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The Synthesis of NPPB and NPBB by Reductive Amination and the Effects of these Compounds on K^+ Channels of the Alga *Nitella hookeri*

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Abstract—This paper communicates a new synthesis of the ion channel inhibitors NPPB and NPBB using a simple reductive amination sequence. The synthesised compounds were found to reduce channel amplitude of a K^+ channel present in cytoplasmic droplets of *Nitella hookeri*.

Many naturally occurring toxins and venoms exhibit their biological effect by blocking ion channels, proteins that facilitate the passive movement of ions across a biological membrane. As such, these compounds are particularly useful tools for studying the biochemistry of ion channels and their associated processes.[1] Synthetic compounds have proven just as useful, for example 5-nitro-2-(4-phenylpropylamino) benzoic acid (NPPB, **1**),[2] is a simple, but important compound that has found wide use in this context. NPPB has typically been used as an inhibitor of anion channels in both plant and animal cells[2–6] but in certain cell types its specificity may not be absolute. In wheat root protoplasts, for example, NPPB has shown greater potency towards K^+ channels[7] compared to Cl^- channels and in Arabidopsis hypocotyls it has limited potency towards an anion channel.[8]

There are, as yet, no reports on the effects of this type of synthetic compound on K^+ channels in other plant systems, nor on how the inhibitor may actually be interacting with the K^+ channels. A K^+ channel of particular interest is the large conductance K^+ channel present in cytoplasmic droplets obtained from characian algae.[9] This channel has similarities with large conductance Ca^{2+} activated K^+ channels (BK or maxi-K channels) of animal cells.[10] Significantly, NPPB has been reported to increase BK current in oocytes expressing cloned human BK channel.[11] Clearly, further testing of NPPB on other cell and channel types is required. It is also important to begin to look into the actions of structurally similar compounds, as these can give clues as to interaction of the inhibitor with the channel.

A general and versatile method for the preparation of NPPB, the related 5-nitro-2-(4-phenylbutylamino) benzoic acid (NPBB, **2**) and their analogues is required to achieve these goals. The existing literature synthesis of NPPB involves the coupling of a 2-halo-5-nitrobenzoic acid and 3-phenylpropylamine in a reaction that requires elevated temperatures and is sensitive to substituent effects.[12] As a consequence, this reaction is somewhat cumbersome and lacks generality. By contrast, the method presented here is based on a simple reductive amination of an aldehyde with 5-nitroanthranilic acid, a reaction that should be amenable to the preparation of a range of related derivatives for activity screening. In this paper we also present the first description of the inhibition by NPPB and NPBB of a K^+ channel present in cytoplasmic droplets of the characian alga *Nitella hookeri*.

Synthesis

NPPB was prepared as shown in Scheme 1. A solution of 5-nitroanthranilic acid and hydrocinnamaldehyde, in

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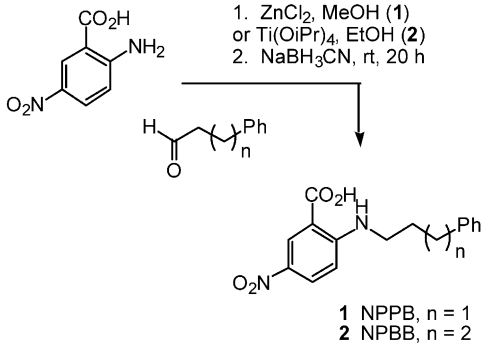

Scheme 1.

methanol, was stirred for 30 min at rt. A suspension of zinc chloride and sodium cyanaborohydride, in methanol, which had itself been stirred for 20 min, was then added and the resulting mixture was stirred at rt for 20 h. The desired product was then isolated in 52% after chromatography on silica. The thus prepared sample of **1** was identical by mp and ^{1}H NMR to a commercial sample obtained from Sigma®.

Next, we tested the versatility of our method with the preparation of NPBB **2**, an important analogue of NPPB that is not commercially available. The literature synthesis of this compound[13] is as reported for NPPB.[12] In this case we chose to use titanium(IV) isopropoxide, rather than zinc chloride, to ascertain if other Lewis acids could be employed in this reaction. As such a solution of 5-nitroanthranilic acid, 4-phenyl-1-butryaldehyde[14] and titanium (IV) isopropoxide was stirred in ethanol, at room temperature, for 1 h. Sodium cyanaborohydride was added and the mixture was stirred for a further 20 h. The desired product was then isolated in an un-optimized yield of 27% after chromatography and recrystallisation from ethanol. Nitroanthranilic acid was also reacted with cinnamaldehyde under these conditions to give NPPB in a comparable yield to that obtained using zinc chloride above.

