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Short communication

NS 1619 activates BK_{Ca} channel activity in rat cortical neurones

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Abstract

Single channel recordings of large conductance Ca^{2+}-activated K^+ (BK_{Ca}) channels were made from neurones isolated from rat motor cortex. Application of levcromakalim, pinacidil or diazoxide had no effect on BK_{Ca} channel activity in excised patches. In contrast, NS 1619 (1-(2′-hydroxy-5′-trifluoromethylphenyl)-5-trifluoromethyl-2(3*H*)benzimidazalone) induced concentration-dependent activation of BK_{Ca} channels with a calculated EC_{50} of 32 μM. The NS 1619-induced activity was dependent on the presence of free Ca^{2+} at the intracellular surface, but was not associated with a change in channel voltage sensitivity. Niflumic acid had no effect on BK_{Ca} activity per se but prevented NS 1619-mediated activation.

Keywords: NS 1619; BK_{Ca} channel; Niflumic acid; Motor cortex

1. Introduction

The potassium channel openers are a heterogeneous group of chemical compounds generally assumed to act upon ATP-sensitive K^+ (K_{ATP}) channels, a view partially supported by single channel recordings and the ability of sulphonylureas to block the potassium currents induced by these agents (Edwards and Weston, 1993). However, it has been reported that pinacidil, one of the established or 'classical' potassium channel openers, can also increase large conductance Ca^{2+}-activated K^+ (BK_{Ca}) channel activity in smooth muscle (Gelband et al., 1989; Ashford et al., 1993). The list of potassium channel openers has recently expanded to include the benzimidazalone derivative NS 1619 (1-(2′-hydroxy-5′-trifluoromethylphenyl)-5-trifluoromethyl-2(3*H*)benzimidazalone), which activates BK_{Ca} channels in a number of tissues (Olesen et al., 1994; Sellers and Ashford, 1994; Edwards et al., 1994), and the structurally related compound niflumic acid, which stimulates BK_{Ca} channels in coronary smooth muscle (Toro et al., 1993).

In the present study the action of three 'classical' potassium channel openers (diazoxide, pinacidil and levcromakalim) and two of the newer agents (NS 1619 and niflumic acid) were examined on BK_{Ca} channels in rat cortical neurones. These BK_{Ca} channels are also activated by application of ATP to the cytoplasmic aspect of excised patches, an effect likely mediated by phosphorylation (Lee et al., 1994a).

2. Materials and methods

2.1. Preparation of acutely dispersed cortical neurones

Coronal slices (350 μm thick) of cerebral cortex were cut from brains of male Sprague-Dawley rats (100–200 g weight) with a vibratome (Oxford Instruments). Portions of the motor cortex were dissected from the coronal sections and were incubated in 1 mg per ml Pronase E (Sigma) in artificial cerebral-spinal fluid gassed with 95% O_2 and 5% CO_2 at 37° C for 10 min and then titurated by use of flame-polished Pasteur pipettes of decreasing internal diameter. The dispersed cells were plated onto tissue culture dishes and left for 30 min–1 h before use by which time the cells had adhered to the plates.

2.2. Recording and analysis

All experiments were performed using either the inside-out or the outside-out configuration of the patch

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clamp technique. Single channel current recordings were made using a List EPC-7 patch clamp amplifier and were stored on Sony digital audio tape (DAT; DTC-1000ES) for subsequent replay into a Gould 2200 chart recorder. The average channel activity ($N_f \cdot P_o$) where N_f is the number of functional channels in the patch and P_o is the open state probability, was determined by measuring the total time spent at each unitary current level and expressed as a proportion of the total time recorded. These parameters were determined off-line using the single channel current analysis program (PAT 6.2) run on an Elonex 386 SX 160 microcomputer as described previously (Lee et al., 1994b). Stretches of data 30 s–120 s in duration were filtered at 1 kHz (8-pole Bessel) and digitised at 5 kHz using a Data Translation 2801A interface.

