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Ca^{2+}-dependent K^+ channels in neuroblastoma hybrid cells activated by intracellular inositol trisphosphate and extracellular bradykinin

Haruhiro Higashida* and David A. Brown

Laboratory of Biochemical Genetics, National Heart, Lung and Blood Institute and **Fogarty International Center, National Institutes of Health, Bethesda, MD 20892, USA*

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Bradykinin (BK) activation of phosphatidylinositide breakdown in NG108-15 neuroblastoma × glioma hybrid cells in the generation of an outward K^+ current through the release of Ca^{2+} by the intermediary messenger inositol 1,4,5-trisphosphate ($InsP_3$). Channels mediating this outward current were identified using cell-attached patch electrodes. Intracellular iontophoretic injection of $InsP_3$ or Ca^{2+}, or extracellular application of BK, evoked bursts of K^+ channel activity coincident with cell hyperpolarization measured with an intracellular recording micropipette. The most frequent channels had a mean single-channel conductance of about 40 pS in symmetrical K^+ solutions; additional openings of lower conductance (18 pS) channels were also detected. Bath application of phorbol dibutyrate (PDBu, 1 μM) increased the number and opening probability of the $InsP_3$-induced channels.

K^+ channel; Inositol trisphosphate; Phorbol dibutyrate; Bradykinin

1. INTRODUCTION

Many transmitters and hormones can initiate the enzymatic breakdown of the membrane phospholipid, phosphatidylinositol 4,5-bisphosphate (PIP_2) [1]. One product of this reaction is inositol 1,4,5-trisphosphate ($InsP_3$), whose principal intracellular effect is to release Ca^{2+} from the endoplasmic reticulum [2]. In the mouse neuroblastoma × rat glioma cell line, NG108-15, this pathway is vigorously activated by the nonapeptide, bradykinin (BK) [3–6]. The Ca^{2+} released by $InsP_3$ increases membrane K^+ permeability, producing an outward K^+ current and consequent cell hyperpolarization [7–9]. Experimentally, the same current can be induced by the intracellular injection of $InsP_3$ or Ca^{2+} [9], thereby bypassing the BK-receptor activation step. Unlike the usual Ca^{2+}-dependent K^+ currents [10–12], this current is relatively insensitive to tetraethylammonium ions (TEA) and instead is reduced by the bee-venom toxin, apamin, or by d-tubocurarine (dTC) [9].

We now report the activation of appropriate single Ca^{2+}-dependent K^+ channels in NG108-15 cells following intracellular injections of $InsP_3$ or Ca^{2+} or extracellular application of BK. These channels have a lower channel conductance than the usual, TEA-sensitive high-conductance Ca^{2+}-activated 'BK' or 'maxi-K' channel [11–13]; some had a similar conductance to that of the apamin-sensitive K^+ channels recently detected in skeletal muscle [14], pituitary tumor [15] and liver [16] cells, but most had a higher (40 pS) conductance. We also find that this channel activity is augmented by the application of phorbol dibutyrate (PDBu), an activator of protein kinase

Correspondence (present) address: D.A. Brown, Department of Pharmacology, University College London, Gower Street, London WC1E 6BT, England

* *Present address:* Department of Biophysics, Neuroinformation Research Institute, Kanazawa University School of Medicine, Kanazawa 920, Japan

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C and hence an imitator of the other product of PIP_2 breakdown, diacylglycerol [17].

