare the best fit values pooled from the concentration-response relationships analysis. The validity of the test is based on the assumption that the best fit values follows the Gaussian distribution and that the sample sizes (number of data points) used

to calculate the degree of freedom are similar between groups. The data are significantly different for $p < 0.05$ or less.

Results

Effects of the Lengthening of the 2-Alkyl Chain Substitutes on the Opening/Blocking Actions of 2*H*-1,4-Benzoxazine Derivatives on the K_{ATP} Channels. The effects of increasing concentrations of the 2-*n*-butyl-1,4-benzoxazine and of the 2-*n*-hexyl-1,4-benzoxazine derivatives **2** and **3** on muscle K_{ATP} currents of excised macropatches recorded at −60 mV (V_m) were investigated. In the presence of internal ATP (10^{-4} M), the exposure of the macropatches to solutions of **2** and **3**, in the range of concentrations from 10^{-11} M to 10^{-8} M, dose dependently enhanced the inward currents with respect to the current levels recorded in the presence of ATP alone (Fig. 2A). The 2-*n*-hexyl-1,4-benzoxazine derivative **3** was more effective in activating the K_{ATP} channels than its analog **2**, producing a significant enhancement of the K_{ATP} current as determined by *t* test. The calculation of the parameters of the eq. 1 by fitting routine showed that the rank order of efficacy of the compounds as openers expressed as A_{max_1} was 2-*n*-hexyl >2-*n*-butyl, whereas the DE_{50_1} and slope values were not statistically different (Tab.1). The 2-*n*-hexyl analog was significantly more effective and potent than the 2-branched alkyl chain and 2-cyclic aromatic analogs in activating the K_{ATP} channels (Tab. 1). As previously observed with other 2-linear alkyl chain structurally related analogs, significant inhibitory responses have been observed also with the 2-*n*-butyl-1,4-benzoxazine and 2-*n*-hexyl-1,4-benzoxazine derivatives **2** and **3** at concentrations $>10^{-8}$ M (Fig. 2A) (Table 1) (Tricarico et al., 2003).

In the absence of internal ATP, the application of increasing concentrations (10^{-9} M–10^{-4} M) of compounds **2** and **3** to the excised patches significantly reduced dose dependently the K_{ATP} currents (Fig. 2B). The calculation of the parameters of the eq. 2 by fitting routine showed that the rank order of efficacy and potency of the blockers expressed as I_{max_2} and IC_{50_2} was 2–*n*-hexyl >2-*n*-butyl, whereas no differences were observed in the relative slopes of the concentration-response curves (Tab.2). The 2-linear alkyl chain analogs were also significantly more effective and potent as blockers than the 2-branched alkyl chain, 2-cyclohexylmethyl, 2-benzyl, and 2-phenylethyl analogs (Table 2).

Effects of 2-Branched Alkyl Chain Substitutes on the Opening/Blocking Actions of 2*H*-1,4-Benzoxazine Derivatives on the K_{ATP} Channels. In the presence of internal ATP (10^{-4} M), the exposure of the macropatches to solutions of the 2-isopropyl-1,4-benzoxazine **4** and of the 2-isobutyl-1,4-benzoxazine derivative **5**, in the range of concentrations from 10^{-9} M to 10^{-6} M, dose dependently enhanced the inward currents in respect with the current levels recorded in the presence of ATP alone (Fig. 3A). The 2-isopropyl-1,4-benzoxazine derivative