The increase in channel P_o was plotted with respect to the concentration of drug ([N]) and the data were fit by non-linear regression to the equation:

$$P_o = (P_{max} - P_c)/1 + (EC_{50}/[N])^n$$

where P_{max} is the maximum increase in channel P_o induced by the drug, P_c is the channel P_o in the absence of drug, EC_{50} is the concentration of the drug producing a P_o half the value of P_{max} and n is the Hill coefficient. Data for the variation of P_o with membrane potential (V) were fitted to the following equation:

$$P_o = P_{max} \cdot (1 + e^{-A(V-V_{0.5})})^{-1}$$

where P_{max} is the maximum open-state probability, $V_{0.5}$ the membrane potential at which P_o is half of P_{max} and A is a constant related to the slope of the curve. This equation is a transformation of the Boltzman distribution for a two-state model and therefore it is assumed that the channel can only be open or closed. The best-fit values for P_{max}, $V_{0.5}$ and A were obtained from non-linear regression analysis using Kaliedagraph 3.0 (Abelbeck Software, USA).

All data in the text and figures are presented as the mean ± S.E.M. The statistical analysis of differences between data groups was assessed by one-way analysis of variance ('Modstat, Modern Microcomputers, USA).

2.3. Drugs and solutions

Before single channel recording, the cells were washed thoroughly with solution A which consisted of (in mM): NaCl 135.0, KCl 5.0, $CaCl_2$ 1.0, $MgCl_2$ 1.0, Hepes 10.0. For inside-out patch recording the patch pipette was filled with solution B (in mM): KCl 140.0, $CaCl_2$ 1.0, $MgCl_2$ 1.0, Hepes 10.0 while the bath contained solution C (in mM): KCl 140.0, $CaCl_2$ 0.79, $MgCl_2$ 1.0, EGTA 1.0, Hepes 10.0 or solution D (in mM) KCl 140.0, $CaCl_2$ 1.0., $MgCl_2$ 1.0, EGTA 1.0, Hepes 10.0. The concentrations of free Ca^{2+} and Mg^{2+} present in the intracellular bath solutions were calculated to be 1.0 μM and 1 mM respectively for solution C and 10.0 μM and 1 mM respectively for solution D using the binding constants determined by Fabiato and Fabiato (1979). For outside-out patch recordings, the pipette solution contained solution C while the bath contained solution B. The pH of all solutions was adjusted to 7.2 with either NaOH or KOH.

Niflumic acid, diazoxide (Sigma), pinacidil (Leo Pharmaceuticals, Bucks., UK), levcromakalim (Beecham Pharmaceuticals, Harlow, Essex, UK), and NS 1619 (Glaxo, Ware, Herts., UK) were added to the bathing solutions from freshly prepared 0.1–1.0 M stock solutions (pinacidil and levcromakalim in ethanol; diazoxide in KOH and niflumic acid and NS 1619 in dimethyl sulphoxide (DMSO), to provide final concentrations as stated in the text. Ethanol (1%) and DMSO (1%) alone were without effect upon BK_{Ca} channel activity ($n = 4$ for each).

All drugs were bath applied to membrane patches by superfusing the bath by a gravity feed system, at a rate of approximately 6 ml min^{-1} which allowed complete solution exchange within 30–60 s. All experiments were performed at room temperature, 22–25° C.

3. Results

In single channel recordings made from isolated cortical neurones, BK_{Ca} channels with mean single channel conductance of 245 ± 6 pS ($n = 6$) were found in all patches ($n = 41$). In excised patches, BK_{Ca} channel open probability increased when the intracellular Ca^{2+} concentration was raised from 0.1 μM to 1–10 μM, by depolarisation or by the addition of 1–5 mM ATP to the intracellular surface (Lee et al., 1994a). The application of diazoxide (200 μM and 500 μM, $n = 4$), pinacidil (200 μM, $n = 4$) or levcromakalim (100 μM, $n = 3$) to the intracellular surface of inside-out patches and levcromakalim (100 μM, $n = 3$) to the extracellular surface of outside-out patches failed to modulate the activity of the BK_{Ca} channel in symmetrical 140 mM KCl and 1 μM free intracellular Ca^{2+} (data not shown). However, stimulation of K_{ATP} channel activity in a variety of tissues is considered to require the presence of Mg · ATP in the intracellular environment (Kozlowski et al., 1989; Edwards and Weston, 1993). Thus the experiments were repeated in the presence of Mg · ATP at the cytoplasmic aspect of membrane patches. In the presence of either a non-stimulatory (100 μM) or stimulatory (2 mM) concentra-

tion of ATP, 100 μM levcromakalim ($n = 4$), 200 μM pinacidil ($n = 3$) or 500 μM diazoxide ($n = 3$) all failed to alter BK_{Ca} channel activity. An example of the lack of effect of levcromakalim is shown in Fig. 1a.

