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

Domenico Tricarico, Antonetta Mele, Giulia Maria Camerino, Antonio Laghezza, Giuseppe Carbonara, Giuseppe Fracchiolla, Paolo Tortorella, Fulvio Loidice, 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

Received February 21, 2008; accepted April 10, 2008

ABSTRACT

The 2H-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 ($D_{50} = 1.08 \times 10^{-10}$ M), whereas the 2-phenylethyl was not effective. The rank order of efficacy of the openers was 2-n-hexyl $\geq$ 2-cyclohexylmethyl $> 2$-isopropyl $= 2$-n-butyl $\geq 2$-phenyl $\geq 2$-benzyl $= 2$-isobutyl analogs. In the absence of ATP, the 2-phenyl analog was the most potent inhibitor ($I_{C50} = 2.5 \times 10^{-11}$ M); the rank order of efficacy of the blockers was 2-phenyl $\geq 2$-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 molecules, which belong to a number of structural classes, including benzopyrans (cromakalim, bimatekalim), benzo-thiadiazines (diazoxide), cyanoguanidines (pinacidil), cyclobutenediones (WAY-151616), nicotinamides (nicorandil), pyrimidines (minoxidil), tertiary carbinoles (ZD-6169), thiadiazines (diazoxide), cyanoguanidines (pinacidil), cyclo-hexanediones (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 $\beta$ cells, neurons, and vascular/nonvascular smooth muscle and cardiac muscle by opening $K_{ATP}$ channels, thereby 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

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; $D_{50}$, drug concentration needed to enhance the current by 50%; $I_{C50}$, drug concentration needed to inhibit the current by 50%; hypoPP, hypokalemic periodic paralyses; MOPS, 3-(N-morpholinopropyl)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\textsubscript{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\textsubscript{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\textsubscript{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 and phenotypes, which corroborates the idea that molecular composition of the skeletal muscle K\textsubscript{ATP} channels is complex (Tricarico et al., 2006). In native skeletal muscle fibers, the K\textsubscript{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\textsubscript{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\textsubscript{ATP} channels, is effective in human skeletal muscle (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\textsubscript{ATP} channels at 10\textsuperscript{-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\textsubscript{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).

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\textsubscript{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\textsubscript{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\textsubscript{ATP} channels, cromakalim-glibenclamide-sensitive and tolbutamide-insensitive, of native fibers of the rat, in the presence or absence of internal ATP (10\textsuperscript{-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\textsuperscript{-3} M NaCl, 5 × 10\textsuperscript{-3} M KCl, 10\textsuperscript{-3} M MgCl\textsubscript{2}, 0.5 × 10\textsuperscript{-3} M CaCl\textsubscript{2}, 5 × 10\textsuperscript{-3} M glucose, and 10\textsuperscript{-2} M MOPS, pH 7.2. The patch pipette solution contained 150 × 10\textsuperscript{-3} M KCl, 2 × 10\textsuperscript{-3} M CaCl\textsubscript{2}, and 10\textsuperscript{-2} M MOPS, pH 7.2. The bath solution contained 150 × 10\textsuperscript{-3} M KCl, 5 × 10\textsuperscript{-3} M EGTA, and 10\textsuperscript{-2} M MOPS, pH 7.2. Stock solution of ATPK\textsubscript{2} (5 × 10\textsuperscript{-3} M) was prepared by dissolving the chemical in the bath solution.

