New Inhibitors of the FVB2 Subunit from Mammalian Kv1 Potassium Channels

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Short communication

New inhibitors of the Kvβ2 subunit from mammalian Kv1 potassium channels

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ABSTRACT

The role of the redox state of Kvβ subunits in the modulation of Kv1 potassium channels has been well documented over the past few years. It has been suggested that a molecule that binds to or inhibits the aldol–ketoreductase activity of Kvβ might affect the modulation of channel properties. Previous studies of possible modulators of channel activity have shown that cortisone and some related compounds are able to physically dissociate the channel components by binding to a site at the interface between α and β subunits. Herein, we describe some new inhibitors of rat brain Kvβ2, identified using an assay based on multiple substrate turnover. This approach allows one to focus on molecules that specifically block NADPH oxidation. These studies showed that, at 0.5 mM, 3,4-dihydroxyphenylacetic acid (DOPAC) was an inhibitor of Kvβ2 turnover yielding a ~40–50% reduction in the aldehyde reductase activity of this subunit. Other significant inhibitors include the bioflavonoid, rutin and the polyphenol resveratrol; some of the known cardioprotective effects of these molecules may be attributable to Kv1 channel modulation. Cortisone or catechol caused moderate inhibition of Kvβ2 turnover, and the aldol-keto reductases inhibitor valproate had an even smaller effect.

Despite the importance of the Kv1 channels in a number of disease states, there have been few Kvβ2 inhibitors reported. While the ones identified in this study are only effective at high concentrations, they could serve as tools to decipher the role of Kvβ2 in vivo and, eventually, inform the development of novel therapeutics.

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1. Introduction

The Shaker voltage-gated K+ channels (Kv1 superfamily) control the efflux of K⁺ through cell membranes and, thereby, dampen membrane excitability. An auxiliary β-subunit (Kvβ), associated with the cytoplasmic face of Kv proteins (Dolly et al., 1994; Parcej et al., 1992; Scott et al., 1994) has sequence homology with aldol–ketoreductase (AKR) enzymes (Gulbis et al., 1999; Long et al., 2005).

The catalytic C-terminal of Kvβ2 has a tightly, but non-covalently, bound nicotinamide (NADPH) cofactor. Most significantly, Kvβ is known to modulate the channel’s activity in a manner that depends on the redox state of the bound cofactor. This mechanism is thought to proceed via redox dependent conformational changes (Weng et al., 2006; Pan et al., 2008a, 2011). The Kvβ subunit is catalytically active as an aldol-keto reductases being able to reversibly oxidise a range of alcohols to their corresponding aldehydes (Alka et al., 2010; Tipparaju et al., 2008).

Allelic deletion of Kvβ is directly linked to severe epilepsy including infantile spasm (Heilstredt et al., 2001) while loss-of-function mutations in pore forming α-subunits are associated with convulsive disorders (Adelman et al., 1995; Guan et al., 2006; Imbrici et al., 2006, 2007). It has been suggested that inhibitors of the action of Kvβ might prove useful as therapeutics (Pan et al., 2008a, 2012). Despite this, there have been few reports of compounds that might inhibit Kvβ.

In 2008, Pan et al., screened a number of potential Kvβ inhibitors and showed that cortisone can bind to Kvβ proteins at two different sites: one close to the enzyme bound NADPH and another at the interface between Kvβ and Kv1 α subunits (Pan et al., 2008a). They further showed that cortisone promotes dissociation of Kvβ from Kv1 subunits, a finding that focused attention on inhibitors that might target the interface between subunits in Kv1 channels. Subsequently, these authors tested 25 cortisone analogues for their ability to potentiate Kv1 channels by binding to Kvβ and promoting its dissociation (Pan et al., 2012). Their work identified fluticasone as a potential lead for designing more efficient small molecules that might influence channel function by dissociating the Kv1–Kvβ assembly. Nevertheless, there is little or no
information on molecules, other than substrates and corticosteroids, that can bind to the active site of the Kvβ subunit. Predicting inhibitors for this protein is difficult since even studies on substrate specificity have proven somewhat confusing (Alka et al., 2010; Tipparaju et al., 2008).

Herein, the interactions of a variety of small molecules with the active site of Kvβ2 were probed. The tightly bound NADPH on Kvβ2 may dissociate and allow measurement of multiple turnovers (Weng et al., 2006). We exploit this activity to screen for Kvβ2 inhibitors that have a direct influence on substrate turnover. This approach utilised a simple HPLC-based assay to monitor the Kvβ2-catalysed reduction of 4-nitrobenzaldehyde (see Alka et al., 2010). This assay was used for screening of small molecule inhibitors that might bind to Kvβ2 and inhibit the redox reaction. In this way, inhibitors were sought for NADPH oxidation rather than modulators of channel function, since this oxidation step is known to influence channel activity.

