Journal of Physiology (1988), **397**, *pp.* 167–184
With 11 *text-figures*
Printed in Great Britain

MEMBRANE CURRENT RESPONSES OF NG108-15 MOUSE NEUROBLASTOMA × RAT GLIOMA HYBRID CELLS TO BRADYKININ

BY DAVID A. BROWN* AND HARUHIRO HIGASHIDA†

*From the *Laboratory of Cell Biology, National Institute of Mental Health and the †Laboratory of Biochemical Genetics, National Heart, Lung, and Blood Institute, National Institutes of Health, Bethesda, MD 20892, U.S.A.*

(*Received 16 December 1986*)

SUMMARY

1. Membrane current responses to focal application of bradykinin (BK) were recorded in voltage-clamped NG108-15 neuroblastoma × glioma hybrid cells.

2. BK produced sequential outward and inward currents at clamp potentials between −60 and −30 mV, designated $I_{BK(out)}$ and $I_{BK(in)}$, respectively.

3. The outward current $I_{BK(out)}$ was accompanied by an increased membrane conductance. Ramp current–voltage (I–V) curves yielded a reversal potential (V_{BK}) of $-80 \pm 5{\cdot}6$ mV (mean ± S.D., $n = 9$) in 5·4 mM $[K^+]_o$. V_{BK} showed a positive shift on raising $[K^+]_o$, compatible with a primary increase in K^+ conductance. Subtracted I–V curves indicated that the underlying conductance was not strongly voltage dependent between −120 and −40 mV.

4. $I_{BK(out)}$ was inhibited by *d*-tubocurarine (dTC, 0·1–0·5 mM) but was insensitive to tetraethylammonium (TEA) below 5 mM.

5. The inward current $I_{BK(in)}$ was accompanied by a fall in membrane conductance. This was associated with the inhibition of a time- and voltage-dependent K^+ current, I_M. In consequence, $I_{BK(in)}$ was strongly voltage dependent and dissipated, usually without reversal, on hyperpolarizing the cell beyond −70 mV in 5·4 mM $[K^+]_o$. Reversal to an outward current negative to −40 mV could be obtained on raising $[K^+]_o$ to 54 mM.

5. Both $I_{BK(in)}$ and $I_{BK(out)}$ persisted when I_{Ca} was blocked with Co^{2+} or Cd^{2+}. $I_{BK(out)}$ slowly diminished in Ca^{2+}-free, Mg^{2+}-substituted solution.

6. The Ca^{2+} spike current I_{Ca} and the Ca^{2+}-activated K^+ current I_{AHP} were inhibited during $I_{BK(out)}$ or after Ca^{2+} injections. BK did not affect the voltage-activated K^+ current $I_{K(V)}$ recorded in Co^{2+} solution.

7. It is concluded that the dual response to BK results from opposing effects on two different species of K^+ current. $I_{BK(out)}$ results from activation of a Ca^{2+}-dependent, voltage-insensitive K^+ conductance, probably mediated by a transient rise in intracellular Ca^{2+}. It is suggested that the Ca^{2+} is released from an intracellular

* Present address: Department of Pharmacology, University College London, Gower Street, London WC1E 5BT.

† Present address: Department of Pharmacology, Cancer Research Institute, Kanazawa University, Kanazawa 920, Japan.

store. $I_{BK(in)}$ results primarily from inhibition of the Ca^{2+}-independent, voltage-gated K^+ current, I_M. This effect is not replicated by a rise of intracellular Ca^{2+} and must therefore be generated by another mechanism.

INTRODUCTION

When differentiated, mouse neuroblastoma and neuroblastoma-derived hybrid cells become electrically excitable and exhibit many of the voltage- and Ca^{2+}-gated ion currents seen in normal adult nerve cells (see e.g. Spector, 1981; Brown & Higashida, 1988*a*). They also show receptor-mediated biochemical and electrical responses to a variety of hormones and neurotransmitters, and hence can be useful models for studying their action on nerve cells (see e.g. Hamprecht, 1977; Kimhi, 1981; Nirenberg, Wilson, Higashida, Rotter, Krueger, Busis, Ray, Kenimer & Adler, 1983).

This report concerns the response of the NG108-15 clone of mouse neuroblastoma × rat glioma hybrid cells to bradykinin (BK). Membrane receptors for BK, with a dissociation constant (K_D) of about 50 pM, have been identified on these cells using radioligand binding (Braas, Manning, Wilson, Perry, Stewart, Vavrek & Snyder, 1985). Activation of these receptors induces an initial hyperpolarization followed by a slow depolarization, accompanied by cessation and activation of repetitive firing respectively (Higashida, Wilson, Adler & Nirenberg, 1978; Reiser & Hamprecht, 1982; Yano, Higashida, Inoue & Nozawa, 1984; Higashida, Streaty, Klee & Nirenberg, 1986). The specific aim of the present experiment was to identify and characterize the membrane currents responsible for this biphasic potential change. We find that they result from the activation and inhibition of two different K^+ currents: a Ca^{2+}-dependent current and a voltage-dependent current, respectively. In a companion paper (Brown & Higashida, 1988*b*), we describe additional experiments aimed at identifying the biochemical transducing mechanisms responsible for these membrane current changes. A short report of some of these observations has been published elsewhere (Higashida & Brown, 1986).

METHODS

These are described in the previous paper (Brown & Higashida, 1988*a*).

RESULTS

Focal application of BK (1–3 μl, 0·1–10 μM) to cells voltage clamped at between −40 and −50 mV usually produced two sequential membrane currents: an initial transient outward current, peaking about 5 s after application, followed by a more prolonged (0·5–3 min) inward current (Fig. 1). These corresponded in time with the sequential hyperpolarizing and depolarizing effects reported previously (Yano *et al.* 1984). These current responses showed strong tachyphyllaxis following repeated BK applications at intervals less than 5–8 min; the initial outward current appeared rather more sensitive in this respect.

Localized ionophoretic application to different regions of fully differentiated cells

indicated that the receptors mediating the outward current response were restricted to the cell soma and to the first 50–100 μm of the cell processes.

Bradykinin-induced outward current ($I_{BK(out)}$)

The initial outward current evoked by BK ($I_{BK(out)}$) was accompanied by an increased input conductance (Fig. 1*C*), as measured by the currents evoked by short

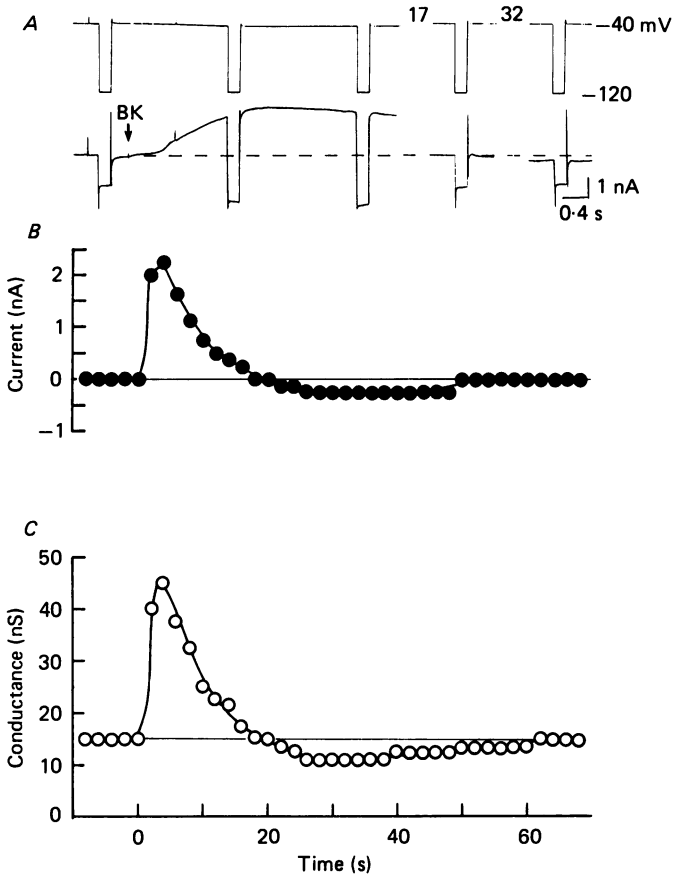

Fig. 1. Response of an NG108-15 cell to bradykinin (BK: 2 μl of 10 μM solution applied at the arrow). *A*, records of membrane potential (upper record) and membrane current (lower record). The membrane potential was stepped from the holding potential of −40 to −120 mV for 200 ms at 2 s intervals to measure input conductance. Intervals of 17 and 32 s separated the last two steps. *B* and *C*, plots of membrane current (outward current upwards) and input conductance against time after BK application. Note that BK produced first an outward current with an increased conductance then a small inward current with a decreased conductance.