2. MATERIALS AND METHODS

NG108-15 cells of 50–150 μm diameter were cultured and perfused as in [3,9]. The usual perfusion temperature was 22°C, but a higher temperature of 33–35°C was necessary to preserve responses to externally applied BK. Channel activity was recorded using cell-attached patch electrodes of tip diameter 1–2 μm (seal resistance > 10 GΩ). Electrodes (borosilicate glass) were filled with 140 mM KCl plus 5 mM Hepes, buffered to pH 7.2 with 3 M KOH ($[K^+]$ = 143 mM), unless otherwise stated. Channel currents were amplified using a List Electronics EPC-7 amplifier (filtered at 1 or 10 kHz) and stored on magnetic tape for analysis. After establishing patch-electrode seals, cells were impaled with (normally) two intracellular microelectrodes, one being filled with 1 M K^+ citrate to monitor membrane potential and the other with either 0.5 mM $InsP_3$ or 0.5 M $CaCl_2$, to allow intracellular iontophoresis of $InsP_3$ or Ca^{2+} [9,18,19]. These electrodes were coupled through an Axoclamp-2 amplifier for voltage recording and current injection. BK was applied focally to the outer cell surface in 1–3 μl volumes, expelled by pressure [9]. PDBu (Sigma) was dissolved at 10 mM in ethanol and added to the perfusion fluid at 1 μM.

3. RESULTS

3.1. *Channels activated by intracellular injections of $InsP_3$*

Fig.1 illustrates the characteristic response to intracellular iontophoresis of $InsP_3$. The injection produces a membrane hyperpolarization, resulting from the macroscopic K^+ current described previously [9,19]. Coincident with this hyperpolarization, a burst of channel activity was recorded through the cell-attached patch electrode. This channel activity was not secondary to the hyperpolarization because (i) it was not induced when the cell was hyperpolarized by passing current through the intracellular recording electrode and (ii) channel activity inside the patch persisted when the cell hyperpolarization was reduced by adding 0.5 mM dTC [9] to the bathing solution outside the patch pipette.

Responses of this type were recorded in 20 out of 29 cells tested. Channels seen most frequently had a conductance of about 40 pS (38 ± 5 pS, mean ± SE, n = 50 patches). A second, lower conductance channel of about 20 pS (18 ± 3 pS, mean ± SE, n = 33), with very long openings lasting up to several seconds, was also frequently induced (as in fig.1A), but this was less closely time-locked to the

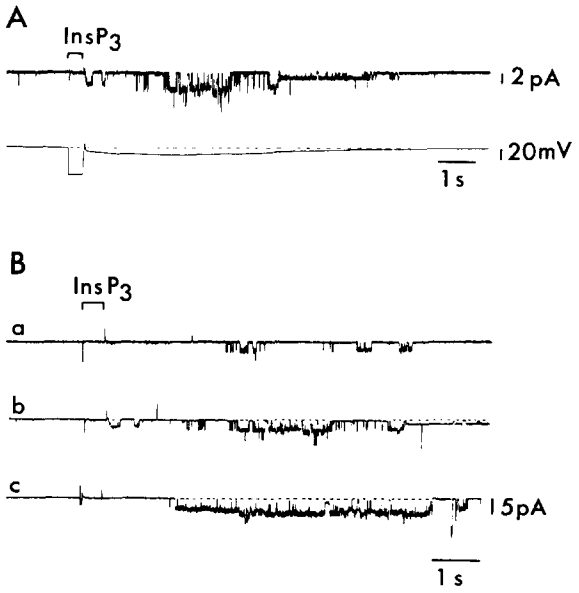

Fig.1. Single-channel currents evoked in an NG108-15 cell by intracellularly injected inositol 1,4,5-trisphosphate ($InsP_3$). $InsP_3$ was injected by iontophoresis through an intracellular microelectrode containing 0.1 mM $InsP_3$ using negative current. Membrane potential was recorded with a second intracellular microelectrode. Single-channel currents were recorded with a cell-attached patch-electrode filled with 140 mM KCl plus 5 mM KOH-buffered Hepes (143 mM $[K^+]$). The cell was bathed with DMEM (5 mM $[K^+]$) at 22°C. (A) Burst of channels recorded at a patch electrode potential of 40 mV following iontophoresis of −10 nA IP_3 for 0.4 s. Upper record, channel currents (inward current downwards); lower record, membrane potential (resting potential about −30 mV). Two classes of channel with current amplitudes 1.2 and 3.2 pA are apparent. Equivalent injections of Cl^- from KCl-filled pipettes or of positive current from IP_3-filled pipettes did not induce channels. (B) Channel currents induced by increasing iontophoretic currents of $InsP_3$ (a, −5 nA; b, −10 nA; c, −20 nA) recorded at 40 mV patch pipette potential.