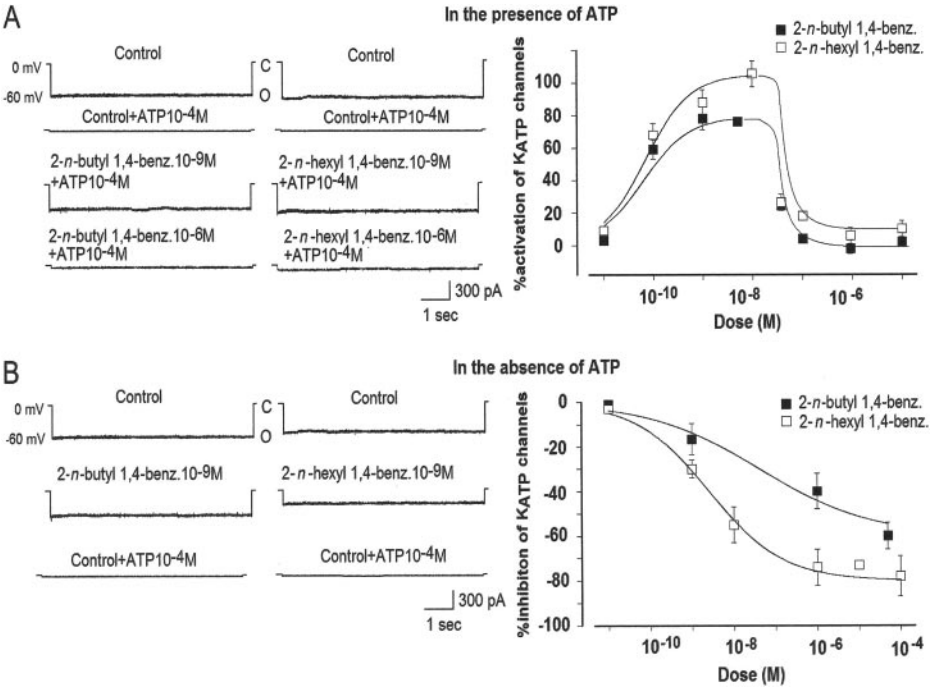


Fig. 2. Effects of the lengthening of the 2-alkyl chain substitutes on the opening/blocking actions of 2*H*-1,4-benzoxazine derivatives on the K_{ATP} channels. The effects of the 2-*n*-hexyl and of the 2-*n*-butyl-1,4-benzoxazine derivatives were tested on K_{ATP} currents recorded in the excised macropatches during voltage step going from 0 mV of holding potential to −60 mV (V_m), in the presence of KCl 150 mM on both sides of the membrane, at 20°C. C and O in the traces indicated closed and open channel levels, respectively. The drug solutions were applied on the internal side of the patches in the presence or absence of ATP. No more than two drug concentrations were applied on the same patches. Sample traces of K_{ATP} currents recorded in control condition (Control), in the presence of internal ATP (Control + ATP 10^{-4} M), in the presence of different concentrations of drug solutions enriched with ATP (10^{-4} M) or in the absence of nucleotide. A, in the presence of internal ATP both compounds, at 10^{-9} M concentration, enhanced the K_{ATP} current in respect with the current levels recorded in the presence of ATP alone. At 10^{-6} M concentration an inhibitory response was observed with both compounds. Concentration-response data were fitted by the sum of a stimulatory and an inhibitory sites function (continuous lines). B, in the absence of ATP, both compounds reduced the K_{ATP} current in respect with the current levels recorded in control condition. Concentration-response relationships analysis showed that the two compounds were effective in inhibiting the K_{ATP} channels. Concentration-response data of both compounds were fitted by a single inhibitory site function. Each experimental point represents the mean ± S.E. of the percentage activation or inhibition of the K_{ATP} currents versus the drug concentrations of a minimum of three and a maximum of six macropatches.

4 was significantly more effective in activating the K_{ATP} channels than its analog **5**. The calculation of the parameters of the eq. 1 by fitting routine showed that the rank order of efficacy of the compounds as openers expressed as A_{max_1} was 2-isopropyl >2-isobutyl, whereas no differences were observed in DE_{50_1} and slope of the concentration-response curves for these compounds (Table 1). No inhibitory responses have been observed with these compounds (Fig. 3A).

In the absence of internal ATP, the application of increasing concentrations (10^{-12} M–5×10^{-4} M) of the 2-branched alkyl chain-1,4-benzoxazine derivatives showed different effects on the K_{ATP} current (Fig. 3B). Concentration-response relationship experiments showed that the 2-isopropyl-1,4-benzoxazine derivative **4** was indeed capable to significantly enhance the K_{ATP} current even in the absence of internal nucleotide, whereas the 2-isobutyl analog **5** in the same range of concentrations did not affect the channel current (Fig. 3B; Tab. 2).

Effects of the Introduction of the Aliphatic or Aromatic Rings at Position 2 of the 1,4-Benzoxazine Nucleus on the Opening/Blocking Actions of the 2*H*-1,4-Benzoxazine Derivatives on the K_{ATP} Channels. In the

TABLE 1

Fitting parameters of the concentration–response curves of 2*H*-1,4-benzoxazine derivatives versus the skeletal muscle K_{ATP} currents in the presence of ATP

The parameters reported in the table were calculated by using the fitting routine based on the sum of the terms 1 and 2 as described under *Materials and Methods*. Compounds are the 2*H*-1,4-benzoxazine derivatives with 2–*n*-hexyl, 2-*n*-butyl, 2-isopropyl, 2-isobutyl, 2-cyclohexylmethyl, 2-benzyl, 2-phenyl, or 2-phenylethyl groups at position 2 of the benzoxazine nucleus. A_{max_1} is the maximal activation of the K_{ATP} currents produced by the molecules under study and it is calculated with respect to the current levels measured in the presence of ATP (10^{-4}M). DE_{50_1} is the concentration of the drug needed to enhance the current by 50% calculated with respect to the maximal activation produced by the compounds in the presence of internal ATP. I_{max_1} is the maximal inhibition of the K_{ATP} currents produced by the molecules under study and it is calculated with respect to the maximal current levels measured in the presence of drugs + ATP. IC_{50_1} is the concentration of the drug needed to reduce the current by 50%, calculated with respect to the maximal inhibition produced by the molecules. *n* is slope factor of the concentration-response relationships. In some cases, the reduced drug responses and the low number of data points did not allow the evaluation of the plateau phase and calculation of the IC_{50_1}, DE_{50_1}, and slopes of the concentration-response relationships of the compounds.

