Molecular Pharmacology Fast Forward. Published on April 10, 2008 as doi:10.1124/mol.108.046615
This article has not been copyedited and formatted. The final version may differ from this version.

MOL #46615

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

DT, AM, GMC, DCC Department of Pharmacobiology, Faculty of Pharmacy, via Orabona no. 4, University of Bari, I-70126 Bari, Italy and AL, GC, GF, PT Department of Medicinal Chemistry, Faculty of Pharmacy, via Orabona no. 4, University of Bari, I-70126 Bari, Italy

Molecular Pharmacology Fast Forward. Published on April 10, 2008 as DOI: 10.1124/mol.108.046615
This article has not been copyedited and formatted. The final version may differ from this version.

Running title: Potassium channel modulators and skeletal muscle

Corresponding author: Domenico Tricarico

address: Dept. of Pharmacobiology, Faculty of Pharmacy, via Orabona n° 4, Bari, Italy

FAX:00390805442801

phone:00390805442795

number of text page:31

number of table: 2

number of figure: 5

number of references: 26

number of words in the abstract: 249

number of words in introduction:733

number of words in the discussion:1468

List of nonstandard abbreviations used in the text:

KCO, potassium channel openers; K_{ATP}, ATP-sensitive K^+-channels; hypoPP, hypokalemic periodic paralysis; DE50, drug concentration needed to enhance the current by 50%; IC50, drug concentration needed to inhibit the current by 50%; MOPS, 3-(N-morpholino)propane-sulphonic acid; DMSO, dimethylsulphoxide; FDB, flexor digitorum brevis

Molecular Pharmacology Fast Forward. Published on April 10, 2008 as DOI: 10.1124/mol.108.046615
This article has not been copyedited and formatted. The final version may differ from this version.

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, while inhibiting it in the absence of nucleotide. To investigate on 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 analogue was the most potent activator (DE50=1.08x10^{-10}M) while 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 analogues. In the absence of ATP the 2-phenyl analogue was the most potent inhibitor(IC50=2.5x10^{-11}M), the rank order of efficacy of the blockers was:2-phenyl≥2-*n*-hexyl > 2-*n*-butyl > 2-cyclohexylmethyl while the 2-phenylethyl, 2-benzyl and 2-isobutyl 1,4-benzoxazine analogues were not effective; the 2-isopropyl analogue 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.

Molecular Pharmacology Fast Forward. Published on April 10, 2008 as DOI: 10.1124/mol.108.046615
This article has not been copyedited and formatted. The final version may differ from this version.

Introduction

Potassium channel openers (KCO) are chemically diverse compounds that belong to a number of structural classes which include 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, hypoglycaemia, neuromuscular disorders and some forms of epilepsy (Longman & Hamilton, 1992; Andersson, 1992). KCO drugs exert their effects on pancreatic beta cells, neurones, vascular and 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 contractility function in humans following neuromuscular blockers induced-paralysis(Saitho J, 2005). Pinacidil is capable to reduce the severity and frequency of paralysis in patients affected by hypokalemic periodic paralyses (hypoPP); however the patients suffer for the severe hypotension which limit its use in this muscle disorder (Links et al, 1992). Cromakalim is able to repolarize the insulin-depolarized muscle fibers of hypoPP patients "in vitro" as well as of K^+ depleted rats, an animal model of hypoPP, by opening the skeletal muscle K_{ATP}

Molecular Pharmacology Fast Forward. Published on April 10, 2008 as DOI: 10.1124/mol.108.046615
This article has not been copyedited and formatted. The final version may differ from this version.

channels(Tricarico et al., 1998; Tricarico et al., 1999). In some myotonic patients it is also able to suppress “in vitro” the abnormal hyper-excitability 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 be instead 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 (Conte Camerino et al., 2007).

Recent data show differences in the biophysical properties, subunits expression and drug-responses between muscles types and phenotypes which corroborate 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 previously shown 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

Molecular Pharmacology Fast Forward. Published on April 10, 2008 as DOI: 10.1124/mol.108.046615
This article has not been copyedited and formatted. The final version may differ from this version.

a 41% inhibition of the K_{ATP} channels at 10^{-4}M concentration which appears 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 behaviour: 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).

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 (FDB) muscles were dissected from male Wistar rats under urethane anaesthesia (1.2 g kg^{-1}). After dissection, the animals were rapidly killed with an overdose of urethane according to the *'Guide for Care and Use of Laboratory Animals'*

Molecular Pharmacology Fast Forward. Published on April 10, 2008 as DOI: 10.1124/mol.108.046615
This article has not been copyedited and formatted. The final version may differ from this version.

prepared by the National Academy of Sciences. Single muscle fibers were prepared by enzymatic dissociation (Tricarico *et al*., 1994).

Drugs and Solutions

The normal Ringer solution contained $145x10^{-3}$ M NaCl, $5x10^{-3}$ M KCl, $1x10^{-3}$ M $MgCl_2$, $0.5x10^{-3}$ M $CaCl_2$, $5x10^{-3}$ M glucose and $1x10^{-2}$ M 3-(N-morpholino)propane-sulphonic acid (MOPS), pH=7.2. The patch pipette solution contained $150x10^{-3}$ M KCl, $2x10^{-3}$ M $CaCl_2$ and $1x10^{-2}$ M MOPS, pH=7.2. The bath solution contained $150x10^{-3}$ M KCl, $5x10^{-3}$ M EGTA and $1x10^{-2}$ M MOPS, pH=7.2. Stock solution of $ATPK_2$ ($5x10^{-3}$ M) was prepared by dissolving the chemical in the bath solution.