hyperpolarization and required larger doses of $InsP_3$, as indicated in fig.1B. In many cells, both channels showed occasional openings at rest (i.e. before IP_3 injection): in such cases, $InsP_3$ injections increased the opening probability several-fold. In some cells (42 out of 215 active patches) infrequent openings of a larger conductance (~150 pS) channel were recorded; the activity of these channels was occasionally increased but sometimes decreased following $InsP_3$ injections.

Current-voltage curves obtained by repeated injections of $InsP_3$ at different patch pipette potentials (fig.2) or, in spontaneously active patches, by

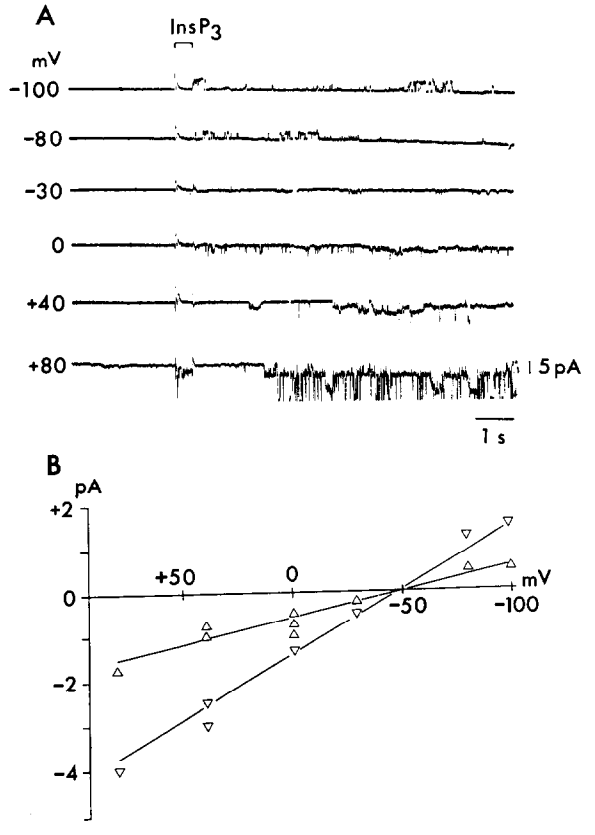

Fig.2. Conductance of $InsP_3$-activated channels. (A) Channel currents recorded following intracellular iontophoresis of −10 nA $InsP_3$ recorded at different patch-pipette potentials. Injections were made at ⩾3-min intervals, and membrane potential changes monitored to ensure approximately equal macroscopic current responses. (B) Amplitudes of single-channel currents recorded following $InsP_3$ injections plotted vs patch pipette potential. Single-channel current-voltage curves accord with two classes of channel of slope conductance 14 and 36 pS; currents through both channels inverted at about −50 mV pipette potential (i.e. near zero membrane potential).

simply varying pipette potential, were approximately linear and reversed at patch potentials between −40 and −60 mV using KCl-filled pipettes, i.e. near 0 mV membrane potential − for both 20 and 40 pS channels (fig.2B). In 17 patches with pipettes filled with 5 mM KCl, a more negative reversal potential near rest potential (i.e. zero pipette potential) was recorded. This suggests that the channels were primarily permeable to K^+, although we cannot exclude the possibility that Ca^{2+}-dependent non-specific cation channels of similar conductance previously described in neuroblastoma cells [20], which conduct K^+ as well as Na^+, might have contributed to some of the 20 pS activity. Since the pipette contained no added Ca^{2+}, $InsP_3$-activated Ca^{2+} channels [21] would not contribute significantly to channel activity.