Compounds	A_{max1}	DE_{501}	*n*	I_{max1}	IC_{501}	*n*
	%	*M*		%	*M*	
Linear						
2-*n*-Hexyl	$+105 \pm 2^{*\dagger\ddagger}$	$1.08 \pm 0.99 \times 10^{-10\dagger\ddagger}$	$0.93 \pm 0.1^{\dagger}$	$-95 \pm 9^{\dagger\S}$	$4.1 \pm 3 \times 10^{-8\dagger\S}$	-0.5 ± 0.1
2-*n*-Butyl	$+75 \pm 2$	$3.24 \pm 0.9 \times 10^{-10}$	0.9 ± 0.2	$-97 \pm 6^{\S}$	$8.0 \pm 1 \times 10^{-8\S}$	-0.6 ± 0.1
Cyclic						
2-Cyclohexylmethyl	$+88 \pm 5^{\dagger\ddagger}$	$1.81 \pm 0.9 \times 10^{-10\dagger\ddagger}$	$0.99 \pm 0.1^{\ddagger}$	$-30 \pm 2^{\dagger\ddagger}$		
2-Benzyl	$+59 \pm 2$	$7.21 \pm 0.1 \times 10^{-10}$	0.95 ± 0.1	-26 ± 5		
2-Phenyl	$+69 \pm 1$	$3.92 \pm 5 \times 10^{-10}$	0.96 ± 0.1	-24 ± 3		
2-Phenylethyl	$+10 \pm 3$					
Branched						
2-Isopropyl	$+76 \pm 0.55^{\P}$	$1.9 \pm 0.9 \times 10^{-9}$	0.71 ± 0.1	-21 ± 5		
2-Isobutyl	$+58 \pm 0.5$	$3.8 \pm 0.7 \times 10^{-9}$	0.72 ± 0.3	-19 ± 6		

Symbols indicates data significantly different for $P < 0.05$ as determined by unpaired *t* test:
* Significantly different from 2-*n*-butyl analog data.
† Significantly different from 2-branched analog data.
‡ Significantly different from 2-cyclic aromatic analogs data.
§ Significantly different from 2-cyclic analog data.
¶ Significantly different from 2-isobutyl analog data.

TABLE 2

Fitting parameters of the concentration–response curves of 2*H*-1,4-benzoxazine derivatives versus the skeletal muscle K_{ATP} currents in the absence of ATP

The parameters reported in the table were calculated by using the fitting routine based on the equation 2 as described under *Materials and Methods*. Compounds are the 2*H*-1,4-benzoxazine derivatives with 2–*n*-hexyl, 2-*n*-butyl, 2-isopropyl, 2-isobutyl, 2- cyclohexylmethyl, 2-benzyl, 2-phenyl, or 2-phenylethyl groups at position 2 of the benzoxazine nucleus. I_{max_2} is the maximal inhibition of the K_{ATP} currents produced by the molecules under study and it is calculated with respect to the maximal current levels measured in the presence of drugs. IC_{50_2} is the concentration of the drug needed to reduce the current by 50%, calculated with respect to the maximal inhibition produced by the molecules; *n* is slope factor of the concentration-response relationships. A_{max_2} is the maximal activation of the K_{ATP} currents produced by the molecules under study. DE_{50_2} is the concentration of the drug needed to enhance the current by 50% calculated with respect to the maximal activation produced by the compounds. In some cases, the reduced drug responses and the low number of data points did not allow the evaluation of the plateau phase and calculation of the IC_{50_1} and slopes of the concentration-response relationships of the compounds.

Compounds	I_{max2}	A_{max2}	IC_{502}	DE_{502}	*n*
	%		*M*		
Linear					
2-*n*-Hexyl*	$-85 \pm 8^{*\dagger}$		$1.0 \pm 0.1 \times 10^{-8*\dagger\ddagger}$		$-0.49 \pm 0.02^{*}$
2-*n*-Butyl	$-62 \pm 6^{*\dagger}$		$9.0 \pm 3 \times 10^{-8\dagger}$		-0.32 ± 0.03
Cyclic					
2-Cyclohexylmethyl	-35 ± 2				
2-Benzyl	-5 ± 0.1				
2-Phenyl	$-98 \pm 5^{\S}$		$2.5 \pm 2 \times 10^{-11\P}$		-0.18 ± 0.09
2-Phenylethyl	-15 ± 3				
Branched					
2-Isopropyl		$+52 \pm 5$		$6 \pm 1 \times 10^{-11}$	0.71 ± 0.1
2-Isobutyl		0			

Symbols indicate data significantly different for $P < 0.05$ as determined by unpaired T test:
* Significantly different from 2-*n*-butyl data.
† Significantly different from 2-cyclohexylmethyl, 2-benzyl, and 2-phenylethyl analogs data.
‡ Significantly different from 2-cyclic aromatic analogs data.
§ significantly different from 2-cyclic analog data.
¶ Significantly different from 2-linear alkyl chain analog.