2. Materials and methods

2.1. Overexpression and purification of Kvβ2

The rat brain Kvβ2 protein was overexpressed in, and purified from, *Escherichia coli* BL21 (DE3, pLysS) cells transformed with the pET15b-Kvβ2 construct containing an N-terminal His-tag, described in Alka et al., 2010. The purified protein was dialysed against 2 L of prechilled 0.2 M potassium phosphate, pH 7.5 for 36 h with three changes.

2.2. HPLC assay for measuring the inhibition of Kvβ2-mediated 4-nitrobenzaldehyde reduction

Two different HPLC separation methods (Isocratic and Gradient) were used to carry out the inhibition studies for the Kvβ2 mediated-reduction of 4-nitrobenzaldehyde. The one chosen depended on the effective separation of the inhibitor from components of the reaction mixture. All inhibition studies were carried out in duplicate in 0.2 M potassium phosphate buffer, pH 7.5 containing 0.2 mM NADPH at 37 °C in the presence of ~0.5 mg of Kvβ2, an appropriate concentration of each test inhibitor and 500 μM of 4-nitrobenzaldehyde as substrate (final volume of 0.25 ml). A 100 mM stock solution of all inhibitors was prepared in DMSO. The concentration of DMSO in the assay mixture was less than or equal to 1% (v/v). DMSO at this concentration was found to have no effect on the 4-nitrobenzaldehyde reductase activity of Kvβ2. The inhibitor was incubated with the enzyme for 20 min before the addition of substrate, which was added last, and the reaction further incubated for 30 min at 22 °C before quenching by adding an equal volume of the HPLC mobile phase consisting of methanol/trifluoroacetic acid/water (60: 0.1: 39.9, v/v/v). Aliquots, (10 μl), of the resultant mixture were analysed on a Nucleosil C18 (3.9 × 150 mm) HPLC column, using Waters 2695 family of Separations modules (Waters Corporation, Milford, USA) with monitoring by a Waters 2998 photodiode array detector at 274 nm. The column temperature was 37 °C. Controls without enzyme were used to monitor any background reaction between the inhibitor and the substrate 4-nitrobenzaldehyde.

2.2.1. Gradient HPLC assay

Solvent A was a mixture of methanol/trifluoroacetic acid/water (60: 0.1: 39.9) while solvent B comprised methanol/trifluoroacetic acid/water (20: 0.1: 79.9). The following linear gradient was used for the separation: 20–100% B (15 min), 100% B (5 min) and 100–20% B (0.1 min) at 0.9 ml/min. The run time was 20 min. Standard curves constructed using each method separately for 4-nitrobenzaldehyde and 4-nitrobenzyl alcohol were used to determine concentrations of product in each of the reaction mixtures.

2.2.2. Isocratic elution method

Solvent A (100%) was used as mobile phase with a total acquisition time of 10 min for the isocratic elution method while the procedure for determination of product concentration was the same as described above (Section 2.2.1).

2.3. Fluorescence measurement of inhibitor binding to Kvβ2

Fluorescence scans of Kvβ2 with bound NADPH were recorded on a Perkin Elmer fluorescence spectrometer LS50B at 22 °C. Binding studies were carried out in a quartz cuvette containing ~2.0 μM Kvβ2-bound NADPH in 0.2 M potassium phosphate buffer, pH 7.5 at 22 °C. The reaction volume was 2 ml. All inhibitor solutions (Stock concentration 100 mM) were prepared in DMSO and then diluted into 0.2 M potassium phosphate buffer, pH 7.5 to the required concentration, keeping the content of DMSO in the reaction mixture equal to or below 1% (v/v). DMSO at this concentration had no effect on enzyme-bound NADPH. The fluorimeter excitation wavelength was set at 360 nm with a slit size of 15 nm and emission spectra were acquired from 300 to 600 nm. After a spectrum was recorded, 10 μl of inhibitor stock solution was added into the Kvβ2 solution and mixed. A spectrum was recorded immediately after mixing (1 min) and at different time points afterwards.

3. Results and discussion

3.1. Inhibitors of the β-subunit from voltage-sensitive potassium channels

Table 1 shows the compounds tested for their ability to inhibit Kvβ2-mediated reduction of 4-nitrobenzaldehyde. Although it has previously proven somewhat difficult to find molecules that bind to Kvβ2 as inhibitors in this study several molecules were found that were capable of binding to, and inhibiting, Kvβ2.