(0·2–1 s) voltage steps (see Fig. 1*A*), and hence resulted from an increased conductance to an ion (K^+ and/or Cl^-) with an equilibrium potential negative to −40 mV. The reversal potential for $I_{BK(out)}$ could not easily be measured directly, since tachyphyllaxis precluded frequent applications of BK to the same cell. Instead, it was estimated from the intersection of current–voltage (*I–V*) curves obtained

before and at (or near) the peak of the BK effect, using rapid (1–4 s) voltage ramps.

Figure 2 shows a series of superimposed ramp current–voltage curves obtained by applying voltage ramps from −40 to −120 mV at −50 mV s^{-1} before and during BK

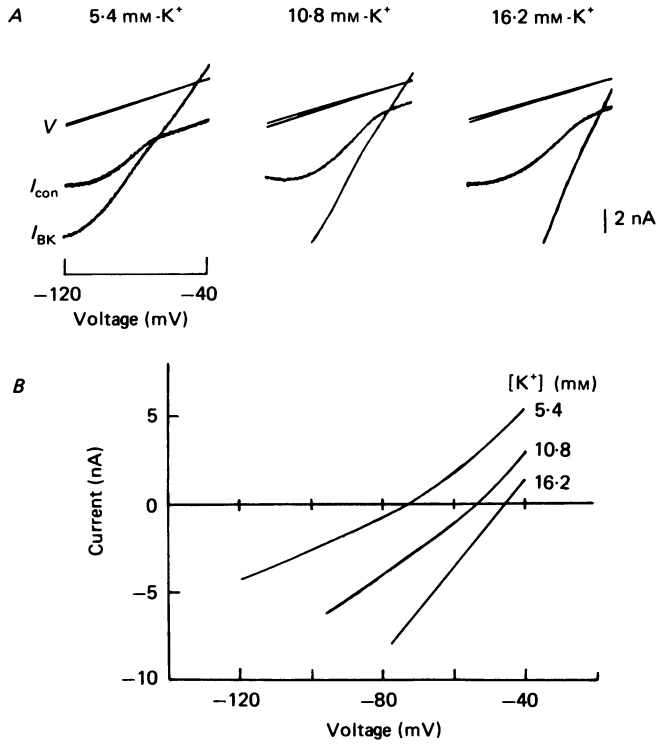

Fig. 2. *A*, current–voltage relationships recorded by applying a 2 s, −80 mV voltage ramp (*V*) from a holding potential of −40 mV before (I_{con}) and during (I_{BK}) the outward current induced by bradykinin (BK) in 5·4, 10·8 and 16·2 mM-K^+ solution. In this experiment the voltage command output was connected to the *X*-plate of the oscilloscope to give a direct plot of current (ordinate) against applied voltage (abscissa). *V* = recorded voltage; *I* = recorded current. In *B*, I_{con} was subtracted from I_{BK} to yield the BK-induced current alone. Curves cross the zero-current line at the reversal potential.

application near the peak of $I_{BK(out)}$ in solutions of differing K^+ concentration. (This voltage ramp-speed was sufficiently slow to allow time-dependent rectifying currents (see Brown & Higashida, 1988*a*) to settle.) The curves intersect implying reversal of $I_{BK(out)}$ with hyperpolarization. The intersection point clearly shifts in a positive direction as $[K^+]_o$ is increased, from −73 mV at 5·4 mM $[K^+]_o$ to −53 and −46 mV at 10·8 and 16·2 mM $[K^+]_o$. This accords quite closely with expected values (−75, −57 and −46 mV) for a K^+ current assuming $[K^+]_i = 95$ mM. The average intersection potential for these ramp currents was −80 ± 5·6 mV (mean ± S.D., $n = 9$ cells) in 5·4 mM $[K^+]_o$.

Subtraction of the control currents from these recorded in the presence of BK (Fig. 2*B*) further shows that $I_{BK(out)}$ display relatively little rectification beyond

that expected from diffusional considerations as expressed in the Goldman–Hodgkin–Katz equation. Thus, the conductance underlying $I_{BK(out)}$ appears to be essentially voltage independent.

Pharmacological characterization of $I_{BK(out)}$. Bradykinin has been reported to increase intracellular Ca^{2+} in NG108-15 hybrid cells (Reiser & Hamprecht, 1985; Osugi, Uchida, Imaizumi & Yoshida, 1986) and to increase transmembrane $^{45}Ca^{2+}$ fluxes (Yano *et al.* 1984; Higashida *et al.* 1986). One possibility, therefore, was that the outward current generated by BK might be a Ca^{2+}-activated K^+ current.

In the previous paper we presented evidence to suggest the presence of two forms of Ca^{2+}-activated K^+ current in these cells – one which deactivates rapidly following a priming Ca^{2+} current and which is blocked by 1 mM-tetraethylammonium (TEA), and which therefore appears analogous to the current in sympathetic neurones termed I_C (Adams, Constanti, Brown & Clark, 1982*c*); and another which deactivates slowly, and which is resistant to TEA at < 5 mM but blocked by apamin (see also Furuya, Furuya & Yamagishi, 1983) or *d*-tubocurarine (dTC), and which therefore is analogous to the current in sympathetic neurones designated I_{AHP} (Pennefather, Lancaster, Adams & Nicoll, 1985).

We therefore tested the effect of dTC and TEA on the outward current produced by BK, at the same time monitoring the amplitude of the Ca^{2+}-activated K^+-current tails (see Brown & Higashida, 1988*a*). *d*-Tubocurarine (0·1–0·5 mM) invariably reduced or abolished $I_{BK(out)}$ in a reversible manner *pari passu* with inhibition of the slow I_{AHP} tail current (Fig. 3). Since dTC did not reduce the late inward current, this effect was not due to block of BK receptors. Conversely, TEA produced some depression at 1 mM, but had a more substantial effect at 5 or 10 mM where I_{AHP} was also depressed. This explains the previous observation that TEA at 10 mM suppressed BK-evoked hyperpolarization (Higashida *et al.* 1986). *En passant*, we also noted that the slow outward current I_{AHP} was depressed and curtailed when recorded during $I_{BK(out)}$ (see also Fig. 10 below).

There was some discrepancy between the sensitivity of I_{AHP} and $I_{BK(out)}$ to 1 mM-TEA. This probably arises because any intrinsic antagonistic activity towards I_{AHP} is offset by an increased Ca^{2+} entry during the preceding voltage step: the Ca^{2+} spike was larger and longer in TEA solution, and could not be completely clamped in our experiments. With this exception, these pharmacological tests strongly suggest that $I_{BK(out)}$ results primarily from the activation of the same species of K^+ conductance as that responsible for the slow Ca^{2+}-activated current I_{AHP}.

If BK activates $I_{K(Ca)}$ (the Ca^{2+}-dependent K^+ current) by a rise in intracellular Ca^{2+}, the question then arises whether this results from an influx of Ca^{2+} from outside or by release from an intracellular source. This is not an easy question to answer, but we have sought a partial answer by testing the effects of inhibiting voltage-activated Ca^{2+} entry on responses to BK.