presence of internal ATP 10^{-4} M, the exposure of the macropatches to drug **6**-**9** solutions, in the range of concentrations from 10^{-11} M to 10^{-7} M, enhanced dose dependently the inward currents with respect to the current levels recorded in the presence of ATP alone, however, showing different efficacy (Fig. 4A). The 2-cyclohexylmethyl-1,4-benzoxazine derivative **6** was significantly more effective and potent than its structurally related analogs in enhancing the K_{ATP} current, whereas the 2-phenylethyl-1,4-benzoxazine derivative **8** was the least effective compound (Fig. 4A). The calculation of the parameters of eq. 1 showed that the rank order of efficacy and potency of the openers expressed as A_{max_1} and DE_{50_1} was 2-cyclohexylmethyl > 2-phenyl ≥ 2-benzyl. No differences were observed in the slopes of the concentration-response curves for these compounds (Table 1). The 2-cyclohexylmethyl analog was also significantly more effective and potent than the 2-branched alkyl chain analogs (Table 1). Slight inhibitory responses have been observed with these compounds at concentrations $>10^{-7}$ M (Fig. 4A; Table 1).

In the absence of internal ATP, in the range of concentration tested (10^{-12}–10^{-4} M), the 2-phenyl-1,4-benzoxazine derivative **9** was significantly more potent and effective as an K_{ATP} channel blocker than its analogs that did not produce more than 35% inhibition of the channel currents (Fig. 4B; Table 2). This compound was also more potent and effective than the 2-linear and 2-branched alkyl chain analogs (Table 2).

Discussion

In the present work, several new molecules belonging to the class of 2*H*-1,4-benzoxazine derivatives were synthesized and tested against the native skeletal muscle K_{ATP} channels with the goal of investigating the molecular determinants for