In contrast, application of the benzimidazolone derivative NS 1619 to the intracellular aspect of inside-out membrane patches from cortical neurones resulted in a rapid and reversible increase in BK_{Ca} channel activity (Fig. 1b). For example application of 40 μM NS 1619 to patches held at a potential of +20 mV in the presence of 1 μM free Ca^{2+} increased BK_{Ca} channel P_o from 0.06 ± 0.02 to 0.63 ± 0.05 ($n = 11$). NS 1619 increased BK_{Ca} channel P_o in a concentration-dependent manner. The EC_{50} for channel activation was calculated as 31.7 ± 0.06 μM with an associated Hill coefficient of 2.6 at a holding potential of +20 mV in 140 mM symmetrical KCl and 1 μM free intracellular Ca^{2+}. The Hill coefficient for the relationship between P_o and intracellular Ca^{2+} concentration at a potential of +20 mV was unaffected by NS 1619; values obtained were between 1.2 and 1.5 in the absence and 1.3–1.5 in the presence of 40 μM NS 1619 respectively (data not shown).

The stimulation of BK_{Ca} channels in isolated patches was dependent upon the concentration of Ca^{2+} at the intracellular membrane surface. At bath concentrations of 0.1 μM Ca^{2+} and below, NS 1619 did not stimulate channel activity, over the voltage range tested, ±60 mV (see Fig. 1c). However, at concentrations of 1 and 10 μM Ca^{2+}, 40 μM NS 1619 induced a large increase in BK_{Ca} channel activity over the same voltage range (Fig. 1c). These data were fitted to the Boltzman equation for a two-state model and the best-fit values obtained for $V_{0.5}$ (the membrane potential at which P_o is half of the maximum fitted value of P_o) were 45.2 ± 1.8 mV ($n = 7$) and 10.3 ± 2.8 mV ($n = 6$) at concentrations of 1 μM and 10 μM Ca^{2+} respectively in the absence of NS 1619, and -32.5 ± 10.8 mV ($n = 4$) and -112.1 ± 2.1 mV ($n = 4$) respectively in the presence of 40 μM NS 1619. Thus in the absence of NS 1619 there is a 34.9 mV change in $V_{0.5}$ per 10-fold increase in calcium concentration whereas in the presence of 40 μM NS1619 the change in $V_{0.5}$ per 10-fold increase in calcium is approximately 80 mV. However, the increase in BK_{Ca} channel activity induced by 40 μM NS 1619 was not associated with a change in the voltage sensitivity of the channel with an e-fold increase in P_o per 31.2 ± 1.6 mV and per 28.2 ± 2.2 mV change in membrane potential in the absence and presence of 40 μM NS 1619 ($n = 4$) respectively (data not shown).

Niflumic acid, structurally related to NS 1619, had no effect on BK_{Ca} channel activity in rat cortical neurones over the concentration range 10–500 μM in the presence of varying concentrations of intracellular Ca^{2+} (0.1–10 μM, $n = 4$ for each) or ATP (100 μM and 2