We found that $I_{BK(out)}$ persisted when voltage-activated Ca^{2+} currents and the consequential Ca^{2+}-activated K^+ currents (see above) were blocked with Cd^{2+} (1–2 mM; two cells) or Co^{2+} (2·5–5 mM; three cells) (Fig. 4). This suggests that BK does not activate voltage-dependent Ca^{2+} channels to produce a Ca^{2+} influx, and indeed the BK-induced outward current was not clearly preceded by any inward current.

On the other hand, removal of external Ca^{2+} (replaced with Mg^{2+}) did suppress

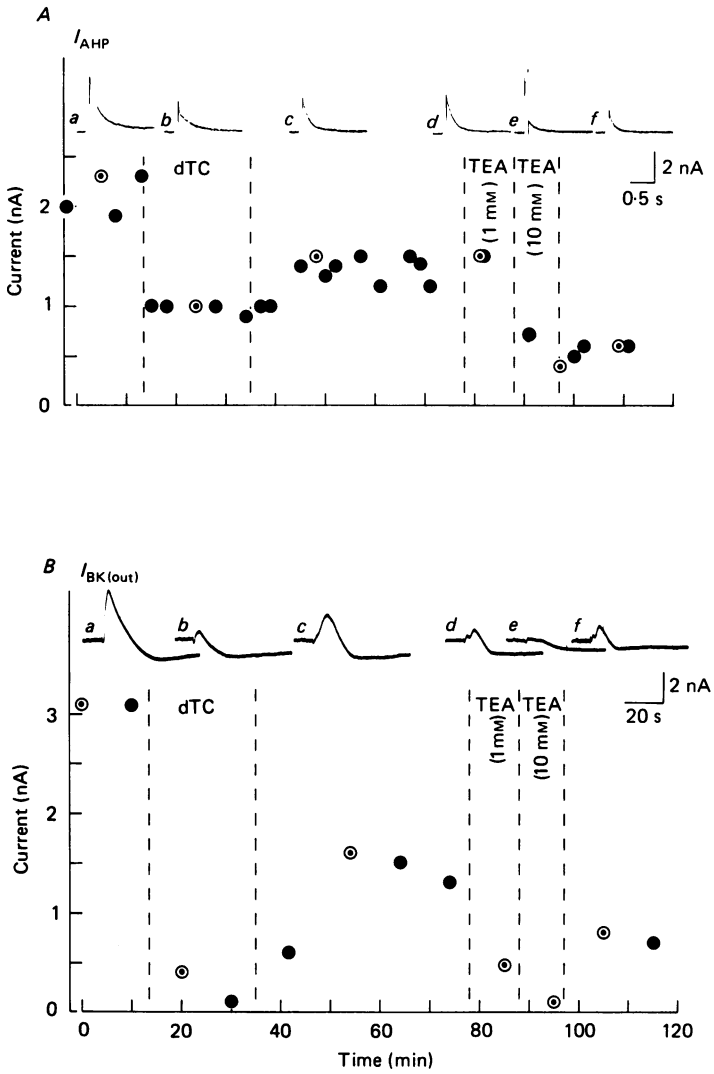

Fig. 3. Effects of *d*-tubocurarine (dTC, 0·2 mM) and tetraethylammonium (TEA, 1 and 10 mM) on slow outward Ca^{2+}-dependent tail currents generated by brief (50 ms) depolarizing voltage commands (*A*, see Brown & Higashida, 1988*a*, Fig. 2), and an outward current generated by repeated focal applications of bradykinin (BK, 2 μl, 0·1 μM) (*B*), both recorded over a period of 2 h in the same cell. Holding potential, −40 mV. Graphs show amplitudes of currents (●,⊙) plotted against time. Records *a*–*f* show selected individual responses denoted by the open circles (⊙).

$I_{BK(out)}$ (Fig. 5). However, this effect required more than 20 min perfusion with Ca^{2+}-free (EGTA) solution, whereas the Ca^{2+}-activated I_{AHP} was inhibited much more rapidly. We therefore suspect that this effect did not stem directly from the inhibition of Ca^{2+} entry but from depletion of an intracellular store. The nature of this source and the mechanism of its release is considered in a subsequent paper (Brown & Higashida, 1988*b*).

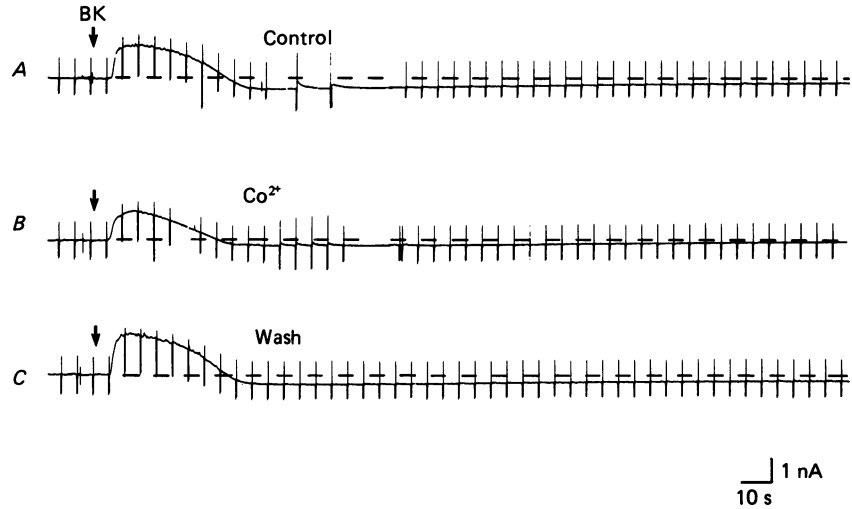

Fig. 4. Effect of adding $CoCl_2$ (Co^{2+}, 5 mM) to the external Tris-HCl-buffered saline (TBS) on responses to focal applications of bradykinin (BK, 3 μl, 100 μM) at −40 mV holding potential. BK was applied before (*A*), 13·5 min after adding Co^{2+} (*B*) and 24 min after Co^{2+} wash-out (*C*). Transient downward current deflections are inward currents generated usually by 0·2 s, −30 mV hyperpolarizing steps, to measure input conductance.

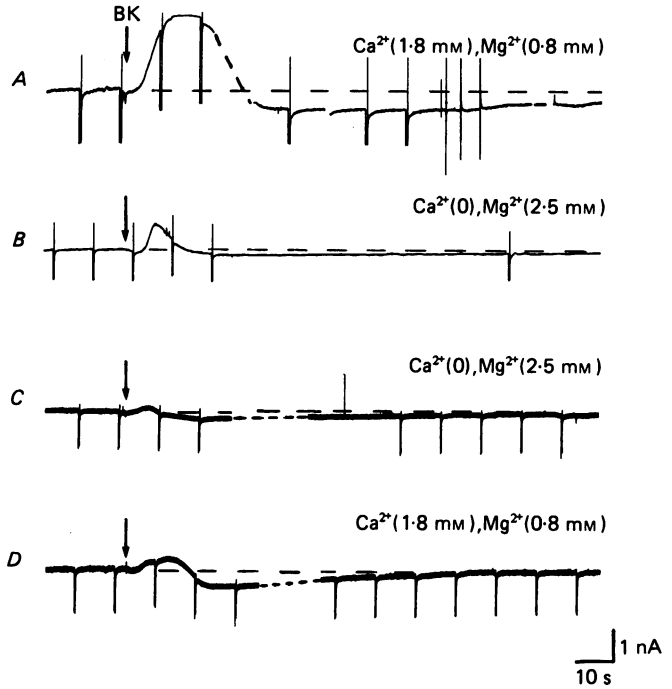

Fig. 5. Current responses to focal applications of bradykinin (BK, 2 μl, 100 μM) recorded in normal Tris-HCl-buffered saline (TBS) (containing 1·8 mM-Ca^{2+} and 0·8 mM-Mg^{2+}) (*A*), then 14 and 24 min after perfusing with a Ca^{2+}-free, 6 mM-Mg^{2+} solution (*B* and *C*), and finally 10 min after returning to normal TBS (*D*). Holding potential −40 mV. (The recorder was changed between *B* and *C*, hence the increased baseline noise.)