Stock solutions of the 2*H*-1,4-benzoxazine derivatives ($30x10^{-3}$M), cromakalim, tolbutamide and glibenclamide ($20x10^{-3}$M) (SIGMA, Mi) were prepared by dissolving the compounds in dimethylsulphoxide (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 $1x10^{-4}$ M (DMSO 0.33 %). Due to the low solubility of these compounds in the aqueous solvent the drugs were tested at concentrations < $1x10^{-4}$ 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 affects 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 (Tricarico et al., 2003; Russ 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-2H-1,4-benzoxazine-3-one which was

Molecular Pharmacology Fast Forward. Published on April 10, 2008 as DOI: 10.1124/mol.108.046615
This article has not been copyedited and formatted. The final version may differ from this version.

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**). Also 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 (benzoxazinones 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 (benzoxazinones 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 name of the 2H-1,4-benzoxazine derivatives **2**, **3**, **4**, **5**, **6**, **7**, **8** and **9** (Fig. 1) were : (R/S)-6-chloro-2-butyl-3-(pyridin-3-yl-amino)-2H-1,4-benzoxazine, (R/S)-6-chloro-2-hexyl-3-(pyridin-3-yl-amino)-2H-1,4-benzoxazine, (R/S)-6-chloro-2-isopropyl-3-(pyridin-3-yl-amino)-2H-1,4-benzoxazine, (R/S)-6-chloro-2-isobutyl-3-(pyridin-3-yl-amino)-2H-1,4-benzoxazine, (R/S)-6-chloro-2-cyclohexylmethyl-3-(pyridin-3-yl-amino)-2H-1,4-benzoxazine, (R/S)-6-chloro-2-benzyl-3-(pyridin-3-yl-amino)-2H-1,4-benzoxazine, (R/S)-6-chloro-2-2-(phenyl)ethyl-3-(pyridin-3-yl-amino)-2H-1,4-benzoxazine, and (R/S)-6-chloro-2-phenyl-3-(pyridin-3-yl-amino)-2H-1,4-benzoxazine, respectively.

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

Molecular Pharmacology Fast Forward. Published on April 10, 2008 as DOI: 10.1124/mol.108.046615
This article has not been copyedited and formatted. The final version may differ from this version.

going from 0 mV of holding potential to -60 mV immediately after excision, at 20°C, in the presence of 150 $x10^{-3}$ 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 (N° of macropatches= 211) were recorded at 1kHz sampling rates (filter = 0.2 kHz) using an Axopatch-1D amplifier equipped with a CV-4 headstage (Axon Instruments, Foster City, CA, U.S.A.). Pipettes having a resistance of 1±0.2 MΩ (N° 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 base-line level of the currents from the open channel level. The base-line level for the K_{ATP} current was measured in the presence of ATP ($5x10^{-3}$ M). Macropatches containing voltage-dependent channels or inward rectifier K^+ channels were excluded from the analysis. Current amplitude was measured using the Clampfit program (Axon Instruments, Foster City, CA, U.S.A.). No correction for liquid junction potential was made, estimated to be < 1.9 mV in our experimental conditions.

Concentration-response relationships were constructed as previously described (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 the 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 than exposed to solutions enriched with the Kir6.2/SUR1 selective blocker tolbutamide (1.5 mM) and/or to glibenclamide (100 nM), an unselective channel blocker. Drug solutions were applied to the patches by using the ValveLink8 fast perfusion system (AutoMate

Molecular Pharmacology Fast Forward. Published on April 10, 2008 as DOI: 10.1124/mol.108.046615
This article has not been copyedited and formatted. The final version may differ from this version.

Scientific, U.S.A.). 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 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 MMFF (Merck Molecular Force Field) conformational analysis as previously described (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; 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, 2005-2006) (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 lower to allow the inter-conversion 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).

Molecular Pharmacology Fast Forward. Published on April 10, 2008 as DOI: 10.1124/mol.108.046615
This article has not been copyedited and formatted. The final version may differ from this version.

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). While 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

$$(I_{drug+ATP}\text{-}1) * 100 = Amax/(1+(DE50/[Drug])^{n}) \quad (1)$$

while the inhibitory component can be described by the term

$$(I_{drug}\text{-}1) * 100 = Imax/(1+([Drug]/IC50)^{n}) \quad (2)$$

For equation (1), $I_{drug+ATP}$ is the K_{ATP} currents 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; Amax is the per cent maximal activation of the K_{ATP} currents produced by the molecules under study; DE50 is the concentration of the drug needed to enhance the current by 50%. For equation (2), I_{drug} is the K_{ATP} currents measured in the presence of the molecules under study, in the absence of ATP (controls) and normalized to that in the absence of drugs; Imax is the per cent maximal inhibition of the K_{ATP} currents produced by the molecules under study; IC50 is the concentration of the drug needed to reduce the current by 50%; [Drug] is the concentration of the drug tested; *n* is the slope factor of the curves calculated in the presence (equation 1) or absence (equation 2) 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(Germany). The data are expressed as mean±standard error unless otherwise specified. The unpaired T test was used to compare the best fit values pooled from the concentration-response

Molecular Pharmacology Fast Forward. Published on April 10, 2008 as DOI: 10.1124/mol.108.046615
This article has not been copyedited and formatted. The final version may differ from this version.

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(Vm) 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 in respect with 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 analogue **2** producing a significant enhancement of the K_{ATP} current as determined by T test. The calculation of the parameters of the equation (1) by fitting routine showed that the rank order of efficacy of the compounds as openers expressed as $Amax_1$ was: 2-*n*-hexyl>2-*n*-butyl, while the $DE50_1$ and slopes values were not statistically different (Tab.1). The 2-*n*-hexyl analogue was significantly more effective and potent than the 2-branched alkyl chain and 2-cyclic aromatic analogues in activating the K_{ATP} channels (Tab. 1). As previously observed with other 2-linear alkyl chain structurally related analogues, 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) (Tab. 1) (Tricarico et al., 2003).