3.1.1. DOPAC and catechol

3,4-Dihydroxyphenylacetic acid (DOPAC), the acid metabolite of dopamine inhibited Kvβ2 in a concentration-dependent and saturable manner (Fig. 1A). Under the conditions used in this study, DOPAC was found to be the most effective inhibitor of Kvβ2 of all the molecules tested, showing ~40% reduction in 4-nitrobenzaldehyde reductase activity (Table 1). The inhibition was found to be concentration dependent (Fig. 1A). Interestingly, catechol, which has a structure like DOPAC but without the acetate group, was found to be far less effective (16% inhibition, see Table 1). While the observed DOPAC inhibition is only 40%, it is of interest to find that this neurotransmitter metabolite is capable of binding to Kvβ2, particularly considering findings regarding the role of Kv1 channels in regulating dopamine release (see Martel et al., 2011).

3.1.2. Cortisone

Structural studies have shown cortisone to bind at two different sites on Kvβ2: one close to the NADPH cofactor binding site and another at an interface site between the α and β subunits (Pan et al., 2008a). It was, therefore, expected that cortisone might inhibit Kvβ2. Indeed, this study shows that cortisone does inhibit Kvβ2 activity but only by ~18% (Table 1). This inhibition was further shown to be concentration dependent (Fig. 1B). Such a relatively modest inhibition by cortisone supports the idea that its main effect on channel activity is likely via dissociation of Kvβ2 from Kv1 (Pan et al., 2008a).
3.1.3. Sodium valproate

This anti-convulsant drug, which is a classical inhibitor of many aldo-keto reductases, (Hinshelwood et al., 2002; Todaka et al., 2000) proved to be a rather poor inhibitor showing a 20% reduction in 4-nitrobenzaldehyde activity of Kvβ2 at 0.5 mM (Table 1).

Table 1
Inhibitors of 4-nitrobenzaldehyde reduction. All data are expressed as mean of triplicate measurements.

<table>
<thead>
<tr>
<th>Inhibitor (0.5 mM)</th>
<th>Description of inhibitor</th>
<th>Percentage inhibition (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sodium valproate</td>
<td>Classical AKR inhibitor/anticonvulsant</td>
<td>10 ± 0.8</td>
</tr>
<tr>
<td>4-Nitrobenzoic acid</td>
<td>Kvβ2 catalysed reaction product</td>
<td>0</td>
</tr>
<tr>
<td>4-Chlorobenzaldehyde</td>
<td>Substrate analogue</td>
<td>6 ± 0.5</td>
</tr>
<tr>
<td>4-Fluorobenzaldehyde</td>
<td>Substrate analogue</td>
<td>3 ± 0.8</td>
</tr>
<tr>
<td>Anisaldehyde</td>
<td>Substrate analogue</td>
<td>0</td>
</tr>
<tr>
<td>Tolualdehyde</td>
<td>Substrate analogue</td>
<td>0</td>
</tr>
<tr>
<td>Chloral hydrate</td>
<td>Sedative and hypnotic drug</td>
<td>0</td>
</tr>
<tr>
<td>4-nitroacetophenone</td>
<td>Aromatic ketone</td>
<td>6 ± 0.6</td>
</tr>
<tr>
<td>Rutin hydrate</td>
<td>Plant flavanoid</td>
<td>38 ± 2</td>
</tr>
<tr>
<td>Resveratrol</td>
<td>Chemopreventive/cardioprotective/antiaging</td>
<td>38 ± 1</td>
</tr>
<tr>
<td>Cortisone</td>
<td>Steroid hormone</td>
<td>18 ± 2</td>
</tr>
<tr>
<td>Pyrazole</td>
<td>Analgesic/anti-inflammatory</td>
<td>0</td>
</tr>
<tr>
<td>Cyproheptadine</td>
<td>Antidepressant</td>
<td>0</td>
</tr>
<tr>
<td>Amtriptyline hydrochloride</td>
<td>Antidepressant</td>
<td>0</td>
</tr>
<tr>
<td>d-Sorbitol</td>
<td>Sugar alcohol</td>
<td>0</td>
</tr>
<tr>
<td>Chlorpromazine hydrochloride</td>
<td>Antipsychotic drug</td>
<td>0</td>
</tr>
<tr>
<td>DOPAC</td>
<td>Dopamine metabolite</td>
<td>40 ± 4</td>
</tr>
<tr>
<td>Catechol</td>
<td>Structural variant of DOPAC</td>
<td>16 ± 0.7</td>
</tr>
<tr>
<td>5-Hydroxyindole-3-acetic acid</td>
<td>Serotonin metabolite</td>
<td>0</td>
</tr>
</tbody>
</table>

* The amount of 4-nitrobenzyl alcohol produced as a result of Kvβ2-mediated multiple turnover of 4-nitrobenzaldehyde with no inhibitor was taken as 100%. Activities in the presence of inhibitor were expressed as a percentage of control 4-nitrobenzaldehyde reductase activity. Enzyme activity was measured as described in Section 2.