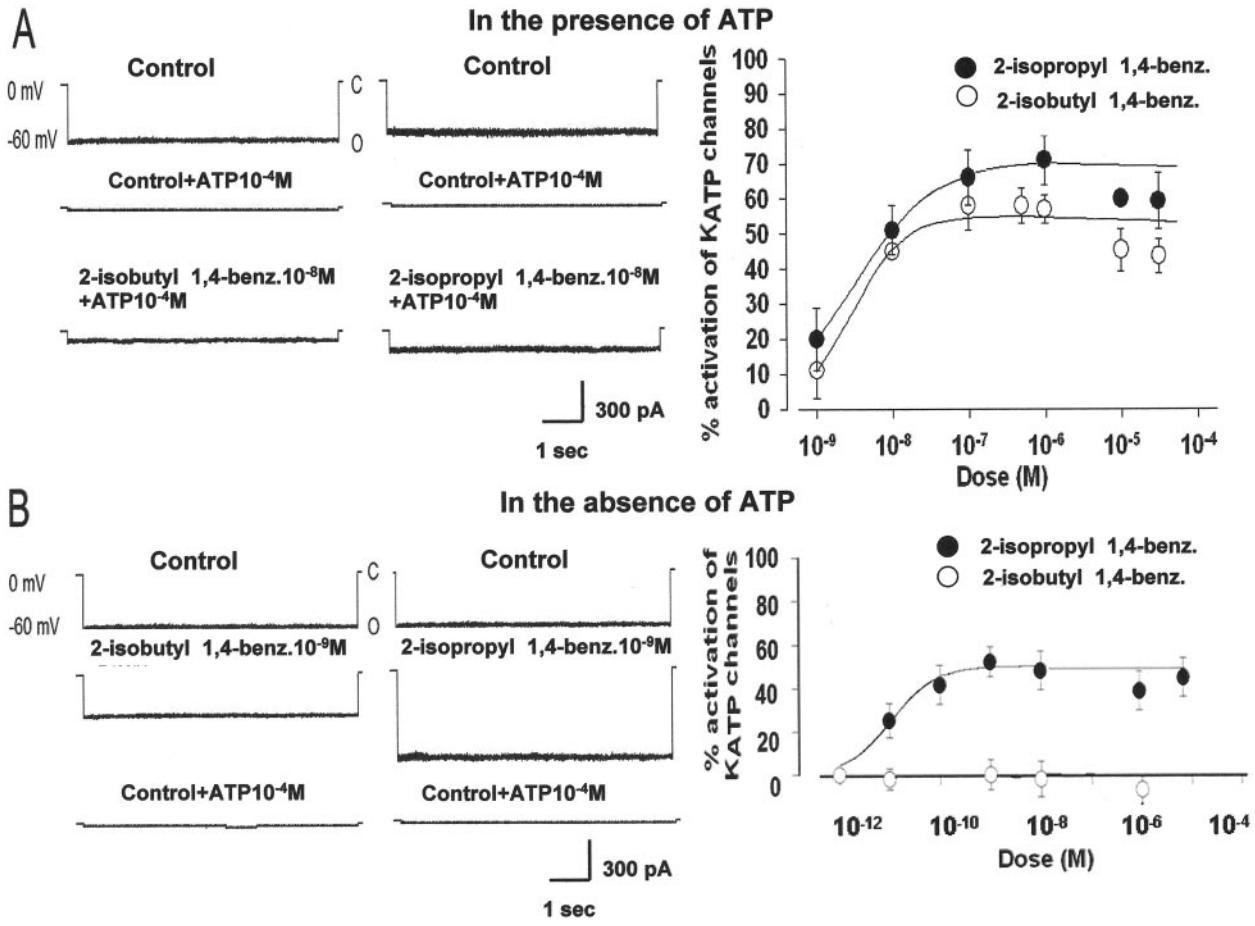


Fig. 3. Effects of the 2-branched alkyl chain substitutes on the opening/blocking actions of 2*H*-1,4-benzoxazine derivatives on the muscle K_{ATP} channels. The effects of the 2-isobutyl and of the 2-isopropyl 1,4-benzoxazine derivatives were tested on K_{ATP} currents recorded in the excised macropatches during voltage step going from 0 mV of holding potential to −60 mV (V_m), in the presence of 150 mM KCl on both sides of the membrane at 20°C. C and O in the traces indicated closed and open channel levels, respectively. The drug solutions were applied on the internal side of the patches in the presence or absence of ATP. No more than two drug concentrations were applied on the same patches. Sample traces of K_{ATP} currents recorded in control condition (Control), in the presence of internal ATP (Control + ATP 10^{-4} M), in the presence of different concentrations of drug solutions enriched with ATP (10^{-4} M) or in the absence of nucleotide. A, in the presence of internal ATP both compounds, at 10^{-8} M concentration, enhanced the K_{ATP} currents in respect with the current levels recorded in the presence of ATP alone. Concentration-response data were fitted by a single stimulatory site function (continuous lines). B, in the absence of ATP, both compounds failed to inhibit the K_{ATP} currents. Concentration-response relationships analysis showed that the 2-isopropyl 1,4-benzoxazine, in the range of concentrations tested, was capable to enhance the K_{ATP} current even in the absence of internal nucleotide, whereas the 2-isobutyl 1,4-benzoxazine derivative did not affects the K_{ATP} current. Concentration-response data of the 2-isopropyl analog were fitted by a single stimulatory site function at concentration (continuous lines). Each experimental point represents the mean ± S.E. of the percentage activation of the K_{ATP} currents versus the drug concentrations of a minimum of three and a maximum of six macropatches.

the activating/blocking actions of these compounds. The strategy used was to replace the H atom at position 2 of the 2*H*-1,4-benzoxazine nucleus with different substitutes, such as linear and branched alkyl chains or groups containing aromatic or aliphatic rings.

We showed here that the compounds most effective at activating the K_{ATP} channels in the presence of ATP were the 2-*n*-hexyl and 2-cyclohexylmethyl −1,4-benzoxazine derivatives, which produced 105% and 88% activation of the channels in excised patches, respectively. Less effective compounds were the analog **2** with shorter linear alkyl chain, the 2-branched alkyl chain analogs **4** and **5**, and the 2-cyclic aromatic analogs **7** and **9**; the 2-phenylethyl analog **8** was not effective. In conclusion, the rank order of efficacy expressed as A_{max_1} of the openers was 2-*n*-hexyl ≥2-cyclohexylmethyl > 2-isopropyl = 2-*n*-butyl ≥ 2-phenyl ≥ 2-benzyl = 2-isobutyl analogs.

In the absence of internal ATP, some of these compounds were effective as K_{ATP} channel blockers in the nanomolar concentration range. The 2-phenyl **9**, 2-*n*-hexyl **3**, and 2-*n*-butyl **5** analogs were the most effective and potent compounds with respect to the other structurally related analogs. The rank order of efficacy of the blockers expressed as I_{max_2} was 2-phenyl ≥ 2-*n*-hexyl > 2-*n*-butyl > 2-cyclohexylmethyl, whereas the 2-arylalkyl analogs **7** and **8** and 2-branched alkyl chain analogs **4** and **5** were not effective as blockers. We were surprised to find that the 2-isopropyl analog **4** was capable of opening the K_{ATP} channels even in the absence of nucleotide.