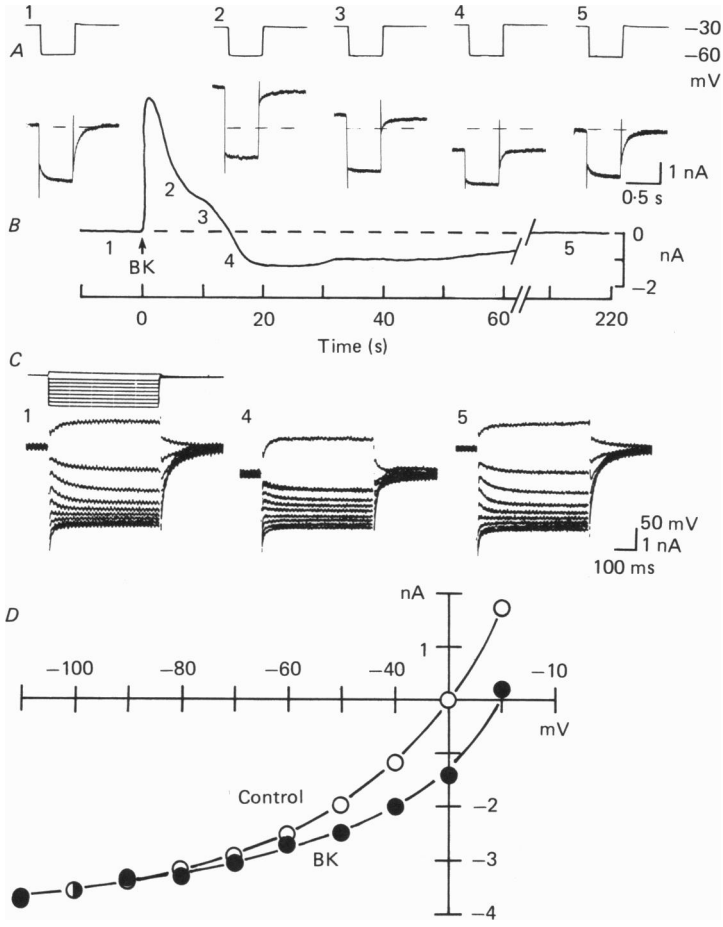

Fig. 6. Response to bradykinin (BK, 2 μl, 10 μM) in an NG108-15 cell clamped at −30 mV. *A*, current transients (lower trace) to −30 mV, 0·5 s voltage commands (upper trace) recorded before BK (1), during the outward component of net BK-induced current (2 and 3), during the inward BK current (4), and on recovery (5). *B*, trace of net current against time, numbers indicating the stages appropriate to the records in *A* and *C*. *C*, families of current transients in response to +10, and −10 to −80 mV, 500 ms voltage steps recorded before (1), during (4) and after (5) the BK-induced inward current. *D*, plots of currents attained at the ends of the voltage steps in *C* against command voltage obtained before (○) and during (●) the BK-induced inward current (i.e. 'steady-state' current–voltage currents). (Zero current = initial holding current; same cell as that illustrated in Fig. 8 of Brown & Higashida, 1988*a*.)

Bradykinin-induced inward current ($I_{BK(in)}$)

As pointed out earlier the initial outward current produced by BK usually reversed to an inward current after a few seconds. Unlike the outward current, this inward current ($I_{BK(in)}$) was accompanied by a fall in input conductance.

Figure 6 illustrates some of the properties of this inward current as revealed from current–voltage curves. Since the inward current lasted longer than the outward current, these curves were constructed from sequences of stepped voltage commands

instead of ramp commands. (Responses to stepped commands are more useful because they also provide information about time-dependent current changes, as well as 'steady-state' currents.) Two points warrant attention. Firstly, unlike $I_{BK(out)}$, $I_{BK(in)}$ showed strong rectification (Fig. 6*D*). Thus, the current–voltage curve obtained during $I_{BK(in)}$ does not cross the control curve to reverse; instead, the two

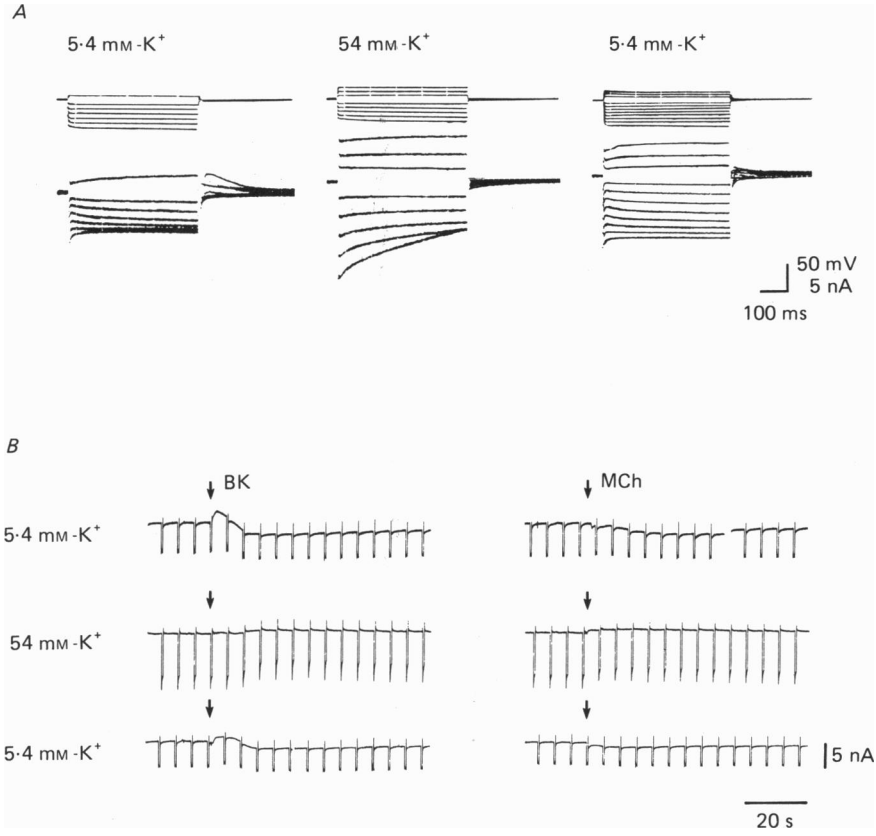

Fig. 7. *A*, families of current transients (lower records) evoked by a series of voltage steps (upper records) in (sequentially) 5·4, 54 and 5·4 mM [K^+] (cf. Fig. 6). Holding potential, −30 mV. *B*, current responses to focal applications of bradykinin (BK, 2 μl, 10 μM) or methacholine (MCh, 2 μl, 300 mM) and recorded sequentially in 5·4, 54 and 5·4 mM [K^+]. Downward deflections are inward currents generated by −20 mV, 1 s hyperpolarizing steps at 0·2 Hz. Note that the reversal potential for the current relaxations in *A* shifted from −80 mV to a value slightly positive to the holding potential on raising [K^+].

curves merge at potentials negative to about −70 mV. In consequence, the inward current produced by BK (measured by subtraction of the two current–voltage curves) decreases with hyperpolarization without reversing to an outward current. The usual absence of true reversal with hyperpolarization (in solution containing the normal K^+ concentration of 5·4 mM) was confirmed using ramp voltage commands. Secondly, the fall in conductance is associated with the reduction of the time-dependent inward current relaxations seen during the hyperpolarizing steps (Fig. 6*A* and *C*).