Stock solutions of the 2H-1,4-benzoxazine derivatives (30 × 10\textsuperscript{-3} M), cromakalim, tolbutamide and glibenclamide (20 × 10\textsuperscript{-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 2H-1,4-benzoxazine derivatives ranging between 10\textsuperscript{-12} and 10\textsuperscript{-4} M (DMSO 0.33%). Because of the low solubility of these compounds in the aqueous solvent, the drugs were tested at concentrations <10\textsuperscript{-4} M. DMSO applied at 0.33% concentration to the excised patches in the presence of ATP (10\textsuperscript{-4} M) did not increase the K\textsubscript{ATP} channel activity (solvent control). DMSO did not affect K\textsubscript{ATP} current even in the absence of internal nucleotide. The drugs were tested in the presence or in the absence of internal ATP (10\textsuperscript{-4} M) with no added Mg\textsuperscript{2+} ions to reduce possible ATPase activity in the patches (Russ et al., 2003; Tricarico et al., 2003).
Synthesis of the New 2H-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-2H-1,4-benzoxazine-3-one, which was condensed with 3-aminopyridine either in refluxing dry toluene in the presence of TiCl4 and anisole as previously reported for derivative 1 (compounds 3 and 9) (Tricarico et al., 2003), or in dry acetonitrile in the presence of triethylamine and POCl3 (compounds 2 and 4–8). In addition, the preparation of benzoxazines followed two different synthetic pathways. The former started from the commercially available α-amino acids, which were converted to the corresponding acyl chloride with SOCl2, and condensed with 2-amino-4-chloro-phenol in the presence of triethanol amine chloride and NaHCO3, in CHCl3, to give a one-pot cyclization reaction (benzoxazine 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 (benzoxazine 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 2H-1,4-benzoxazine derivatives (Fig. 1) were: (R/S)-6-chloro-2-butylyl-3-(pyridin-3-yl- aminomethylene)2H-1,4-benzoxazine (2), (R/S)-6-chloro-2-hexylyl-3-(pyridin-3-yl-aminomethylene)2H-1,4-benzoxazine (3), (R/S)-6-chloro-2-isopropyl-3-(pyridin-3-yl-aminomethylene)2H-1,4-benzoxazine (4), (R/S)-6-chloro-2-isobutyl-3-(pyridin-3-yl-aminomethylene)2H-1,4-benzoxazine (5), (R/S)-6-chloro-2-cyclohexylmethyl-3-(pyridin-3-ylaminomethylene)2H-1,4-benzoxazine (6), (R/S)-6-chloro-2-benzyl-3-(pyridin-3-ylaminomethylene)2H-1,4-benzoxazine (7), (R/S)-6-chloro-2-phenyl-3-(pyridin-3-yl-aminomethylene)2H-1,4-benzoxazine (8), and (R/S)-6-chloro-2-phenethyl-3-(pyridin-3-yl-aminomethylene)2H-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 KC1 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 Hz) 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 KC1 on both sides of the membrane patches were used to measure the currents sustained by multiple KATP 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 KATP 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 Kir2.2/SUR1 selective blocker tolbutamide (1.5 mM) and/or to glibenclamide (100 nM), an nonsensitive channel blocker. Drug solutions were applied to the patches by using the ValvoLink® 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 2H-1,4-benzoxazine derivatives were first constructed by fragments, and the molecular geometry was optimized to DPT 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-Truhlar 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 KATP 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 KATP 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:

\[
I_{\text{KATP,ATP}} = I_{\text{max}} \times \frac{pDE50 |\text{Drug}|}{1 + pDE50 |\text{Drug}|}
\]

whereas the inhibitory component can be described by the term:

\[
I_{\text{KATP,ATP}} = I_{\text{max}} \times \frac{pIC50 |\text{Drug}|}{1 + pIC50 |\text{Drug}|}
\]

For eq. 1, \(I_{\text{KATP,ATP}}\) is the KATP 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, \(A_{\text{max}}\) is the percentage maximal activation of the KATP currents produced by the molecules under study, \(DE50\) is the concentration of the drug needed to enhance the current by 50%, \(IC50\) is the concentration of the drug tested, and \(n\) is the slope factor of the concentration-response relationships. For eq. 2, \(I_{\text{KATP,ATP}}\) is the KATP 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; \(I_{\text{max}}\) is the percentage maximal inhibition of the KATP currents produced by the molecules under study; \(IC50\) 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 2H-1,4-Benzoxazine Derivatives on the \( \text{K}_{\text{ATP}} \) 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 \( \text{K}_{\text{ATP}} \) 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 \( \text{K}_{\text{ATP}} \) channels than its analog 2, producing a significant enhancement of the \( \text{K}_{\text{ATP}} \) 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 \( A_{\text{max}} \) was 2-\( n \)-hexyl > 2-\( n \)-butyl, whereas the \( D_{50} \) 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 \( \text{K}_{\text{ATP}} \) 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\(^{-9}\) M (Fig. 2A) (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 \( \text{K}_{\text{ATP}} \) 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 \( I_{\text{max}} \) and IC\(_{50}\) 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 2H-1,4-Benzoxazine Derivatives on the \( \text{K}_{\text{ATP}} \) 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 2H-1,4-benzoxazine derivatives on the \( \text{K}_{\text{ATP}} \) channels.](image-url)
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 $A_{\text{max1}}$ was 2-isopropyl > 2-isobutyl, whereas no differences were observed in $DE_{501}$ 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 – $5 \times 10^{-4}$ 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 2H-1,4-Benzoxazine Derivatives on the $K_{ATP}$ Channels.** In the absence of internal ATP, the application of increasing concentrations ($10^{-12}$ M – $5 \times 10^{-4}$ 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).