These findings indicate that, first, the lengthening of the

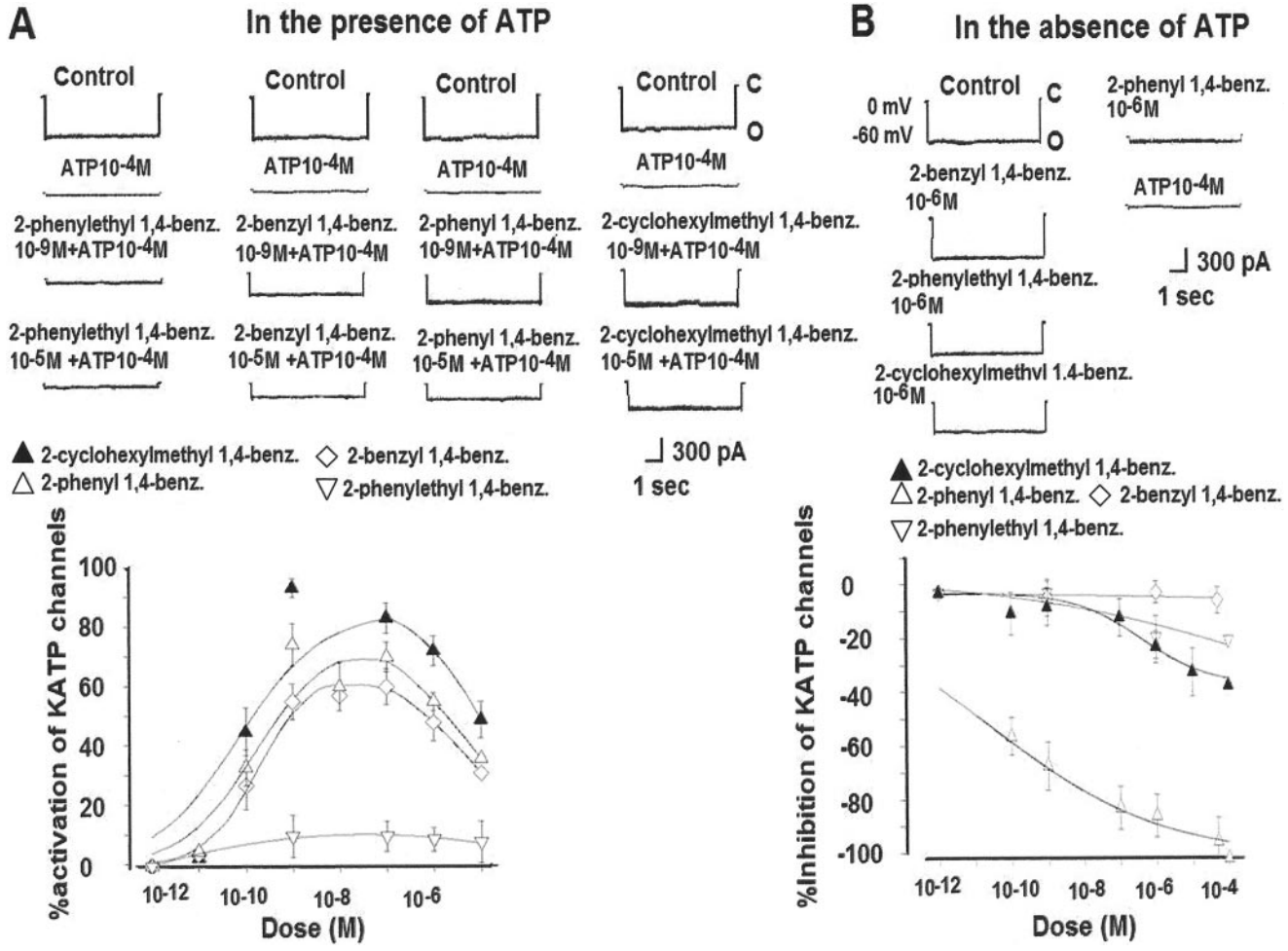


Fig. 4. Effects of the introduction of the aliphatic or aromatic rings at position 2 of the 1,4-benzoxazine nucleus on the opening/blocking actions of the 2*H*-1,4-benzoxazine derivatives on the muscle K_{ATP} channels. The effects of the 2-phenylethyl, 2-benzyl, 2-phenyl and 2-cyclohexylmethyl 1,4-benzoxazine derivatives were tested on K_{ATP} currents recorded in the excised macropatches during voltage step going from 0 mV of holding potential to −60 mV (V_m), in the presence of KCl 150 mM on both sides of the membrane at 20°C. C and O in the traces indicated closed and open channel levels, respectively. The drug solutions were applied on the internal side of the patches in the presence or absence of ATP. No more than two drug concentrations were applied on the same patches. Sample traces of K_{ATP} currents recorded in control condition (Control) in the presence of internal ATP (Control + ATP 10^{-4} M), in the presence of different concentrations of drug solutions enriched with ATP (10^{-4} M) or in the absence of nucleotide. A, in the presence of internal ATP all compounds, at 10^{-9} M concentration, enhanced the K_{ATP} current in respect with the current levels recorded in the presence of ATP alone however with different efficacy. The 2-ciclohexylmethyl 1,4-benzoxazine was the most effective compound in activating the K_{ATP} channel. At concentrations $>10^{-6}$ M all compounds showed slight inhibitory responses. Concentration-response data were fitted by the sum of a single stimulatory and an inhibitory sites function (continuous lines). B, in the absence of ATP, the compounds under investigations reduced the K_{ATP} currents in respect with the current levels recorded in control condition but with different efficacy. The 2-phenyl 1,4-benzoxazine was the most effective compound in inhibiting the K_{ATP} currents in respect with the other analogs. Concentration-response data of the 2-cyclohexylmethyl, 2-phenyl, and 2-phenylethyl-1,4-benzoxazine derivatives were fitted by a single inhibitory site function. Each experimental point represents the mean ± S.E. of the percentage activation or inhibition of the K_{ATP} channels versus the drug concentrations of a minimum of three and a maximum of six macropatches.