As pointed out in the preceding paper (Brown & Higashida, 1988*a*), these inward relaxations probably result from time-dependent deactivation of the voltage-dependent K^+ current, I_M (cf. Brown & Adams, 1980). Reduction of these relaxations therefore implies that I_M is partly blocked during $I_{BK(in)}$. If inhibition of I_M was responsible for $I_{BK(in)}$, this could explain why $I_{BK(in)}$ does not normally reverse to an outward current with hyperpolarization, because at normal K^+ concentrations, I_M fully deactivates before the K^+ reversal potential (E_K) is attained (see Adams, Brown & Constanti, 1982*a*). A corollary to this is that reversal might be possible if E_K is raised to within the activation range of I_M. We tested this by raising $[K^+]_o$ to 54 mM

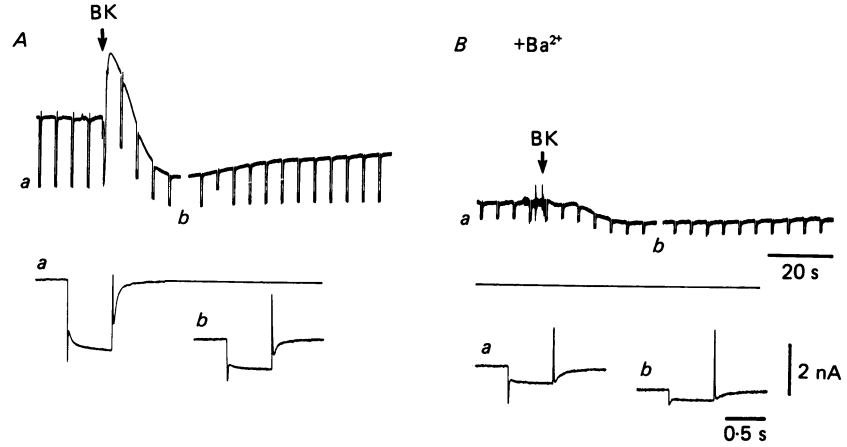

Fig. 8. Responses to bradykinin (BK, 2 μl, 10 μM) recorded before (*A*) and after (*B*) adding 4 mM-$BaCl_2$ to the perfusion fluid. Holding potential, −30 mV. Upper traces show continuous records of steady current, with superimposed inward current transients generated by −20 mV, 0·5 s voltage steps delivered at 0·2 Hz. Lower traces show expanded records of current transients obtained at the indicated times (*a* and *b*); records are offset from the initial baseline (horizontal line) by the amount of inward current.

(Fig. 7). This raised the reversal potential for I_M from about −80 mV to about −20 mV – i.e. 10 mV or so positive to the original holding potential. Under these conditions the original inward currents produced by BK (or methacholine, see Brown & Higashida, 1988*a*) at the holding potential reversed to a small outward current (Fig. 7*B*). Since the conductance was still reduced, the envelope of outward current at the hyperpolarized command potential of −50 mV became larger.

The inward relaxations seen in 54 mM $[K^+]$ during hyperpolarizing steps (Fig. 7*A*) were larger and slower than these expected for I_M deactivation alone. These slow relaxations were frequently encountered in high-$[K^+]$ solution, and sometimes in normal (5·4 mM) $[K^+]$. They appear to relate to some form of additional inward rectifier current.

Ba^{2+} ions. As in sympathetic neurones (Constanti, Adams & Brown, 1981) M-currents in NG108-15 cells are inhibited by Ba^{2+} ions (Brown & Higashida, 1988*a*). Additions of Ba^{2+} (1–4 mM) reduced both inward and outward current components of the response to BK (Fig. 8).

Role of Ca^{2+} in $I_{BK(in)}$. The initial outward current induced by BK appears to be a species of Ca^{2+}-dependent K^+ current, probably activated by Ca^{2+} released from an

intracellular source, and can be imitated by injecting Ca^{2+} (see above). The obvious question is therefore whether $I_{BK(in)}$ (and hence I_M inhibition) is also due to the release of Ca^{2+}. Calcium-injection experiments suggest not. As pointed out previously (Brown & Higashida, 1988*a*), the outward current produced by Ca^{2+} injections was frequently followed by an inward current, but this was accompanied by an *increased* conductance, not a decrease. Further, I_M deactivation relaxations could still be detected, superimposed on the instantaneous current steps, when long hyperpolarizing steps were applied during both the initial outward current and the subsequent inward current induced by Ca^{2+} injections. (Some loss of relaxation amplitude was sometimes seen, particularly during large Ca^{2+}-evoked currents. This

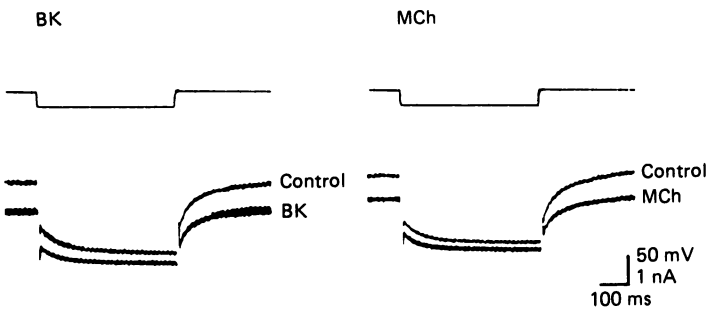

Fig. 9. Inward current responses to bradykinin (BK, 2 μl, 10 μM) and methacholine (MCh, 2 μl, 200 mM) recorded in 2 mM-$CoCl_2$ solution (same cell as that illustrated in Fig. 10 in Brown & Higashida, 1988*a*). Records show superimposed current responses to −30 mV voltage steps recorded before and after adding BK and MCh. Holding potential −30 mV. Note that the inward current relaxations are preserved in Co^{2+} solution and are depressed by both BK and MCh (cf. Fig. 6).

is a common feature of 'leaky' ganglion cells (see for example, Jones, Adams, Brownstein & Rivier, 1984): it might reflect rapid perineuronal K^+ concentration changes, and hence changes in E_K, during the larger current flows encountered in leaky cells.)

A secondary BK-induced inward current accompanied by a conductance increase, which corresponded more clearly to that seen after Ca^{2+} injections, was recorded in a few cells following large BK-induced inward currents. However, this was infrequent and was not studied in detail.

Unlike I_{AHP} and I_C, I_M is not a Ca^{2+}-activated current in the sense of requiring a priming Ca^{2+} current, and hence persists when I_{Ca} is blocked with Cd^{2+} or Co^{2+} (Adams, Brown & Constanti, 1982*b*). We confirmed that I_M relaxations persisted in NG108-15 cells in the presence of Cd^{2+} (0·5–2 mM) or Co^{2+} (2–5 mM) (Brown & Higashida, 1988*a*). As seen in Fig. 4, BK continued to produce an inward current in Co^{2+} (or Cd^{2+}) solution and – as shown in Fig. 9 – this was accompanied by an inhibition of I_M, just as in normal solution. The inward current also persisted in Ca^{2+}-free solution to a greater extent than the outward current, although somewhat attenuated (Fig. 5).

In some cells, the inward currents produced by BK were smaller than those recorded before adding Cd^{2+} or Co^{2+}, suggesting that BK was capable of inhibiting

a component of membrane conductance other than the M-conductance. In keeping with this, in a small proportion of cells, ramp current–voltage curves suggested a reversal of the inward BK-induced currents to an outward current at potentials between −50 and −80 mV. Methacholine and Ba^{2+} ions sometimes produced the same effect (see Brown & Higashida, 1988*a*).