Molecular Pharmacology Fast Forward. Published on April 10, 2008 as DOI: 10.1124/mol.108.046615
This article has not been copyedited and formatted. The final version may differ from this version.

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 equation (2) by fitting routine showed that the rank order of efficacy and potency of the blockers expressed as $Imax_2$ and $IC50_2$ was: 2– *n*-hexyl > 2– *n*-butyl, while no differences were observed in the relative slopes of the concentration-response curves (Tab.2). The 2-linear alkyl chain analogues were also significantly more effective and potent as blockers than the 2-branched alkyl chain, 2-cyclohexylmethyl, 2-benzyl and 2-phenylethyl analogues (Tab. 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 2-isopropyl-1,4-benzoxazine **4** and of 2-isobutyl-1,4-benzoxazine derivatives **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 **4** was significantly more effective in activating the K_{ATP} channels than its analogue **5**. The calculation of the parameters of the equation (1) by fitting routine showed that the rank order of efficacy of the compounds as openers expressed as Amax1 was: 2-isopropyl> 2-isobutyl, while no differences were observed in $DE50_1$ and slope of the concentration-response curves for these compounds (Tab.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-5x10^{-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

Molecular Pharmacology Fast Forward. Published on April 10, 2008 as DOI: 10.1124/mol.108.046615
This article has not been copyedited and formatted. The final version may differ from this version.

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, while the 2-isobutyl analogue **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 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 in respect with 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 analogues in enhancing the K_{ATP} current, while the 2-phenylethyl-1,4-benzoxazine derivative **8** was the less effective compound (Fig 4A). The calculation of the parameters of the equation (1) showed that the rank order of efficacy and potency of the openers expressed as $Amax_1$ and $DE50_1$ was: 2-cyclohexylmethyl>2-phenyl $\underline{\geq}$ 2-benzyl. No differences were observed in the slopes of the concentration-response curves for these compounds (Tab.1). The 2-cyclohexylmethyl analogue was also significantly more effective and potent than the 2-branched alkyl chain analogues (Tab. 1). Slight inhibitory responses have been observed with these compounds at concentrations >10^{-7}M (Fig. 4A; Tab.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 K_{ATP} channel blocker than its analogues that did not produce more than 35% inhibition of the channel currents (Fig. 4B; Tab.2). This compound was also more potent and effective than

Molecular Pharmacology Fast Forward. Published on April 10, 2008 as DOI: 10.1124/mol.108.046615
This article has not been copyedited and formatted. The final version may differ from this version.

the 2-linear and 2-branched alkyl chain analogues (Tab. 2).

Discussion

In the present work, several new molecules belonging to the class of 2*H*-1,4-benzoxazine derivatives were synthesised and tested against the native skeletal muscle K_{ATP} channels at aim to investigate on 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 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 most effective compounds in 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 analogue **2** with shorter linear alkyl chain, the 2-branched alkyl chain analogues **4** and **5,** and the 2-cyclic aromatic analogues **7** and **9**; while the 2-phenylethyl analogue **8** was not effective. In conclusion, the rank order of efficacy expressed as $Amax_1$ of the openers was: 2-*n*-hexyl≥2-cyclohexylmethyl>2-isopropyl=2-*n*-butyl≥2-phenyl≥2-benzyl=2-isobutyl analogues.

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** analogues were the most effective and potent compounds in respect to the other structurally related analogues. The rank order of efficacy of the blockers expressed as $Imax_2$ was: 2-phenyl≥2-*n*-hexyl > 2-*n*-butyl > 2-cyclohexylmethyl while the 2-arylalkyl analogues **7** and **8,** and 2-branched alkyl chain analogues **4** and **5** were not effective as

Molecular Pharmacology Fast Forward. Published on April 10, 2008 as DOI: 10.1124/mol.108.046615
This article has not been copyedited and formatted. The final version may differ from this version.

blockers. Surprisingly, the 2-isopropyl analogue **4** was capable to open the K_{ATP} channels even in the absence of nucleotide.

These findings indicate that: first, the lengthening of the 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 $Amax_1/Imax_2$ and $DE50_1/IC50_{1\text{-}2}$ ratios which are close to the unity for both 2-*n*-hexyl and 2-*n*-butyl analogues **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 analogues **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 analogue **9**. An additional factor is the intra-molecular distance of the substitutes from the 1,4-benzoxazine nucleus which is inversely related with the effectiveness of the molecules as blockers/openers as observed with the 2-arylalkyl analogues **7** and **8** and with the 2-branched alkyl chain analogue **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 folds (NBD1 and NBD2) of the K_{ATP} channel complexes (Haider et al., 2007; Nichols, 2006). 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

Molecular Pharmacology Fast Forward. Published on April 10, 2008 as DOI: 10.1124/mol.108.046615
This article has not been copyedited and formatted. The final version may differ from this version.

of the 2*H*-1,4-benzoxazines overlaps with that of the adenine nucleotide tri and di-phosphates. 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 analogues, and particularly with the 2-*n*-hexyl analogue **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 $IC50_1$ and $IC50_2$ of the 2-*n*-hexyl and 2-*n*-butyl analogues **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 to inhibit the truncated Kir6.2 channel expressed in Hek293 cell line (Rolland et al., 2006). Conformational analysis also show that molecules with a reduced 3D area of superimposition with the nucleotide may loose the capability to inhibit the Kir6.2 subunit as in the case of the 2-isopropyl analogue **4,** or may show a different inhibitory profile as in the case of the 2-phenyl analogue **9** which potently inhibit the K_{ATP} channel however 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 an hydrophobic pocket at the interface between the N and C domain of the Kir6.2 (Nichols, 2006). While the substitutes at the position 2 of the 1,4-benzoxazine nucleus could adopt conformations similar with that of the phosphate group of nucleotides determining the actions of these compounds as blockers and openers.