3.1.4. Rutin

Rutin, a naturally-occurring plant flavanoid known to inhibit aldose reductase from rat brain (Wermuth et al., 1982), reduced Kvβ2 aldehyde reductase activity by ~38% at 0.5 mM (Table 1). A concentration-dependent increase in inhibition was observed

Fig. 1. Concentration dependent inhibition of Kvβ2 by various inhibitors. The data show the percentage inhibition as a function of increasing concentration of (A) DOPAC (0–700 µM), (B) cortisone (0–1000 µM), (C) rutin (0–2000 µM), (D) Resveratrol (0–2000 µM). The assay mixture contained ~0.5 mg Kvβ2 in 0.2 M potassium phosphate buffer (pH 7.5), along with 500 µM 4-nitrobenzaldehyde as substrate, 200 µM NADPH and various concentrations of inhibitor in a final reaction volume of 250 µL at 37 °C. After the addition of inhibitor to Kvβ2 containing NADPH, the reaction was initiated by the addition of the substrate (4-nitrobenzaldehyde) and incubated for 30 min. The amounts of the reduced product (4-nitrobenzylalcohol) formed were analysed by HPLC, as described in Section 2. Percentage inhibition of the 4-nitrobenzaldehyde reductase activity of Kvβ2 at different starting concentrations of inhibitor was plotted against inhibitor concentration. Data are expressed as the mean of triplicate measurements. The points were fitted to a rectangular hyperbolic curve using prism 6.0.
3.1.5. Resveratrol
Kvβ2 inhibition by resveratrol was examined since it has been shown to have chemopreventive, cardioprotective, and anti-aging properties which may be mediated by inhibition of a reductase (Buryanovsky et al., 2004). Resveratrol (0.5 mM) inhibited Kvβ2 activity in a concentration-dependent manner but to a maximal level of ~40% (Fig. 1D). It is interesting to speculate that some of the cardioprotective effects attributed to resveratrol might be mediated by Kvβ2 inhibition.

3.1.6. Other compounds tested
4-Nitrobenzoic acid showed no inhibition showing that Kvβ2 was not liable to product inhibition under these conditions. Anisaldelyde, pyrrolysidine, choline hydrate, n-sorbitol, chlorpromazine and 5-hydroxyindole-3-acetic acid all failed to inhibit Kvβ2. The antidepressants amitriptyline and cyproheptadine (a known potassium channel blocker, Wooltorton and Mathie, 1993) were, likewise, devoid of effect on Kvβ2. The antipsychotic drug, chlorpromazine and the serotonin metabolite 5-hydroxyindole-3-acetic acid showed no measurable inhibition of Kvβ2.

3.1.7. Summary of inhibition findings
As with the substrate specificity studies mentioned above it is difficult to deduce common features that will define a Kvβ2 inhibitor. A variety of aldehydes might be expected to be inhibitors or substrates. However, the fully hydrated aldehyde, choline hydrate, did not inhibit neither did a several aromatic aldehydes although 6% inhibition was observed with 4-chlorobenzaldoxime. Sodium valproate did show some inhibition (10%) indicating that a carboxylate might be necessary for inhibition, however, 4-nitrobenzoate did not inhibit. On the other hand, 5-nitroacetophenone did inhibit albeit by only 6%. Catechol inhibited Kvβ2 by 16% but DOPAC which consists of catechol bearing an acetyl group inhibited by 40%. This again suggests that a carboxylate is desirable for inhibition. Large multi ring structures such as amitriptyline, chlorpromazine and cyproheptadine were not inhibitory presumably due to their bulk. On the other hand the multi ring structure of rutin was inhibitory. These findings probably reflect an active site with a high degree of specificity. The most significant inhibition was observed with DOPAC, rutin hydrate and resveratrol.