**TABLE 1**

Fitting parameters of the concentration–response curves of 2H-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 2H-1,4-benzoxazine derivatives with 2-n-hexyl, 2-n-butyl, 2-isopropyl, 2-isobutyl, 2-cyclohexymethyl, 2-benzyl, 2-phenyl, or 2-phenylethyl groups at position 2 of the benzoxazine nucleus. $A_{\text{max1}}$ 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 ($10^{-5}$ M). $DE_{501}$ 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. $I_{\text{max1}}$ 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. $IC_{501}$ 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 $IC_{501}$, $DE_{501}$, and slopes of the concentration-response relationships of the compounds. (Fig. 3B; Tab. 2).

<table>
<thead>
<tr>
<th>Compounds</th>
<th>$A_{\text{max1}}$</th>
<th>$DE_{501}$</th>
<th>$n$</th>
<th>$I_{\text{max1}}$</th>
<th>$IC_{501}$</th>
<th>$n$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Linear</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2-n-Hexyl</td>
<td>+105 ± 2*‡</td>
<td>1.08 ± 0.99$ \times 10^{10-11}$</td>
<td>0.93 ± 0.1$\dagger$</td>
<td>$-95 \pm 9^$</td>
<td>4.1 ± 3 $\times 10^{-8}$</td>
<td>0.5 ± 0.1$\dagger$</td>
</tr>
<tr>
<td>2-n-Butyl</td>
<td>+75 ± 2</td>
<td>3.24 ± 0.9$ \times 10^{-10}$</td>
<td>0.9 ± 0.2</td>
<td>$-97 \pm 0$</td>
<td>8.0 ± 1 $\times 10^{-8}$</td>
<td>0.6 ± 0.1</td>
</tr>
<tr>
<td>Cyclic</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2-Cyclohexymethyl</td>
<td>+88 ± 5$\dagger$</td>
<td>1.81 ± 0.9$ \times 10^{10-11}$</td>
<td>0.99 ± 0.1$\dagger$</td>
<td>$-30 \pm 2^$</td>
<td>26 ± 5</td>
<td></td>
</tr>
<tr>
<td>2-Benzyl</td>
<td>+59 ± 2</td>
<td>7.21 ± 0.1$ \times 10^{-10}$</td>
<td>0.95 ± 0.1</td>
<td>24 ± 3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2-Phenyl</td>
<td>+69 ± 1</td>
<td>3.92 ± 5$ \times 10^{-10}$</td>
<td>0.96 ± 0.1</td>
<td>21 ± 5</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2-Phenylethyl</td>
<td>+10 ± 3</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Branched</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2-Isopropyl</td>
<td>+76 ± 0.55$\dagger$</td>
<td>1.9 ± 0.9$ \times 10^{-9}$</td>
<td>0.71 ± 0.1</td>
<td>21 ± 5</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2-Isobutyl</td>
<td>+58 ± 0.5</td>
<td>3.8 ± 0.7$ \times 10^{-9}$</td>
<td>0.72 ± 0.3</td>
<td>19 ± 6</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Symbols indicate 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-isoalkyl analog data.
- †Significantly different from 2-cyclic analog data.

**TABLE 2**

Fitting parameters of the concentration–response curves of 2H-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 2H-1,4-benzoxazine derivatives with 2-n-hexyl, 2-n-butyl, 2-isopropyl, 2-isobutyl, 2-cyclohexymethyl, 2-benzyl, 2-phenyl, or 2-phenylethyl groups at position 2 of the benzoxazine nucleus. $I_{\text{max}}$ 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. $IC_{501}$ 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. $A_{\text{max2}}$ is the maximal activation 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 absence of drugs. $DE_{502}$ 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 $IC_{501}$, $DE_{501}$, and slopes of the concentration-response relationships of the compounds. (Fig. 3B; Tab. 2).