Molecular Pharmacology Fast Forward. Published on April 10, 2008 as DOI: 10.1124/mol.108.046615
This article has not been copyedited and formatted. The final version may differ from this version.

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 to activate 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 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; Teramoto et al., 2001; Bienengraeber et al., 2000; Tricarico et al, 2003; Russ et al., 2003; Alekseev et al., 2005). The direct interaction 2-*n*-hexyl and 2-*n*-butyl analogues **3** and **2** with the inhibitory site possibly located on Kir6.2 would explains 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

Molecular Pharmacology Fast Forward. Published on April 10, 2008 as DOI: 10.1124/mol.108.046615
This article has not been copyedited and formatted. The final version may differ from this version.

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 an 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 appear 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 analogue **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 analogues 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; since deficiency of skeletal muscle K_{ATP} channels is associated with reduced muscle contractility in the rat (Saitho J, 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).

Molecular Pharmacology Fast Forward. Published on April 10, 2008 as DOI: 10.1124/mol.108.046615
This article has not been copyedited and formatted. The final version may differ from this version.

References

Alekseev AE, Hodgson DM, Karger AB, Park S, Zingman LV and Terzic A (2005) ATP-sensitive K^+ channel channel/enzyme multimer: Metabolic gating in the heart. *J Mol Cell Cardiol* **38:** 895–905.

Allard B and Lazdunski M (1992) Nucleotides diphosphates activate the ATP-sensitive potassium channels in mouse skeletal muscle. *Pflugers Arch*. **422:** 185 – 192.

Andersson KE (1992). Clinical pharmacology of potassium channel openers. *Pharmacol. Toxicol.* **70:** 244 – 254.

Ashcroft FM (2006) From molecule to malady. *Nature* **440:** 440-447. Review.

Bienengraeber M, Alexey E, Alekseev M, Abraham R, Carrasco AJ, Moreau C, Vivaudou M, Dzeja P P and Terzic A (2000) ATPase activity of the sulfonylurea receptor: a catalytic function for the KATP channel complex. *The FASEB J* **14:**1943-1952.

Conte Camerino D, Tricarico D and Desaphy JF (2007) Ion channel pharmacology. *Neurotherapeutics.* **4(2)**:184-98 Review.

Cecchetti V, Tabarrini O and Sabatini S (2006) From cromakalim to different structural classes of K_{ATP} channel openers. *Curr Top Med Chem* 6:1049-1068.

Cifelli C, Bourassa F, Gariepy L, Banas K, Benkhalti M, and Renaud JM (2007) KATP channel deficiency in mouse flexor digitorum brevis causes fibre damage and impairs Ca^{2+} release and force development during fatigue *in vitro. J Physiol* **582(2) :** 843–857

Flechtner I, De Lonlay P, and Polak M (2006) Diabetes and hypoglycaemia in young children and mutations in the Kir6.2 subunit of the potassium channel: therapeutic consequences. *Diabetes Metab* **32(6):**569-80

Haider S, Syma Khalid S, Tucker SJ, Ashcroft FM and Sansom MSP (2007) Molecular Dynamics Simulations of Inwardly Rectifying (Kir) Potassium Channels: A Comparative Study. *Biochemistry* **46:** 3643-3652.

Jahangir A and Terzic A (2005) KATP channel therapeutics at the bedside.
J Mol Cell Cardiol **39:** 99–112.

Koster JC, Remedi MS, Dao C and Nichols C G (2005) ATP and Sulfonylurea Sensitivity of Mutant ATP-Sensitive K^+ Channels in Neonatal Diabetes. Implications for Pharmacogenomic Therapy. *Diabetes* **54:**2645–2654

Links T, Smit A J, and Oosterhuis, HJGH (1992) A pilot study with pinacidil, an opener of the ATP-sensitive potassium channels in hypokalemic periodic paralysis. *In: Familial Hypokalemic Periodic Paralysis.* ed. Haren & Drukkerij Niemeijer, pp. 149 – 151.

Longman S D and Hamilton T C (1992) Potassium channel activator drugs: mechanism of action, pharmacological properties, and therapeutic potential. *Med Res Rev* **12:** 73 – 148.

Lu T, Hong M and Lee H (2005) Molecular Determinants of Cardiac KATP Channel Activation by Epoxyeicosatrienoic Acids. *J Biol Chem*. **280/19** :19097–19104

Mannhold R(2004) KATP Channel Structure-Activity Relationships and Therapeutic Potential. *Med Res Rev* **24/2:** 213-266.

Nichols CG (2006) KATP channels as molecular sensors of cellular metabolism. *Nature.* **440(7083):** 470-476. Review.

Rolland JF, Tricarico D, Laghezza A, Loiodice F, Tortorella V and Camerino DC.(2006) A new benzoxazine compound blocks KATP channels in pancreatic beta cells: molecular basis for tissue selectivity in vitro and hypoglycaemic action in vivo. *Br J Pharmacol.* **149(7)**:870-9. Epub 2006 Oct 23.

Rovati E G, Nicosia S (1994) Lower efficacy: interaction with an inhibitory receptor or partial agonism? *TiPS* **15:** 141 – 144.

Teramoto N, Yunoki T, Takano M, Yonemitsu Y, Masaki I, Sieishi K, Brading A F and Ito Y (2001) Dual action of SZ6169, a novel K^+ channel opener, on ATP-sensitive K^+ channels in pig urethral myocytes *Br J Pharmacol* **133:**154-164.