Quantitative assessment of opening probabilities was difficult using repeated $InsP_3$ injections because of the transient nature of the $InsP_3$ effect and possible variations in the amounts of $InsP_3$ injected. However, it is clear from fig.2 that the opening probability of the 40 pS channel was not strongly dependent on voltage: if anything, activity was more pronounced at positive patch potentials (i.e. at hyperpolarized membrane potentials). Thus, these channels do not require depolarization to open. This accords with the properties of the macroscopic K^+ current induced by $InsP_3$ injection, which is readily induced at rest potential or hyperpolarized potentials [9,19].

3.2. *$InsP_3$-activated channels are also activated by intracellular Ca^{2+}*

To test whether the channel activation produced by $InsP_3$ might be due to the release of intracellular Ca^{2+}, a third intracellular pipette filled with 0.5 M $CaCl_2$ was inserted into the cell and the response to iontophoretic injection of Ca^{2+} and $InsP_3$ compared (fig.3). As reported in [9], both substances induced membrane hyperpolarization, and both induced a burst of comparable (40 pS) channels recorded with the cell-attached pipette.

3.3. *Action of extracellular bradykinin*

BK induces a rapid formation of $InsP_3$ and a transient rise in intracellular $[Ca^{2+}]$ in NG108-15 cells [3–6]. Focal application of BK produced a membrane hyperpolarization as reported previously [9], during which a pronounced increase in channel activity was recorded with cell-attached patch electrodes (fig.4). Such effects were observed in 17 out of 24 patches tested. With smaller doses of BK a clear increase in the opening probability of channels corresponding to the 40 pS channels activated by $InsP_3$ was observed (fig.4B). In some patches (13 cells) additional openings of lower conductance (10–20 pS) channels were observed. Channel activity induced by BK was strongly reduced during perfusion with Ca^{2+}-free solution: this accords with the decline in the BK-induced macroscopic