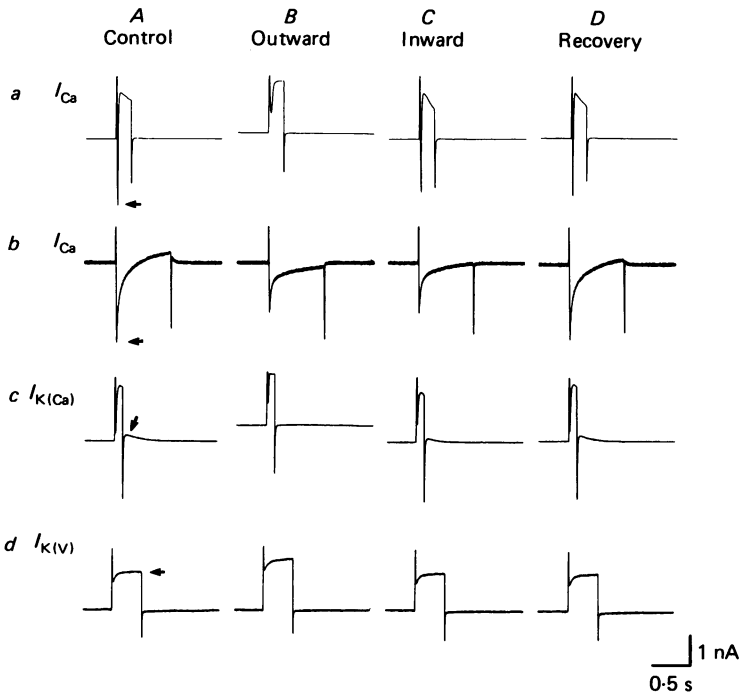

Fig. 10. Membrane currents recorded before bradykinin (BK, 2 μl, 10 μM) (*A*), during the outward phase of a BK-induced current (*B*), during the inward phase of the BK-induced current (*C*), and after BK (*D*) in four cells. *a*, cell 1 (I_{Ca}): the current was recorded during perfusion with a high-Ca^{2+}, 0 mM [Na^+] plus 1 μM-tetrodotoxin solution (20 mM-$CaCl_2$ and 150 mM-choline chloride instead of NaCl). Holding potential, −70 mV; voltage step, +70 mV, 150 ms. *b*, cell 2 (I_{Ca}): current recorded from a cell treated for about 30 min in the same solution as for cell 1 but additionally supplemented with 10 mM-tetraethylammonium and 20 μM-quinine. Holding potential, −50 mV; +70 mV, 1 s depolarization. *c*, cell 3 ($I_{K(Ca)}$): recorded in the medium for cell 2 but without quinine. Holding potential, −40 mV; +50 mV, 20 ms depolarization step. (The recorder saturated during the outward current in panel *B*.) *d*, cell 4 ($I_{K(V)}$): the current recorded in a 0 mM [Na^+], 0 mM [Ca^{2+}] solution with 1 μM-tetrodotoxin and 2 mM-$CoCl_2$. Holding potential, −50 mV; depolarizing step, +70 mV, 0·5 s.

Effects of bradykinin on endogenous membrane currents

In this section we describe some experiments testing whether bradykinin affected some of the other endogenous voltage-dependent and Ca^{2+}-dependent currents in NG108-15 cells. These currents include a fast Na^+ current (I_{Na}), Ca^{2+} currents (I_{Ca}), a voltage-activated K^+ current ($I_{K(V)}$), and two forms of Ca^{2+}-activated K^+ current ($I_{K(Ca)}$) (see for examples, Kostyuk, Krishtal, Pidoplichko & Veselovsky, 1978; Moolenaar & Spector, 1978, 1979*a*, *b*; Fishman & Spector, 1981; Spector, 1981;

Brown & Higashida, 1988*a*). We could not clamp I_{Na} adequately, so it was eliminated with Na^+-free solution and tetrodotoxin (1 μM) and we concentrated attention on I_{Ca}, $I_{K(Ca)}$ and $I_{K(V)}$. The principal effects of BK on these currents are summarized in Fig. 10.

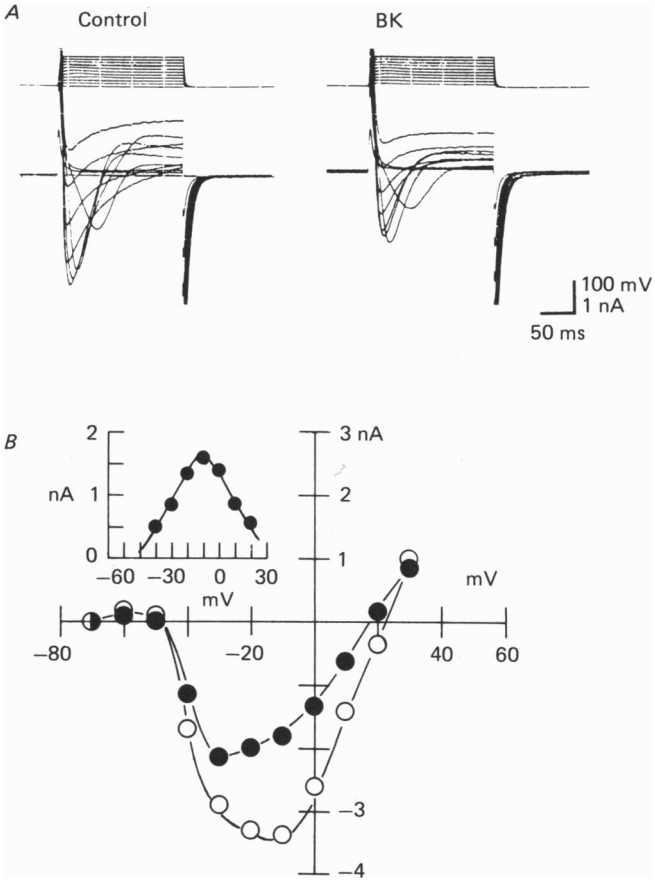

Fig. 11. *A*, family of Ca^{2+} currents recorded before and after applying bradykinin (2 μl, 10 μM). Currents (lower records) were evoked by 200 ms voltage steps (upper records) of +10 to +100 mV from a holding potential of −70 mV in a cell perfused with a same solution as used for recording cell 2 of Fig. 10. *B*, plots of peak inward current against command voltage from the records in *A*. Control, ○; BK, ●. Inset shows the difference current (BK minus control).

I_{Ca}. Two forms of Ca^{2+} current have been reported in N1E-115 neuroblastoma and NG108-15 hybrid cells: a rapidly inactivating low-threshold current, and a more slowly inactivating high-threshold current (see Moolenaar & Spector, 1979*b*; Fishman & Spector, 1981; Bodewi, Hering, Schubert & Wollenberger, 1985; Docherty, 1986; Narahashi, Tsunoo & Yoshii, 1987). To activate these two current components we used protocols based on those of Moolenaar & Spector (1979*b*) and Fishman & Spector (1981): namely, depolarizing steps from a hyperpolarized potential (−70 mV) to activate the rapidly inactivating 'low-threshold' component

(Fig. 10, row *a*; Fig. 11), and from a more depolarized potential (−50 mV) in the added presence of 10 mM-TEA and 100 μM-quinidine to activate a more slowly decaying form of I_{Ca} (Fig. 10, row *b*). (We have no firm evidence to show whether these are different currents, or whether the larger outward current in the absence of quinidine simply masked the residual current.) Both currents were readily blocked by Cd^{2+} or Co^{2+}.

I_{Ca} was strongly depressed during the outward phase of BK-induced current (Fig. 10*B* and *C*). This depression carried over to a variable extent into the second (inward current) phase of BK action, but was clearly less. The depression did not appear to result from shunting by the BK-induced outward current since the outward currents generated during the depolarizing steps were actually reduced (Fig. 11). Examination of current–voltage curves showed that the depression was approximately proportional to the peak Ca^{2+} current amplitude, further suggesting an effect on I_{Ca} itself (Fig. 11*B*).

I_{AHP}. This slow component of $I_{K(Ca)}$ was recorded as a slow outward tail current following brief (50–100 ms) depolarizing commands to between 0 and +20 mV from a holding potential of −50 mV, as described previously (Brown & Higashida, 1988*a*; see also Fig. 3). I_{AHP} showed a pronounced depression during the BK-induced outward current (Fig. 10 row *c*), which usually reversed during the BK-induced inward current.