3.2. Inhibitor binding studies
It was possible that some of the compounds tested might be binding to sites other than the active site of Kvβ2. In 2008, Pan et al., identified an interface site that promotes dissociation of Kvβ2 from Kv1. It is not known if this site will bind cortisone in the absence of Kv1 α subunits. Moreover, because some of the compounds tested might be substrates for Kvβ2, the binding of the inhibitors to the Kvβ2-active site was examined by monitoring the quenching of the cofactor fluorescence peak at 450 nm. This effect on fluorescence intensity is different from that induced by a substrate, which oxidises the Kvβ2-bound NADPH and, thereby, eliminates the fluorescence peak (Alka et al., 2010; Pan et al., 2008a). Binding studies carried out for cortisone, DOPAC, catechol, rutin and resveratrol yielded reductions in the fluorescence intensity at 450 nm of 16 ± 0.7%, 7 ± 0.3%, 12 ± 0.8% and 16 ± 1.1%, respectively immediately after their addition; the decreased signals remained almost constant for 20 min. The data for DOPAC and cortisone are illustrated in Fig. 2A and B; findings with the latter agree well with a report from Pan et al., 2009. In the same year, Tipparaju et al., reported cortisone as a poor substrate for Kvβ2; this was not observed in the present study. Indeed, none of the inhibitors tested identified in this study were Kvβ2 substrates.

4. Conclusion
Previous studies of modulators of Kv1 channel activity have focused on cortisone and its analogues (Pan et al., 2008a, 2012). The present study advances the search for inhibitors of Kvβ2 by identifying several new compounds that bind to this subunit that specifically inhibit subunit turnover and, therefore, are likely to have a quite different mechanism of action from cortisone which appears to exert its influence in large part by dissociating Kvβ from Kv1.

It is surprising that, given the sequence homology between Kvβ proteins and AKR enzymes, there is very little inhibition of this subunit activity by classical AKR inhibitors; in fact, there are few compounds that appear to bind tightly to this protein and this includes substrates (see Pan et al., 2012; Alka et al., 2010).

Interestingly, DOPAC (the acid degradation product of dopamine) was found to be an effective inhibitor of Kvβ2. Dopamine is released by several areas of the brain and these findings make DOPAC a possible in vivo effector of brain Kvβ2. However, the concentration at which inhibition was observed is quite high. DOPAC is only the second physiological compound shown to inhibit Kvβ2 activity. Physiologically, Dopamine undergoes catabolism by monoamine oxidase to 3,4-dihydroxyphenylacetaldehyde (DOPAL), which is further oxidised to 3,4-dihydroxyphenylacetic acid (DOPAC) by aldehyde dehydrogenase. It is unlikely that DOPAC levels in vivo will approach those used in this study (Jing et al., 2007) unless in localised microenvironments.

Cortisone was found to influence aldehyde turnover as predicted, but rutin and resveratrol were at least as effective in this regard. Resveratrol, a polyphenolic compound found in red wine, is well known for its cardioprotective (Markus and Morris, 2008; Nicholson et al., 2008) and anti-cancer effects (Chen et al., 2004; He et al., 2011). Its observed inhibition of the catalytic activity of Kvβ2 may relate to some of the therapeutic properties exhibited by resveratrol.

It is acknowledged that the concentrations used to achieve Kvβ2 inhibition are high and the degree of inhibition does not exceed 40% in some cases. The lack of inhibition studies in the literature for this subunit may be due to difficulties in identifying any molecules that can bind to Kvβ2. It is important to note that cortisone does not greatly inhibit aldehyde turnover by this subunit but it has nonetheless been shown to have a significant effect on channel activity (see Pan et al., 2008a).

It is important to note that while the inhibitors examined here showed concentration dependence they did not give rise to complete inhibition in any case. This precluded the measurement of IC50 values. Moreover, a plot of enzyme activity versus inhibitor concentration saturation was saturable. Inhibition of this kind is normally regarded as partial and can be explained by assuming that inhibitor binding does not fully exclude substrate turnover (Scheme 1).

An alternative explanation, where inhibition is caused by binding at a site other than the active site in a manner that constrains substrate binding, is also possible but was discounted on the basis of the fluorescent quenching studies. Moreover, direct binding of cortisone to the active site was observed previously (Pan et al., 2008a).

Of course, such an explanation might be considered where such binding gave rise to perturbation of the active site in a way that caused fluorescence quenching. Further kinetic studies are needed to distinguish these possibilities.

The compounds described here may help to provide a starting point to stimulate further exploration of Kvβ2 inhibitors. In summary, a number of molecules were identified that bind to and inhibit Kvβ2-mediated p-nitrobenzaldehyde reduction, including the steroid cortisone.

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