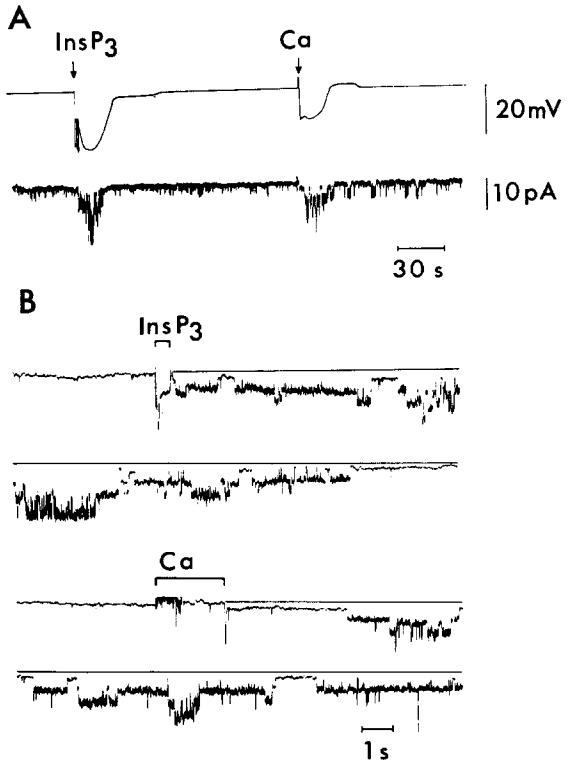

Fig.3. Ca^{2+} dependence of $InsP_3$-induced channels. A single cell was impaled with three microelectrodes: one containing 1 M K^+ citrate, to record membrane potential; one containing 0.5 mM $InsP_3$; and the third containing 0.5 M $CaCl_2$. A fourth patch electrode filled with 140 mM KCl + 5 mM Hepes was sealed to the external surface and held at 60 mV to record channel activity. Records in (A) show membrane potential change (upper trace, hyperpolarization downwards) and patch-electrode channel activity (lower trace, inward current downwards) following intracellular iontophoretic injections of $InsP_3$ and Ca^{2+} from the intracellular $InsP_3$- and Ca^{2+}-containing microelectrodes with currents of −20 nA for 0.4 s and 20 nA for 2 s, respectively (at arrows). Records in (B) show channel activity in (A) recorded at a faster speed; upper and lower traces in each segment are continuous. Note that both injections induced the opening of up to 3 channels of identical (44 pS) conductance and that this coincided with the period of membrane hyperpolarization.

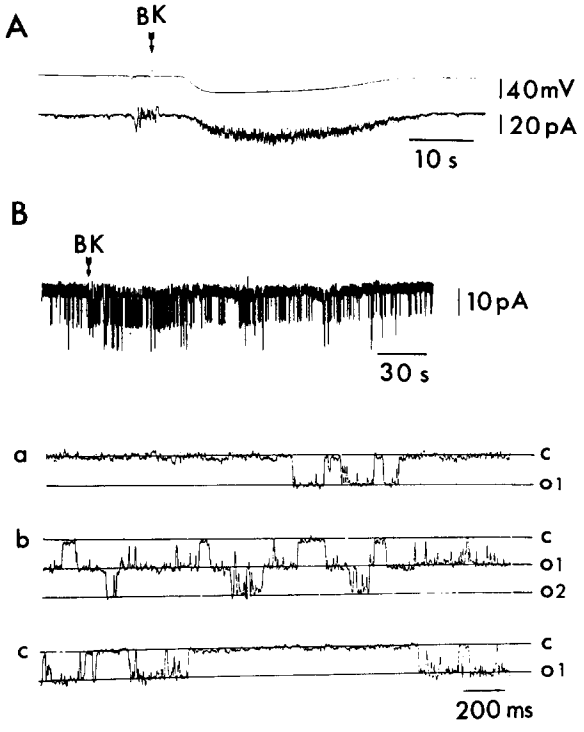

Fig.4. Channel activity in cell-attached patches induced by extracellular application of bradykinin (BK) to NG108-15 cells. (A) Membrane potential (upper trace, resting potential −35 mV) and patch pipette current (lower trace, pipette potential 0 mV) responses to a focal application by pressure of 2 μl of 20 μM BK solution. Perfusion temperature 33°C. Single-channel current vs voltage curves, obtained from spontaneously active channels seen before BK application reversed at −40 mV pipette potential and gave a slope conductance of 44 pS. BK increased the frequency of these channels and induced multiple channel activity. (B) Responses of another patch in another cell to a lower concentration of BK (2 μl of 1 μM solution). Perfusion temperature 35°C. Patch pipette potential, 80 mV. The upper record shows a continuous trace of channel activity; the lower records show faster-speed playbacks of currents recorded before (a) and 11 (b) and 50 s (c) after BK application. Single-channel current vs voltage curves reversed at −50 mV pipette potential and yielded a channel conductance of 56 pS. BK increased the opening frequency of these channels, inducing the appearance of a second superimposed channel current. (A higher recording temperature was used in these experiments because the outward current produced by BK was strongly attenuated at the temperature of 22°C used for recording responses to $InsP_3$ or Ca^{2+}. This may explain the higher single-channel conductance in these tests.)

outward current observed under these conditions [9] and probably reflects depletion of intracellular Ca^{2+} stores. Activation of channels in sealed patches by remote (extra-patch) application of BK confirms that the effect of BK is mediated by a soluble second messenger such as $InsP_3$.