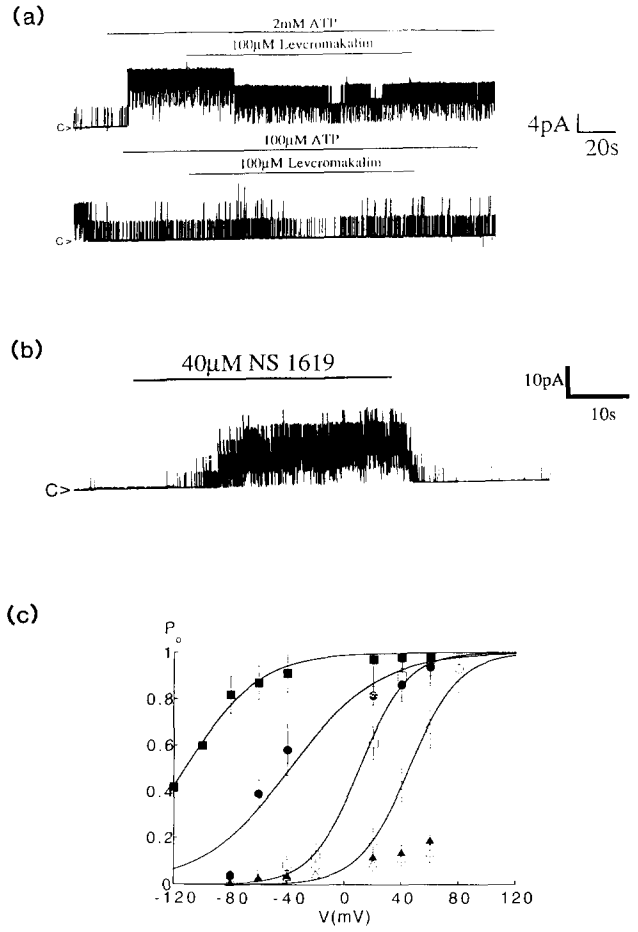

Fig. 1. (a) Lack of effect of levcromakalim upon BK_{Ca} channel activity in the presence of varying concentrations of ATP. Continuous recording made from an excised inside-out patch held at +20 mV in the presence of symmetrical 140 mM KCl whilst the intracellular surface of the patch was continuously perfused with 1 μM free Ca^{2+}. Channel activity was low ($N_f \cdot P_o$ 0.08), but was stimulated by the application of 2 mM ATP ($N_f \cdot P_o$ 1.86). This level of activity was not significantly affected by the application of 100 μM levcromakalim ($N_f \cdot P_o$ 1.66), but was reversed following nucleotide washout ($N_f \cdot P_o$ 0.29). The application of 100 μM ATP ($N_f \cdot P_o$ 0.26) and 100 μM ATP in the presence of 100 μM levcromakalim ($N_f \cdot P_o$ 0.24) both failed to affect this level of channel activity. (b) 40 μM NS 1619 increases BK_{Ca} channel activity when applied to the intracellular surface of an excised inside-out patch held at +20 mV in the presence of 140 mM symmetrical KCl and 1 μM free intracellular Ca^{2+}. When 40 μM NS 1619 was applied to the intracellular surface of the excised patch, channel $N_f \cdot P_o$ increased from 0.09 to 1.55. The effect of NS 1619 was rapidly reversed following its removal from the bath solution ($N_f \cdot P_o$ 0.05). (c) The effect of 40 μM NS 1619 (filled symbols) upon channel P_o in the presence of varying concentrations of intracellular Ca^{2+} at various membrane holding potentials. Data were obtained using excised inside-out patches in the presence of 140 mM symmetrical KCl and increasing concentrations of intracellular Ca^{2+} (i.e. 0.1 μM Ca^{2+} triangles, 1 μM free Ca^{2+} circles, 10 μM free Ca^{2+} squares) in the bath. Each point is the mean of between four and eight determinations with the S.E.M. indicated by the associated error bar. Where no bars are present, the error was within the size of the symbol. The fitted lines are the lines of best fit to a Boltzman distribution.