Tricarico D, Barbieri M, Laghezza A, Tortorella P, Loiodice F and Conte Camerino D(2003) Dualistic actions of cromakalim and new potent 2H-1,4-benzoxazine derivatives on the native skeletal muscle KATP channel. *Br J Pharmacol* **139(2)**:255-62.

Tricarico D, Barbieri M, Mele A, Carbonara G, and Camerino DC (2004) Carbonic anhydrase inhibitors are specific openers of skeletal muscle BK channel of K^+-deficient rats. *FASEB J.* **18(6):**760-1. Epub 2004 Feb 6.

Tricarico D, Mele A, Lundquist AL, Desai RR, George, Jr. AL and Conte Camerino(2006) Hybrid assemblies of ATP-sensitive K^+ channels determine their muscle-type-dependent biophysical and pharmacological properties. *Proc Natl Acad Sci U S A* **103(4)**:1118-23. Epub 2006 Jan 17.

Ulrich Russ U, Lange H, Lo¨ ffler-Walz C, Hambrock A and Quast U (2003) Binding and effect of KATP channel openers in the absence of Mg^{2+}. *Br J of Pharmacol.* **139:** 368–380.

Quastoff S, Spuler A, Spittelmeister W, Lehmann-Horn F and Grafe P (1990) K^+ channel openers suppress myotonic activity of human skeletal muscle in vitro. *Eur J Pharmacol* **186:** 125 – 128.

Yuhji Saitoh (2005) Drugs to facilitate recovery of neuromuscular blockade and muscle strength. *J Anesth* **19:**302–308.

Acknowledgments

This work was supported by Telethon Grant GGP04140

Molecular Pharmacology Fast Forward. Published on April 10, 2008 as DOI: 10.1124/mol.108.046615
This article has not been copyedited and formatted. The final version may differ from this version.

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

Figure 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 (Vm), 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 ± SE of the % activation or inhibition of the K_{ATP} currents vs the drug concentrations of a minimum of three and a

Molecular Pharmacology Fast Forward. Published on April 10, 2008 as DOI: 10.1124/mol.108.046615
This article has not been copyedited and formatted. The final version may differ from this version.

maximum of six macropatches.

Figure 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 (Vm), 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^{-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, while the 2-isobutyl 1,4-benzoxazine derivative did not affects the K_{ATP} current. Concentration-response data of the 2-isopropyl analogue were fitted by a single stimulatory site function at concentration (continuous lines). Each experimental point represents the mean ± SE of the % activation of the K_{ATP} currents vs the drug concentrations of a minimum of three and a maximum of six macropatches.

Figure 4 Effects of the introduction of the aliphatic or aromatic rings at position 2 of the

Molecular Pharmacology Fast Forward. Published on April 10, 2008 as DOI: 10.1124/mol.108.046615
This article has not been copyedited and formatted. The final version may differ from this version.

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 (Vm), 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 analogues. 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 ± SE of the % activation or inhibition of the K_{ATP} channels vs the drug concentrations of a minimum of three and a maximum of six macropatches.

Figure 5 Conformational analysis of the 2*H*-1,4-benzoxazine derivatives and comparison

Molecular Pharmacology Fast Forward. Published on April 10, 2008 as DOI: 10.1124/mol.108.046615
This article has not been copyedited and formatted. The final version may differ from this version.

with nucleotide tri-phosphate. (**A, 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 2-3 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 inter-conversion 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 color), 2-phenyl (green color), and of the 2-isopropyl(red color) 1,4-benzoxazine derivatives matches with that of the adenine ring of the ATP(violet color) molecule. In the case of 2-hexyl and 2-isopropyl analogues the 3-amino-pyridine substitute overlaps with the ribose ring of the nucleotide, however in the 2-phenyl analogue **9** does not. Moreover, the 2-linear alkyl chain of the 2-*n*-hexyl analogue **3** may occupies the same area of the phosphate group of ATP, while this is not observed for the substitutes of the 2-phenyl and 2-isopropyl analogues **9** and **4**. Therefore the best molecular overlay is observed with the 2-*n*-hexyl analogue **3** followed by the 2-isopropyl and 2-phenyl analogues **4** and **9**.

Molecular Pharmacology Fast Forward. Published on April 10, 2008 as DOI: 10.1124/mol.108.046615
This article has not been copyedited and formatted. The final version may differ from this version.

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

Compounds	$Amax_1$%	$DE50_1$ (M)	n	$Imax_1$ %	$IC50_1$ (M)	n
Linear						
2-*n*-hexyl	+105±2 * □ °	$1.08\pm0.99\times10^{-10}$ □ °	0.93±0.1□	-95±9 ▼□	$4.1\pm3\times10^{-8}$▼□	-0.5±0.1
2-*n*-butyl	+75±2	$3.24\pm0.9\times10^{-10}$	0.9±0.2	-97±6 ▼	$8.0\pm1\times10^{-8}$▼	-0.6±0.1
Cyclic						
2-cyclohexylmethyl	+88±5 ° □	$1.81\pm0.9\times10^{-10}$ ° □	0.99±0.1°	-30±2° □	/	/
2-benzyl	+59±2	$7.21\pm0.1\times10^{-10}$	0.95±0.1	-26±5	/	/
2-phenyl	+69±1	$3.92\pm5\times10^{-10}$	0.96±0.1	-24±3	/	/
2-phenylethyl	+10±3	/	/	/	/	/
Branched						
2-isopropyl	+76±0.55 Δ	$1.9\pm0.9\times10^{-9}$	0.71±0.1	-21±5	/	/
2-isobutyl	+58±0.5	$3.8\pm0.7\times10^{-9}$	0.72±0.3	-19±6	/	/