$I_{K(V)}$. This was recorded as the residual outward current produced by large depolarizing commands to +20 mV after eliminating $I_{K(Ca)}$ with 10 mM-Co^{2+}. No change in $I_{K(V)}$ was to be detected after applying BK: instead the additional BK-induced current simply added an extra 'instantaneous' component to the evoked currents (Fig. 10, row *d*).

DISCUSSION

The main conclusion from these experiments is that bradykinin (BK) produces sequential outward and inward currents in neuroblastoma and neuroblastoma-derived hybrid cells, and that these result from changes in two quite different K^+ conductances: the outward current ($I_{BK(out)}$) results from activation of a Ca^{2+}-dependent but voltage-insensitive K^+ conductance, whereas the inward current seems to result, in part at least, from inhibition of a Ca^{2+}-independent but voltage-dependent K^+ conductance similar to (and probably identical with) the M-conductance originally described in sympathetic neurones (see Brown & Adams, 1980; Adams *et al.* 1982*a*).

Outward current ($I_{BK(out)}$)

$I_{BK(out)}$ is probably a K^+ current since it reverses with hyperpolarization and the reversal potential varies in an approximately Nernstian manner with external [K^+]. Two features characterize the current: firstly, the underlying conductance seems not to vary strongly with voltage; and secondly, the current is preferentially blocked by *d*-tubocurarine (dTC) rather than by tetraethylammonium (TEA). These features suggest that the current is analogous to that subspecies of Ca^{2+}-activated K^+ current

termed (in sympathetic neurones) I_{AHP} (Pennefather *et al.* 1985), to distinguish it from the TEA-sensitive, voltage-dependent current I_C (Adams *et al.* 1982*c*; Brown, Constanti & Adams, 1983). A current of this type can be readily induced in NG108-15 cells either after a priming Ca^{2+} current or by injecting Ca^{2+} ions (Brown & Higashida, 1988*a*). The absence of strong voltage dependence is an important feature in explaining why BK can induce a hyperpolarization at resting potentials. This effect of BK seems analogous to its action on intestinal smooth muscle which is also inhibited by dTC (Gater, Haylett & Jenkinson, 1985).

Concerning the source of Ca^{2+} for this response, Reiser & Hamprecht (1985) and Osugi *et al.* (1986) have previously reported that BK produces a transient rise in $[Ca^{2+}]_i$ of 2–3 times, as measured by quin-2 fluorescence, with a time course comparable to that of the outward current. Since $I_{BK(out)}$ was not preceded by an inward current and was not blocked by the Ca^{2+} channel blockers Cd^{2+} or Co^{2+}, it seems unlikely that the outward current depends on prior activation of a Ca^{2+} current by BK. In agreement with this, Osugi *et al.* (1986) noted that the transient Ca^{2+} signal induced by BK persisted in the presence of verapamil and nifedipine, which can inhibit $^{45}Ca^{2+}$ fluxes and the slow Ca^{2+} current in NG108-15 cells (Nirenberg *et al.* 1983; Freedman, Dawson, Villereal & Miller, 1984; R. J. Docherty & R. J. Miller, unpublished observations). The effects of removing external Ca^{2+} appear more complex. Reiser & Hamprecht (1985) reported that the rise in intracellular Ca^{2+} was inhibited in a Ca^{2+}-free solution containing 50 μM-EGTA. Osugi *et al.* (1986) reported that the initial transient rise persisted down to 10 μM $[Ca^{2+}]_o$ but was blocked at 1 μM $[Ca^{2+}]_o$. We find that $I_{BK(out)}$ gradually diminishes in a 'Ca^{2+}-free', Mg^{2+}-substituted medium. A possible reason for the slow and variable effects of reducing external $[Ca^{2+}]$ is that BK releases Ca^{2+} from an internal store which becomes depleted in Ca^{2+}-free solutions.

Both the voltage-activated Ca^{2+} current and the subsequent Ca^{2+}-activated K^+ current were strongly depressed during the outward current induced by BK. Ca^{2+} injections could produce similar effects (Brown & Higashida, 1988*a*, and unpublished), so a plausible explanation is that the Ca^{2+} current might be inhibited by the rise in internal $[Ca^{2+}]$. Ca^{2+}-induced I_{Ca} inactivation has been reported in a number of cells (see Eckert & Chad, 1984). Although Quandt & Narahashi (1984) could not find evidence for such a form of I_{Ca} inactivation in neuroblastoma cells, their method of internal dialysis presumably buffered $[Ca^{2+}]_i$ to a constant low level.

Application of BK did not fully reproduce the effect of Ca^{2+} injections because the latter often induced a secondary inward current which (unlike that produced by BK) was accompanied by an increased conductance (Brown & Higashida, 1988*a*). We presume it was associated with higher Ca^{2+} concentrations following Ca^{2+} injections than those attained by applying BK.

Inward current ($I_{BK(in)}$)

Our principal evidence that $I_{BK(in)}$ might arise from M-current inhibition is that (a) the time-dependent current relaxations associated with M-current deactivation and activation (see Adams *et al.* 1982*a*) were depressed during the inward current; (b) $I_{BK(in)}$ was voltage dependent and did not usually reverse with hyperpolarization at

normal values of $[K^+]_o$ (because I_M deactivates positive to E_K); (c) the inward current was replicated by applying muscarinic acetylcholine receptor agonists or Ba^{2+} (Brown & Higashida, 1988*a*), which inhibit I_M in ganglion cells (Adams *et al.* 1982*b*): and (d) $I_{BK(in)}$ was strongly reduced after inhibiting I_M with Ba^{2+}.

In some cells, the inhibition of I_M was sufficient in itself to account fully for the inward current. In others, however, the current–voltage curves obtained before and during BK application appeared to cross, implying reversal to an outward current at potentials where I_M is deactivated, and suggesting an additional component of current inhibition at hyperpolarized potentials as in sympathetic neurones (see Brown & Selyanko, 1985). Notwithstanding, I_M inhibition was the most consistent feature during $I_{BK(in)}$.

It should be emphasized that I_M is a quite different current from the Ca^{2+}-dependent current I_{AHP} activated during the initial phase of BK action. (a) The conductance underlying I_M is strongly voltage sensitive whereas that underlying I_{AHP} is not. (b) I_M is not blocked by dTC. (c) I_M is not Ca^{2+} dependent in the sense of requiring a priming Ca^{2+} current: it persisted after addition of Cd^{2+} or Co^{2+} (Brown & Higashida, 1988*a*). (Occasionally divalent cations produced some inhibition of I_M but this probably results from a degree of non-specificity in their effect, unrelated to I_{Ca} inhibition: see Adams *et al.* 1982*b*.)

I_M was not strongly inhibited following Ca^{2+} injections. Further, and as noted previously (Brown & Higashida, 1988*a*), an inward current after such injections was accompanied by an increased conductance, not a decrease. This suggests that I_M inhibition, and hence $I_{BK(in)}$, is not due to the rise in intracellular $[Ca^{2+}]$, unlike $I_{BK(out)}$, but must be triggered by a different mechanism. This is reinforced by the observation that cholinergic agonists replicate $I_{BK(in)}$ without inducing a preceding outward current (Brown & Higashida, 1988*a*).

Receptors

One relatively straightforward explanation for the ability of BK to differentially change two separate K^+ currents would be their mediation by two different populations of BK receptors. Although difficult to totally exclude, we think this unlikely. Thus, in parallel tests on unclamped cells (H. Higashida, unpublished results), where BK produces a hyperpolarization–depolarization sequence (Yano *et al.* 1984), both components of response persisted in the presence of the BK antagonists [des-Arg^9]-BK and [Leu^8, des-Arg^9]-BK (Regoli, Barabe & Park, 1977). This suggests that both responses are mediated by the general class of BK_2 receptors (see Regoli & Barabe, 1980). This accords with the observation that the hydrolysis of polyphosphoinositides (which probably triggers both neuroblastoma responses: see Brown & Higashida, 1988*b*) is resistant to 'BK_1' antagonists in other tissue (Derian & Maskowitz, 1986). Notwithstanding, a separation of the two responses could sometimes be obtained following repeated applications of BK: the initial outward current showed stronger tachyphyllaxis than the inward current, eventually leaving a 'pure' inward current. This differential tachyphyllaxis may, however, reside in the different components of the transduction mechanism, rather than in different receptor desensitization.