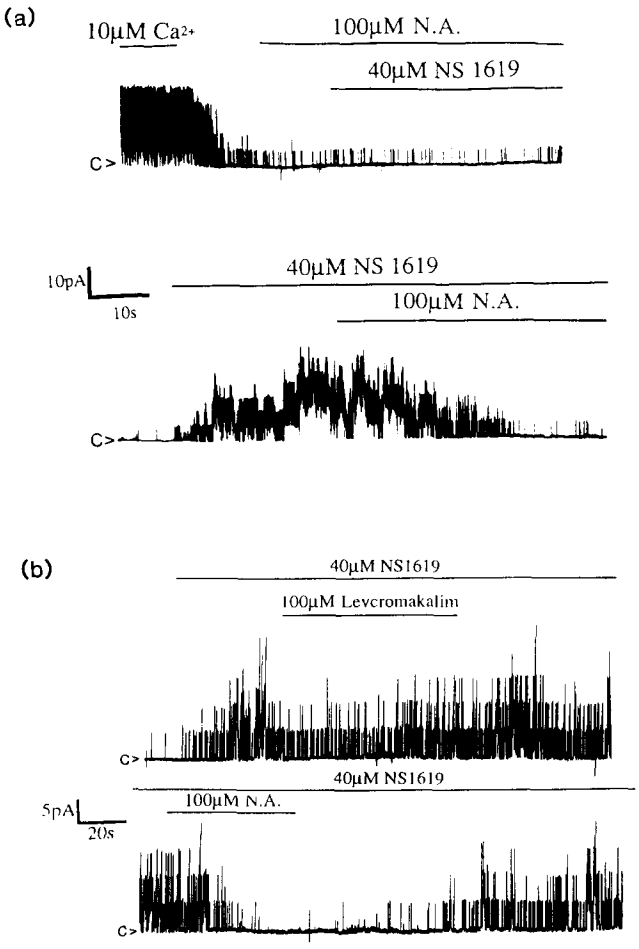

Fig. 2. (a) The effect of 100 μM niflumic acid (N.A.) and 40 μM NS 1619 upon BK_{Ca} channel activity when applied to the intracellular surface of an excised inside-out patch. The two traces form a continuous recording made at +20 mV in symmetrical 140 mM KCl. The intracellular surface of the membrane patch was perfused with various solutions as indicated in the figure. At the start of the recording, the intracellular bath solution contained 10 μM free Ca^{2+} (the preceding 20 s of data at this Ca^{2+} concentration have been omitted from the figure) and there were five BK_{Ca} channels active in the patch ($N_f \cdot P_o$ 3.41). However, when the solution was replaced by one containing 1 μM Ca^{2+}, the level of channel activity was reduced ($N_f \cdot P_o$ 0.11). The subsequent application of 100 μM niflumic acid alone and 100 μM niflumic acid in the presence of 40 μM NS 1619 failed to affect this level of channel activity ($N_f \cdot P_o$ 0.14). When both agents were subsequently removed from the bath solution and 40 μM NS 1619 reapplied, five channels became active in the membrane patch ($N_f \cdot P_o$ 2.61). This increase in channel activity was reversed upon the reapplication of 100 μM niflumic acid in the continued presence of 40 μM NS 1619 ($N_f \cdot P_o$ 0.28). (b) Levcromakalim does not antagonise the NS 1619-mediated stimulation of BK_{Ca} channel activity. Continuous recording made from an excised inside-out patch held at +30 mV. In the presence of symmetrical 140 mM KCl and 1 μM free intracellular Ca^{2+} channel activity was low ($N_f \cdot P_o$ 0.08), but was stimulated by the application of 40 μM NS 1619 ($N_f \cdot P_o$ 1.13). This level of activity was not significantly affected by the application of 100 μM levcromakalim ($N_f \cdot P_o$ 0.87), but was reversed following the application of 100 μM niflumic acid (N.A., $N_f \cdot P_o$ 0.06). The effect of niflumic acid was slowly reversible following its washout ($N_f \cdot P_o$ 0.75).