Table 1 The parameters reported in the table were calculated by using the fitting routine based on the sum of the term 1 and 2 as described in the 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. $Amax_1$ is the per cent maximal activation of the K_{ATP} currents produced by the molecules under study and it is calculated in respect to the current levels measured in the presence of ATP (10^{-4}M). $DE50_1$ is the concentration of the drug needed to enhance the current by 50% calculated in respect to the maximal activation produced by

Molecular Pharmacology Fast Forward. Published on April 10, 2008 as DOI: 10.1124/mol.108.046615
This article has not been copyedited and formatted. The final version may differ from this version.

the compounds in the presence of internal ATP. $Imax_1$ is the per cent maximal inhibition of the K_{ATP} currents produced by the molecules under study and it is calculated in respect to the maximal current levels measured in the presence of drugs + ATP. $IC50_1$ is the concentration of the drug needed to reduce the current by 50%, calculated in respect to the maximal inhibition produced by the molecules; n is slope factor of the concentration-response relationships. Symbols indicates data significantly different for $p<0.05$ as determined by unpaired T test: * significantly different from 2-*n*-butyl analogue data; □ significantly different from 2-branched analogue data; ▼ significantly different from 2-cyclic analogue data; ° significantly different from 2-cyclic aromatic analogues data; Δ significantly different from 2-isobutyl analogue data. In same cases, the reduced drug-responses and the low number of data points did not allow to evaluate the plateau phase and to calculate the $IC50_1$, $DE50_1$ and slopes of the concentration-response relationships of the compounds.

Molecular Pharmacology Fast Forward. Published on April 10, 2008 as DOI: 10.1124/mol.108.046615
This article has not been copyedited and formatted. The final version may differ from this version.

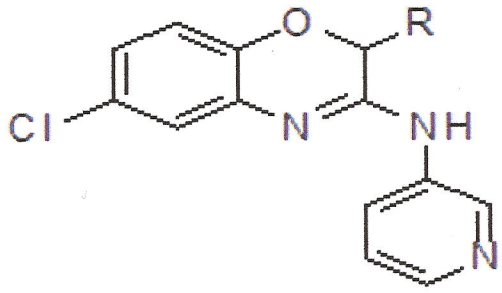
Table 2 Fitting parameters of the concentration–response curves of 2*H*-1,4-benzoxazine derivatives versus the skeletal muscle K_{ATP} currents in the absence of ATP

Compounds	$Imax_2$%	$IC50_2$ (M)	n
Linear			
2-*n*-hexyl *	-85±8 * ▼	$1.0±0.1x10^{-8}$ * ▼ °	-0.49±0.02*
2-*n*-butyl	-62±6* ▼	$9.0±3x10^{-8}$ ▼	-0.32±0.03
Cyclic			
2-cyclohexylmethyl	-35±2	/	/
2-benzyl	-5±0.1	/	/
2-phenyl	-98±5 □	$2.5±2x10^{-11}$ ■	-0.18±0.09
2-phenylethyl	-15±3	/	/
	$Amax_2$%	$DE50_2$ (M)	n
Branched			
2-isopropyl	+52±5	$6±1x10^{-11}$	0.71±0.1
2-isobutyl	0	/	/

Table 2 The parameters reported in the table were calculated by using the fitting routine based on the equation 2 as described in the 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. $Imax_2$ is the per cent maximal inhibition of the K_{ATP} currents produced by the molecules under study and it is calculated in respect to the maximal current levels measured in the presence of drugs. $IC50_2$ is the concentration of the drug needed to reduce the current by 50%, calculated in respect to the maximal inhibition

Molecular Pharmacology Fast Forward. Published on April 10, 2008 as DOI: 10.1124/mol.108.046615
This article has not been copyedited and formatted. The final version may differ from this version.

produced by the molecules; n is slope factor of the concentration-response relationships. $Amax_2$ is the per cent maximal activation of the K_{ATP} currents produced by the molecules under study. $DE50_2$ is the concentration of the drug needed to enhance the current by 50% calculated in respect to the maximal activation produced by the compounds. 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 analogues data; □ significantly different from 2-cyclic analogue data, ■ significantly different from 2-linear alkyl chain analogue. In same cases, the reduced drug-responses and the low number of data points did not allow to evaluate the plateau phase and to calculate the $IC50_1$, and slopes of the concentration-response relationships of the compounds.



2H-1,4 benzoxazine nucleus

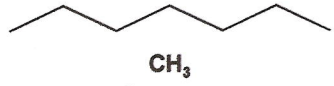
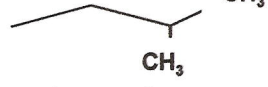
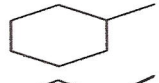
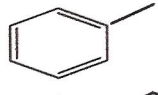
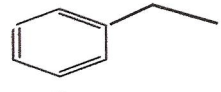
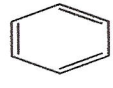
Compound	R	
1	*n*-propyl	
2	*n*-butyl	
3	*n*-hexyl	
4	*i*-propyl	
5	*i*-butyl	
6	cyclohexylmethyl	
7	benzyl	
8	2-(phenyl)ethyl	
9	phenyl	