linear alkyl chain at position 2 of the 1,4-benzoxazine nucleus determines the efficacy and potency of these molecules as openers and blockers. This is demonstrated by the calculated A_{max_1}/I_{max_2} and $DE_{50_1}/IC_{50_{1-2}}$ ratios, which are close to the unity for both 2-*n*-hexyl and 2-*n*-butyl analogs **3** and **2**. Second, the molecular switch from blocking to opening actions for the 2*H*-1,4-benzoxazine derivatives can be achieved by replacing the linear alkyl chain with 2-branched alkyl chain substitutes as observed for the analogs **4** and **5**. Third, the introduction of an aromatic cycle in place of the linear alkyl chain at position 2 of the 1,4-benzoxazine nucleus conferred pronounced blocking action to the molecule in the absence of ATP, as in the case of the analog **9**. An additional factor is the intramolecular distance of the substitutes from the 1,4-benzoxazine nucleus, which is inversely related to the effectiveness of the molecules as blockers/openers as observed with the 2-arylalkyl analogs **7** and **8** and with the 2-branched alkyl chain analog **5**.

The observed differences in the molecular requisites responsible for the action of these compounds as openers and blockers would suggest that distinct high-affinity binding sites modulate their dual actions on the K_{ATP} channels. Possible sites of interactions for these compounds can be located on the nucleotide binding sites on the K_{ATP} channel subunits. Recognition sites for ATP and ADP are located on the Kir6.2 subunit and on the SUR subunits such as the nucleotide-binding -fold (NBD1 and NBD2) of the K_{ATP} channel complexes (Nichols, 2006; Haider et al., 2007). This is supported by the observed structural similarities of the 2*H*-1,4-benzoxazine derivatives with the ATP and ADP molecules. Preliminary conformational analysis would indeed suggest that the planar area of the 2*H*-1,4-benzoxazines overlaps with that of the adenine nucleotide tri- and diphosphates. The electronic distribution profile of this area is also similar with that of the adenine nucleotides being electrons reached suggesting that these compounds share a common area of interaction with ATP and ADP on the receptor sites. We found that the best overlay with the ATP conformer, which interacts with the inhibitory site on Kir6.2, is observed with the linear alkyl chain analogs, and particularly with the 2-*n*-hexyl analog **3,** explaining the blocking action of these compounds in the absence and in the presence of ATP (Fig. 5). This is corroborated by the finding that the IC_{50_1} and IC_{50_2} of the 2-*n*-hexyl and 2-*n*-butyl analogs **3** and **2**, respectively, evaluated in the presence and absence of ATP, did not differ significantly indicating a common site of interaction possibly on Kir6.2 subunit. Furthermore, we have shown that the 2-*n*-propyl-1,4-benzoxazine derivative, in the absence of ATP, is capable of inhibiting the truncated Kir6.2 channel expressed in Hek293 cell line (Rolland et al., 2006). Conformational analysis also shows that molecules with a reduced three-dimensional area of superimposition with the nucleotide may loose the capability to inhibit the Kir6.2 subunit as in the case of the 2-isopropyl analog **4** or may show a different inhibitory profile as in the case of the 2-phenyl analog **9**, which potently inhibit the K_{ATP} channel, but only in the presence of ATP. Therefore, it is likely that the hydrophobic planar area of the 2*H*-1,4-benzoxazine derivatives may fits into the hydrophobic pocket of the Kir6.2, which is the area in which the ATP binds. Ligand docking investigations indeed showed that the ATP site is located into a hydrophobic pocket at the interface between the N and C domain of the Kir6.2 (Nichols, 2006). Although the substitutes at the position 2 of the 1,4-benzoxazine nucleus could adopt conformations similar to that of the phosphate group of nucleotides determining the actions of these compounds as blockers and openers.

The opening action of the 2*H*-1,4-benzoxazine derivatives could be mediated by the nucleotide-binding-folds of SUR2, and it may be also dependent on the binding of these compounds to the TMD13–17/NBD2 regions of this subunit. This is supported by the previously reported tissue-selective opening action of the 2-*n*-propyl-1,4-benzoxazine derivative, which is capable of activating the skeletal muscle K_{ATP} channels but in contrast fails to activate the pancreatic channel type, which is composed by the SUR1 subunit (Rolland et al., 2006). The TMD13–17/NBD2 regions indeed distinguish the muscle type SUR2 from the pancreatic SUR1 subunit and are involved in the binding/actions of several KCO.

The reduced efficacy of the 2*H*-1,4-benzoxazine derivatives in activating the K_{ATP} channels observed in the presence of internal ATP at micromolar concentrations has been described also for other KCO and ADP molecules that are capable to activate the recombinant and native K_{ATP} channels at low concentrations and to inhibit it at high concentrations. This generates bell-shaped concentration-response relationships for these drugs and ADP molecules as also observed in our experiments. This phenomenon has been associated with several mechanisms including interaction