mM, $n = 3$ for each). Although niflumic acid did not per se directly affect BK_{Ca} channel activity (i.e. there was no increase or decrease in channel activity), it did inhibit the stimulatory effects of NS 1619 ($n = 10$). In inside-out patches addition of 40 μM NS 1619 increased BK_{Ca} channel activity ($N_f \cdot P_o$) in the presence of 1 μM Ca^{2+} from 0.12 ± 0.05 to 1.78 ± 0.34 ($n = 6$). However, the subsequent co-application of 100 μM niflumic acid resulted in a reduction in channel $N_f \cdot P_o$ to 0.18 ± 0.07 (Fig. 2a; lower trace). Furthermore, pre-application of 100 μM niflumic acid (Fig. 2a; upper trace) prevented the increase in channel activity associated with the introduction of 40 μM NS 1619 ($N_f \cdot P_o$ unchanged at 0.09 ± 0.05 ($n = 4$)). The inhibition of NS 1619-induced BK_{Ca} channel activity by niflumic acid was not mimicked by the 'classical' potassium channel openers, diazoxide (500 μM, $n = 3$), levcromakalim (100 μM, $n = 3$, Fig. 2b) or pinacidil (100 μM, $n = 3$).

4. Discussion

The potassium channel openers are a group of compounds which are currently the centre of intense scientific and clinical investigation owing to their enormous therapeutic potential (Cook, 1988). Many of these agents (which we have termed 'classical' as no simple structural differentiation is possible) have been shown to activate K_{ATP} channels, though in some tissues they have been reported to stimulate BK_{Ca} channel activity (Gelband et al., 1989; Ashford et al., 1993). However, in the present study the three 'classical' potassium channel openers tested (pinacidil, levcromakalim and diazoxide) failed to affect BK_{Ca} channel activity in the presence of a non-stimulatory and stimulatory range of Ca^{2+} and ATP concentrations.

Although the 'classical' potassium channel openers failed to modulate BK_{Ca} channel activity, the benzimidazolone compound NS 1619 clearly increased BK_{Ca} channel P_o. The increase in BK_{Ca} channel activity induced by NS 1619 does not appear to be via an alteration in voltage sensitivity of the channel. NS 1619 had no affect on the single channel current amplitude, and therefore is also unlikely to interfere with the BK_{Ca} channel conduction pathway.

However, the effect of NS 1619 was dependent upon the presence of Ca^{2+} ions at the intracellular surface, for at free Ca^{2+} ion concentrations of 0.1 μM and below (a range where the BK_{Ca} channel P_o in this tissue is relatively insensitive to Ca^{2+}), the compound was ineffective. The absolute requirement of NS 1619 for intracellular Ca^{2+} suggests that the drug may act to increase channel sensitivity to this divalent cation. This is supported by the data presented in Fig. 1c which shows that 40 μM NS 1619 increases the BK_{Ca} channel

Ca^{2+} sensitivity. Such a process might also explain the unusually large Hill coefficient associated with the NS 1619-mediated activation of channel activity since the observed response (increase in P_o) would not necessarily be directly proportional to the concentration of NS 1619 if increasing concentrations of this agent produced a non-linear increase in Ca^{2+} sensitivity. Similar findings have been reported by Olesen et al. (1994) for BK_{Ca} channels in bovine aortic smooth muscle cells. In contrast, the BK_{Ca} channel present in rat ventromedial hypothalamus neurones was reported to be activated by NS 1619 at much lower concentrations of internal Ca^{2+} (Sellers and Ashford, 1994). However, this may simply reflect the much higher intrinsic Ca^{2+} sensitivity of the BK_{Ca} channels in these hypothalamic neurones (Treherne and Ashford, 1991).

In addition to NS 1619, niflumic acid has also been shown to activate BK_{Ca} channels from coronary smooth muscle inserted into lipid bilayers (Toro et al., 1993). In the present study niflumic acid failed to affect BK_{Ca} channel activity per se at either low or high values of P_o, although it did significantly inhibit the effects of 40 μM NS 1619. These findings suggest that the two compounds may interact with a particular site associated with BK_{Ca} channels and furthermore induce differential effects upon specific members of the BK_{Ca} channel family. Thus NS 1619 and niflumic acid may ultimately act as templates for the development of selective activators of subgroups of the BK_{Ca} channel family. However, it will also be important to determine the actions of these compounds on other cellular conductances as it has been reported that the related molecule, NS 004, inhibits Ca^{2+} currents in rat ventricular myocytes (Sargent et al., 1993) and recently Edwards et al. (1994) have shown that NS 1619 inhibits a delayed rectifier K^+ current and the L-type Ca channel in vascular smooth muscle.

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