Figure 1

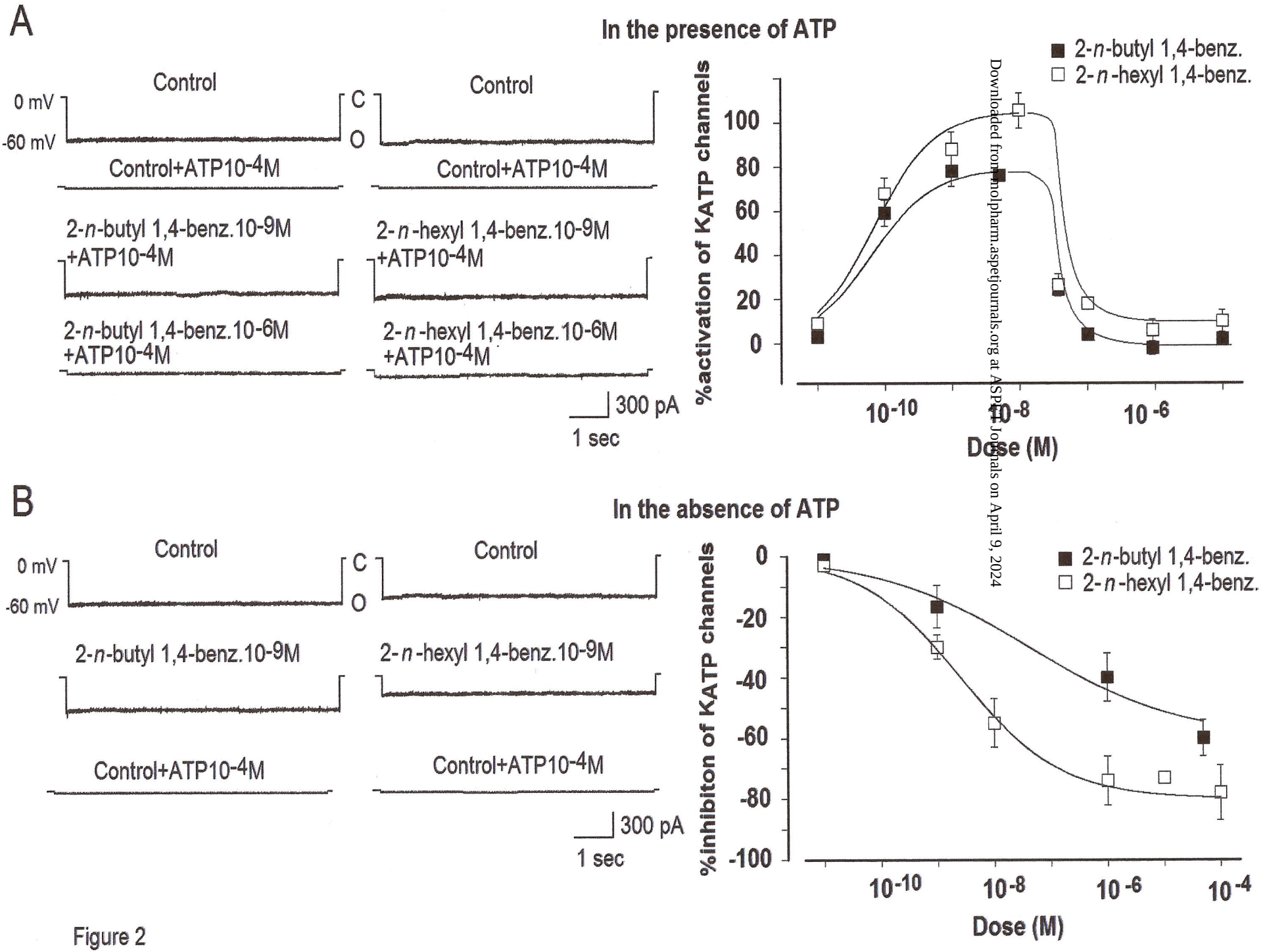


Figure 2

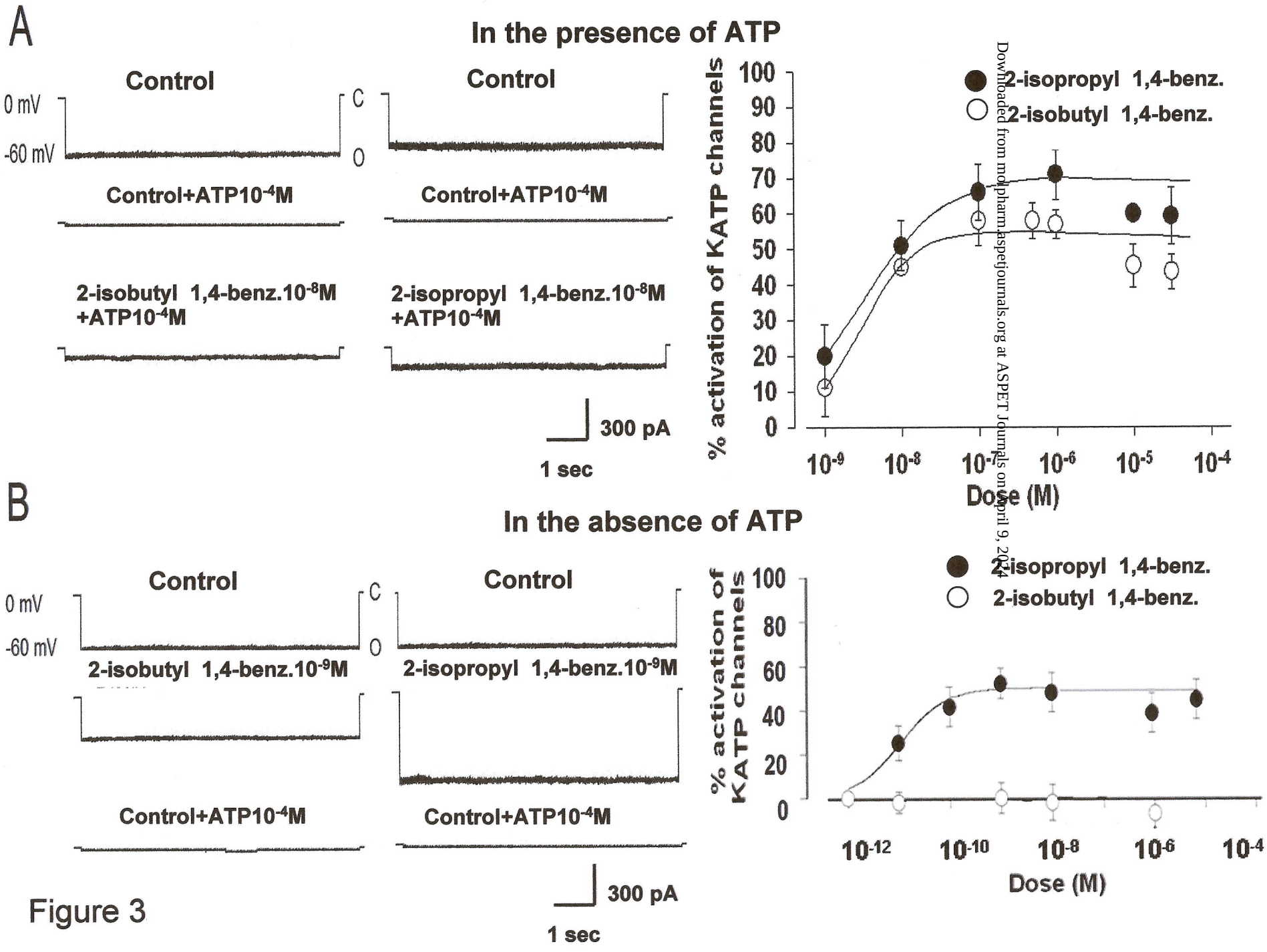