<table>
<thead>
<tr>
<th>Compounds</th>
<th>$I_{\text{max}}$</th>
<th>$A_{\text{max2}}$</th>
<th>$IC_{501}$</th>
<th>$DE_{502}$</th>
<th>$n$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Linear</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2-n-Hexyl</td>
<td>-85 ± 8$\dagger$</td>
<td>1.0 ± 0.1$ \times 10^{-8}$</td>
<td>-0.49 ± 0.02$\dagger$</td>
<td>-0.32 ± 0.03</td>
<td></td>
</tr>
<tr>
<td>2-n-Butyl</td>
<td>-62 ± 6$\dagger$</td>
<td>9.0 ± 3$ \times 10^{-8}$</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cyclic</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2-Cyclohexymethyl</td>
<td>-35 ± 2</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2-Benzyl</td>
<td>-5 ± 0.1</td>
<td>2.5 ± 2$ \times 10^{-11}$</td>
<td>-0.18 ± 0.09</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2-Phenyl</td>
<td>-98 ± 5$\dagger$</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Branched</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2-Isopropyl</td>
<td>+52 ± 5</td>
<td>6 ± 1$ \times 10^{-11}$</td>
<td>0.71 ± 0.1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2-Isobutyl</td>
<td>0</td>
<td>6 ± 1$ \times 10^{-11}$</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Symbols indicate 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-cyclic aromatic analogs data.
- §Significantly different from 2-cyclic analog data.
- †Significantly different from 2-isoalkyl analog data.
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 $A_{\text{max}}$ and $D_{E_{50}}$ 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 2H-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
the activating/blocking actions of these compounds. The strategy used was to replace the H atom at position 2 of the 2H-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\textsubscript{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\textsubscript{max} of the openers was 2-n-hexyl > 2-cyclohexylmethyl > 2-isopropyl = 2-n-butyl ≥ 2-phenyl ≥ 2-benzyl = 2-isobutyryl analogs.

In the absence of internal ATP, some of these compounds were effective as K\textsubscript{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\textsubscript{max} 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\textsubscript{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 2H-1,4-benzoxazine derivatives on the muscle K\textsubscript{ATP} channels. The effects of the 2-phenylethyl, 2-benzyl, 2-phenyl and 2-cyclohexylmethyl 1,4-benzoxazine derivatives were tested on K\textsubscript{ATP} currents recorded in the excised macropatches during voltage step going from 0 mV of holding potential to -60 mV (V\textsubscript{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\textsubscript{ATP} currents recorded in control condition (Control) in the presence of internal ATP (Control + ATP 10\textsuperscript{-4} M), in the presence of different concentrations of drug solutions enriched with ATP (10\textsuperscript{-4} M) or in the absence of nucleotide. A, in the presence of internal ATP all compounds, at 10\textsuperscript{-4} M concentration, enhanced the K\textsubscript{ATP} current in respect with the current levels recorded in the presence of ATP alone however with different efficacy. The 2-cyclohexylmethyl 1,4-benzoxazine was the most effective compound in activating the K\textsubscript{ATP} channel. At concentrations $>10$ $\mu$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\textsubscript{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\textsubscript{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\textsubscript{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 $A_{max}/I_{max}$ and $DE_{50}/IC_{50}$ 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 2H-1,4-benzoxazine derivatives can be achieved by replacing the linear alkyl chain with 2-branched alkyl chain substituents 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, which is inversely related to the effectiveness of the molecules as blockers/openers as observed with the 2-aryalkyl 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 2H-1,4-benzoxazine derivatives with the ATP and ADP molecules. Preliminary conformational analysis would indeed suggest that the planar area of the 2H-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$_{50}$ and IC$_{50}$ 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 lose 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 2H-1,4-benzoxazine derivatives may fit 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 2H-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 2H-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...
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 Laduniski, 1992; Bienenraeber 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 2H-1,4-benzoxazine derivatives observed in our experiments at micromolar concentrations.

We should stress that our experiments were performed on the cromakalin and glibenclamide-sensitive K<sub>ATP</sub> 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<sub>ATP</sub> 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 how-

scribed that channels showing these properties are composed by Kir6.2/SUR2, 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<sub>ATP</sub> 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 how-

References


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