Channel Inhibition

The effects of the inhibitors on K^+ channel activity of cytoplasmic droplets of the alga *N. hookeri* was investigated using the patch clamp technique.[15] Preparation of droplets, pipette fabrication, patch clamp methodology and data analysis were carried out as described previously.[16] Solutions used were as follows: bath solution (in mM) KCl 150; MES 5, pH 7 with KOH; pipette solution (in mM) KCl 150, $CaCl_2$ 1, MES 5, Choline chloride 70, pH 7 with KOH. Inhibitors, at a concentration of 50 μM, were added by perfusion to the bath solution. Statistical analyses were carried out using the software package Statistix 7 (Analytical Software). Means were compared using one-way ANOVA and Tukey tests.

Representative I/V curves, obtained from inside out patch preparations, showing the effect of synthesised inhibitors, are presented in Figure 1. The data indicate

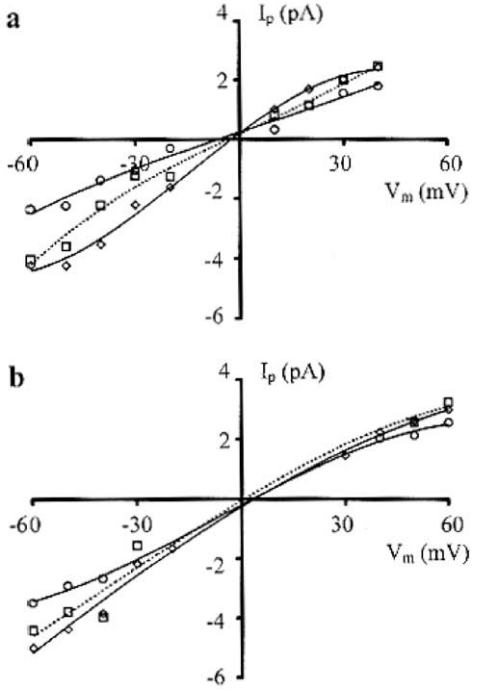

Figure 1. Representative I/V curves showing the effect of (a) NPPB and (b) NPBB synthesised in our laboratory. Diamonds: currents before addition of the inhibitor. Circles: currents 2 min after addition of the inhibitor. Squares: current after inhibitor washout.

that both inhibitors reduce single channel amplitude at both positive and negative membrane potentials with the effect greater at negative values.

For statistical analyses we have compared the respective currents at +40 and −40 mV before and after the addition of inhibitor. NPPB, at 40 mV, significantly reduced ($p<0.001$, $n=3$) the mean amplitude (±SD) of 2.4 ± 0.01 to 1.88 ± 0.1 pA. At −40 mV the mean amplitude (±SD) of -3.8 ± 0.22 pA was significantly reduced ($p<0.01$, $n=3$) to -2 ± 0.56 pA. The reductions observed with synthesised NPBB showed no significance difference to those observed with NPPB ($p>0.05$). At 40 mV in the presence of NPBB the mean amplitude (±SD) of 2.52 ± 0.02 pA was significantly reduced ($p<0.01$, $n=4$) to 1.94 ± 0.23 pA and at −40 mV the mean amplitude (±SD) of -3.95 ± 0.4 pA was significantly reduced ($p<0.001$, $n=4$) to -2.26 ± 0.22 pA. With both NPPB and NPBB, currents returned to pre-inhibited levels upon inhibitor wash-out. Ethanol controls (at the highest concentrations required to solubilise the inhibitors) had no effect on channel amplitude. As expected, synthetic and commercial samples of NPPB gave the same channel activity.

These data suggest that NPPB, in addition to inhibiting the voltage-dependent $I_{K,out}$ current of wheat root protoplasts[7] may also affect the large conductance K^+ channels present in cytoplasmic droplets of characian algae. There is, to the best of our knowledge, no sequence data available for the K^+ channel of the characian droplets. If these channels show structural (as well the previously mentioned biophysical[10]) similarities to the BK channels then their architectural form may

differ from that of voltage-dependent channels, for which there is an increasing body of structural information.[17] This raises the possibility that NPPB may inhibit a wide range of different K^+ channels, irrespective of overall structure, and its interaction with the channels may be based on highly conserved regions such as the K-channel signature sequence responsible for channel selectivity.[1] It should, however, be noted that the effect of NPPB on the large conductance K^+ channel in this study differs from the previously reported effect of increasing current in oocytes expressing cloned human BK channel,[11] which may indicate that their structural similarity may not be that high.

Further investigation of the interaction of NPBB with K^+ channels is possible through the use of inhibitors with closely related structures. One such compound, NPBB, differs from NPPB by a simple methylene extension. Similar extensions in Na^+ channel inhibitors (for example the difference between phenamil and benzamil) increase the efficacy of the inhibitor.[18] We found no significant difference in the effect of NPPB and NPBB on channel amplitude; thus, the methylene extension does not appear to evoke greater efficacy toward the large conductance K^+ channel. Clearly, this is an area in which further studies are warranted and our synthetic technique should enable to production of a range of potentially interesting compounds.

In summary, we present a simple method for the preparation of NPPB and NPBB which should be amenable to a range of related compounds where a range of the required aldehydes and arylamines is available. We also show that these two inhibitors can reduce channel amplitude of the large conductance K^+ channel present in cytoplasmic droplets of the alga *N. hookeri*.

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