Figure 3

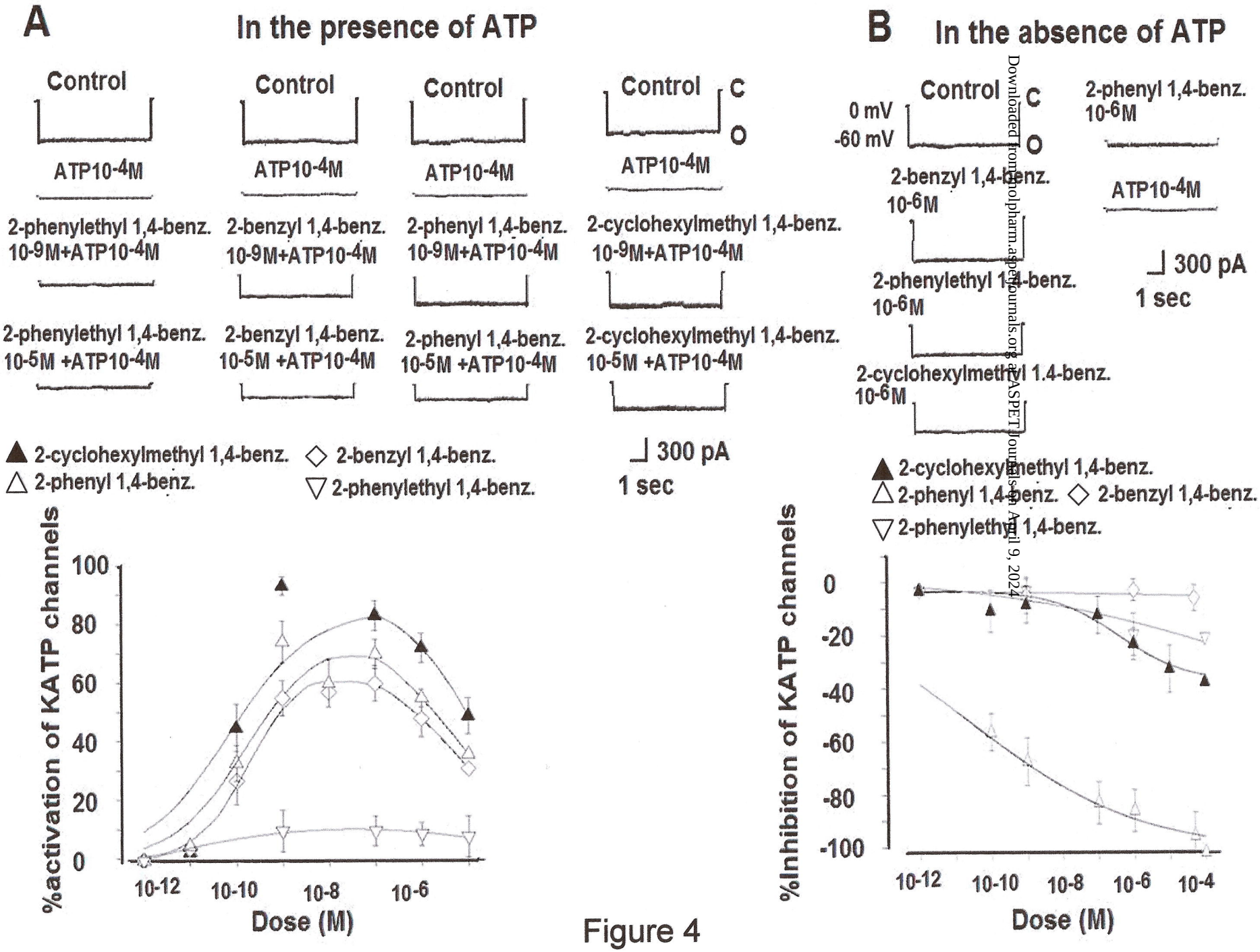


Figure 4

Figure 5