3.4. *Action of phorbol dibutyrate*

A further point of correspondence between the macroscopic $InsP_3$-induced current and the 40 pS channel activity concerns the action of PDBu. It has been noted that the macroscopic current is strongly enhanced on adding PDBu to the bathing

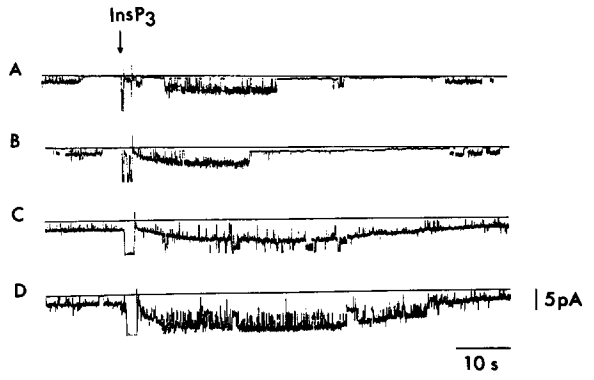

Fig.5. Effect of bath-applied phorbol dibutyrate (PDBu, 1 μM) on channel activity induced by intracellular iontophoresis of $InsP_3$ (−10 nA, 0.2 s at 3-min intervals). Patch pipette potential, 20 mV (membrane potential not measured). Records show responses to $InsP_3$ recorded (a) before adding PDBu, and (b–d) 2, 11 and 17 min, respectively, after adding PDBu. Note that addition of PDBu increased the duration of channel activity induced by $InsP_3$ and, in (c,d) led to persistent channel opening, on which the opening of a second channel is superimposed following $InsP_3$ injections. (The outward current shift and apparent increase in channel current amplitude following $InsP_3$ injections are probably the result of cell hyperpolarization – which is augmented after PDBu [18,19] – and consequently increased current driving force.)

solution [18,19]. In keeping with this, channel activity in cell-attached patches induced by $InsP_3$ injections showed a progressive increase in opening probability plus recruitment of additional channels, after adding PDBu to the bathing fluid (fig.5); eventually the activity became persistent. There was no clear increase in single-channel conductance, though channel current amplitudes sometimes increased because of the increased $InsP_3$-induced hyperpolarization (and hence increased driving force).

4. DISCUSSION

The evidence presented above suggests that the outward current activated by the stimulus pathway BK-IP_3-Ca^{2+} in NG108-15 cells is carried primarily by a set of voltage-independent but Ca^{2+}-dependent K^+ channels of approx. 40 pS conductance. Similar channel activity was induced by all three components of this pathway and coincided temporally with the hyperpolarization induced by the macroscopic outward current. Some contribution to the overall current might also be made by the lower conductance (18 pS) channels frequently recorded. On the other hand, large 'BK' channels were infrequent, and were rarely activated by $InsP_3$; this explains the resistance of the macroscopic current to TEA [9]. We cannot exclude a contribution by very small (<2 pS) [14,16] channels to the inward current sometimes seen by the patch electrode following agonist application, but this current was usually rather small compared with the total current carried by the 40 pS channels except at very high agonist concentrations.

The sensitization of the $InsP_3$-induced K^+-channel activity by PDBu accords with the increased macroscopic currents reported previously [18,19] and suggests a possible interaction between the two products of PIP_2 breakdown when these are formed together under the influence of natural stimuli such as activation of BK receptors. Since PDBu also increases the macroscopic current response to Ca^{2+} injections [19], it seems probable that the effect is mediated by a change in the properties of the Ca^{2+}-dependent channels themselves rather than a change in $InsP_3$ metabolism or Ca^{2+}-releasing activity. This effect of PDBu differs from the more usual inhibitory effect on K^+ currents [22]. In contrast, increased Ca^{2+}-channel activity by phorbol esters has been reported, resulting from the incorporation of new channels [23]. We cannot totally exclude this possibility in the present experiments, particularly as a contributory factor to the increased spontaneous channel activity, but the major factor responsible for the increased response to $InsP_3$ appeared to be an increased opening probability and number of channels normally activated by $InsP_3$. The mechanism of this effect of PDBu (and, by inference, of protein kinase C) is not yet clear.

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