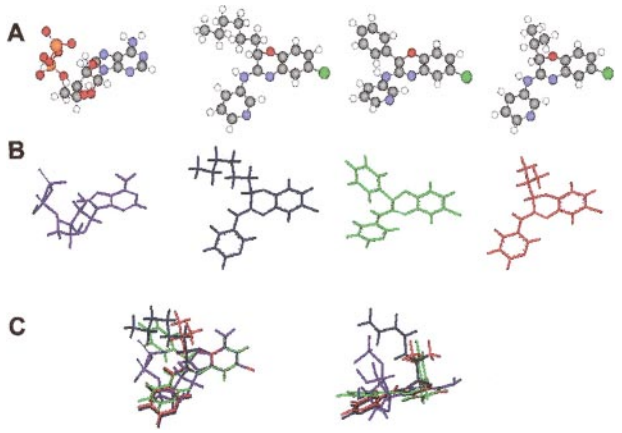


Fig. 5. Conformational analysis of the 2*H*-1,4-benzoxazine derivatives and comparison with nucleotide triphosphate. (A and B) Stick and balls representations of the lower energy conformers, from left to right, of ATP anion, 2-*n*-hexyl, 2-phenyl, and 2-isopropyl 1,4-benzoxazine derivatives. Conformers representation were selected on the basis of their energy levels not exceeding 2 to 3 kcal/mol for the 2*H*-1,4-benzoxazine derivatives and 7 kcal/mol for the ATP molecule. Moreover, within the low-energy conformers, we identified those showing a conformation matching with that adopted by the ATP molecule into the Kir6.2 task (Haider et al., 2007). The low calculated differences in the energy levels between conformers for each molecule suggest that the interconversion between conformers is favored in physiological condition. C, optimized molecular superimposition of 2-*n*-hexyl, 2-phenyl, and 2-isopropyl 1,4-benzoxazine derivatives with ATP. Two different orientations of the molecules on the *z*-axis are represented. The planar area of 2-*n*-hexyl (blue), 2-phenyl (green), and of the 2-isopropyl (red), 1,4-benzoxazine derivatives matches with that of the adenine ring of the ATP (violet) molecule. In the case of 2-hexyl and 2-isopropyl analogs, the 3-amino-pyridine substitute overlaps with the ribose ring of the nucleotide, however in the 2-phenyl analog **9** does not. Moreover, the 2-linear alkyl chain of the 2-*n*-hexyl analog **3** may occupy the same area of the phosphate group of ATP, whereas this is not observed for the substitutes of the 2-phenyl and 2-isopropyl analogs **9** and **4**. Therefore, the best molecular overlay is observed with the 2-*n*-hexyl analog **3** followed by the 2-isopropyl and 2-phenyl analogs **4** and **9**.

with inhibitory site/s on the channel subunits, loss of second messengers regulating channel openings in excised patches and with a reduced drug-dependent ATP-ase activity of NBD2/SUR2 (Allard and Lazdunski, 1992; Bienengraeber et al., 2000; Teramoto et al., 2001; Russ et al., 2003; Tricarico et al., 2003; Alekseev et al., 2005). The direct interaction 2-*n*-hexyl and 2-*n*-butyl analogs **3** and **2** with the inhibitory site possibly located on Kir6.2 would explain the inhibitory actions of the 2*H*-1,4-benzoxazine derivatives observed in our experiments at micromolar concentrations.

We should stress that our experiments were performed on the cromakalim and glibenclamide-sensitive K_{ATP} channels but insensitive to tolbutamide. We have indeed demonstrated that channels showing these properties are composed by Kir6.2/SUR2A, Kir6.2/SUR2B subunits and by a hybrid assembly of Kir6.2/2A-B subunits. These channels represent the mayor channel populations found in skeletal muscle and are therefore valuable drug targets in this tissue (Tricarico et al., 2006). Possible drug opening action of other subtypes of K_{ATP} channels such as those composed by SUR1 subunit characteristic of the pancreatic and neuronal tissues and also found in skeletal muscle were not evaluated in the present work. Inhibitory actions of the Kir6.2/SUR1 channel are however still possible, indeed our blockers seem to target the Kir6.2 subunit, which is shared by the diverse subtypes of channels. This is corroborated by the observation that the 2-*n*-propyl analog **1** blocked the pancreatic K_{ATP} channel without showing activating action (Rolland et al., 2006).

In conclusion, the molecular determinants responsible for the opening action of the 2*H*-1,4-benzoxazine derivatives have been found. The most effective compounds reported here were 100-fold more potent than their structural analogs as well as than the first generation KCO such as cromakalim, pinacidil, nicorandil, and minoxidil (Tricarico et al., 2003; Mannhold, 2004; Cecchetti et al., 2006). Modulators of the K_{ATP} channels are promising in those conditions associated with impaired skeletal muscle functionality. Openers restore muscle contraction in humans affected by periodic paralysis, myotonia, and in neuromuscular disorders associated with impaired fiber excitability and contraction; deficiency of skeletal muscle K_{ATP} channels is associated with reduced muscle contractility in the rat (Saitoh, 2005; Cifelli et al., 2007). Blockers targeting the Kir6.2 subunit may be effective in the insulin-resistant state and diabetes type II with neuromuscular symptoms associated with abnormal openings of the skeletal muscle, neuronal, and pancreatic K_{ATP} channels (Koster et al., 2005; Flechtner et al., 2006).

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Address correspondence to: Domenico Tricarico, Dept. of Pharmacobiology, Faculty of Pharmacy, via Orabona no. 4, Bari, Italy.