We thank Dr Marshall Nirenberg for his support and for the use of laboratory facilities. This work was undertaken as part of a Fogarty Scholar-in-residence programme during tenure of a Fogarty Scholarship by D.A.B.

REFERENCES

ADAMS, P. R., BROWN, D. A. & CONSTANTI, A. (1982*a*). M-currents and other potassium currents in bullfrog sympathetic neurones. *Journal of Physiology* **330**, 537–572.

ADAMS, P. R., BROWN, D. A. & CONSTANTI, A. (1982*b*). Pharmacological inhibition of the M-current. *Journal of Physiology* **332**, 223–262.

ADAMS, P. R., CONSTANTI, A., BROWN, D. A. & CLARK, R. B. (1982*c*). Intracellular Ca^{2+} activates a fast voltage-sensitive K^+ current in vertebrate sympathetic neurones. *Nature* **296**, 746–749.

BODEWEI, R., HERING, S., SCHUBERT, B. & WOLLENBERGER, A. (1985). Sodium and calcium currents in neuroblastoma × glioma hybrid cells before and after morphological differentiation by dibutyryl cyclic AMP. *General Physiology and Biophysics* **4**, 113–127.

BRAAS, K. M., MANNING, D. C., WILSON, V. S., PERRY, D. C., STEWART, J. M., VAVREK, R. J. & SNYDER, S. H. (1985). Characterization of bradykinin antagonists in a cultured neuronal cell line. *Society for Neuroscience Abstracts* **11**, 414.

BROWN, D. A. & ADAMS, P. R. (1980). Muscarinic suppression of a novel voltage-sensitive K^+ current in a vertebrate neurone. *Nature* **283**, 673–676.

BROWN, D. A., CONSTANTI, A. & ADAMS, P. R. (1983). Ca-activated potassium current in vertebrate sympathetic neurones. *Cell Calcium* **4**, 407–420.

BROWN, D. A. & HIGASHIDA, H. (1988*a*). Voltage- and calcium-activated potassium currents in mouse neuroblastoma × rat glioma hybrid cells. *Journal of Physiology* **397**, 149–165.

BROWN, D. A. & HIGASHIDA, H. (1988*b*). Inositol 1,4,5-trisphosphate and diacylglycerol mimic bradykinin effects on mouse neuroblastoma × rat glioma hybrid cells. *Journal of Physiology*, **397**, 185–207.

BROWN, D. A. & SELYANKO, A. A. (1985). Two components of muscarine-sensitive membrane current in rat sympathetic neurones. *Journal of Physiology* **358**, 335–363.

CONSTANTI, A., ADAMS, P. R. & BROWN, D. A. (1981). Why do barium ions imitate acetylcholine? *Brain Research* **206**, 244–250.

DERIAN, C. K. & MOSKOWITZ, M. A. (1986). Polyphosphoinositide hydrolysis in endothelial cells and carotid artery segments. Bradykinin-2 receptor stimulation is calcium-independent. *Journal of Biological Chemistry* **261**, 3831–3837.

DOCHERTY, R. J. (1986). Gadolinium specifically blocks a component of voltage-sensitive Ca current in neuroblastoma × glioma hybrid (NG108-15) cells. *Journal of Physiology* **381**, 69*P*.

ECKERT, R. & CHAD, J. E. (1984). Modulation of Ca channels. *Progress in Biophysics and Molecular Biology* **44**, 215–267.

FISHMAN, M. C. & SPECTOR, I. (1981). Potassium current suppression by quinidine reveals additional calcium currents in neuroblastoma cells. *Proceedings of the National Academy of Sciences of the U.S.A.* **78**, 5245–5249.

FREEDMAN, S. B., DAWSON, G., VILLEREAL, M. L. & MILLER, R. J. (1984). Identification and characterization of voltage-sensitive calcium channels in neuronal clonal cell lines. *Journal of Neuroscience* **4**, 1453–1467.

FURUYA, K., FURUYA, S. & YAMAGISHI, S. (1983). Developmental time courses of Na and Ca spikes in neuroblastoma × glioma hybrid cells. *Developmental Brain Research* **11**, 229–234.

GATER, P. R., HAYLETT, D. G. & JENKINSON, D. H. (1985). Neuromuscular blocking agents inhibit receptor-mediated increases in the potassium permeability of intestinal smooth muscle. *British Journal of Pharmacology* **86**, 861–868.

HAMPRECHT, B. (1977). Structural, electrophysiological and pharmacological properties of neuroblastoma–glioma cell hybrids in cell culture. *International Review of Cytology* **49**, 99–170.

HIGASHIDA, H. & BROWN, D. A. (1986). Two polyphosphatidylinositide metabolites control two K^+ currents in a neuronal cell. *Nature* **323**, 333–335.

HIGASHIDA, H., STREATY, R. A., KLEE, W. & NIRENBERG, M. (1986). Bradykinin-activated transmembrane signals are coupled via No or Ni to production of inositol 1,4,5-trisphosphate, a

second messenger in NG108-15 neuroblastoma–glioma hybrid cells. *Proceedings of the National Academy of Sciences of the U.S.A.* **83**, 942–946.

HIGASHIDA, H., WILSON, S. P., ADLER, M. & NIRENBERG, M. (1978). Synapse formation by neuroblastoma hybrid cell lines. *Society for Neuroscience Abstracts* **4**, 591.

JONES, S. W., ADAMS, P. R., BROWNSTEIN, M. J. & RIVIER, J. E. (1984). Teleost luteinizing hormone-releasing hormone: action on bullfrog sympathetic ganglia is consistent with role as neurotransmitter. *Journal of Neuroscience* **4**, 420–429.

KIMHI, Y. (1981). Nerve cells in clonal systems. In *Excitable Cells in Tissue Culture*, ed. NELSON, P. G. & LIEBERMAN, M., pp. 173–245. New York. Plenum Press.

KOSTYUK, P. G., KRISHTAL, O. A., PIDOPLICHKO, V. I. & VESELOVSKY, N. S. (1978). Ionic currents in the neuroblastoma cell membrane. *Neuroscience* **3**, 327–332.

MOOLENAAR, W. H. & SPECTOR, I. (1978). Ionic currents in cultured mouse neuroblastoma cells under voltage-clamp conditions. *Journal of Physiology* **278**, 265–286.

MOOLENAAR, W. H. & SPECTOR, I. (1979*a*). The calcium action potential and a prolonged calcium dependent after-hyperpolarization in mouse neuroblastoma cells. *Journal of Physiology* **292**, 297–306.

MOOLENAAR, W. H. & SPECTOR, I. (1979*b*). The calcium current and the activation of a slow potassium conductance in voltage-clamped mouse neuroblastoma cells. *Journal of Physiology* **292**, 307–323.

NARAHASHI, T., TSUNOO, A. & YOSHII, M. (1987). Characterization of two types of calcium channels in mouse neuroblastoma cells. *Journal of Physiology* **383**, 231–249.

NIRENBERG, M., WILSON, S., HIGASHIDA, H., ROTTER, A., KRUEGER, K., BUSIS, N., RAY, R., KENIMER, J. G. & ADLER, M. (1983). Modulation of synapse formation by cyclic adenosine monophosphate. *Science* **222**, 794–799.

OSUGI, T., UCHIDA, S., IMAIZUMI, T. & YOSHIDA, H. (1986). Bradykinin-induced intracellular Ca^{2+} elevation in neuroblastoma × glioma hybrid NG108-15 cells; relationship to the action of inositol phospholipids metabolites. *Brain Research* **379**, 84–89.

PENNEFATHER, P., LANCASTER, B., ADAMS, P. R. & NICOLL, R. A. (1985). Two distinct Ca-dependent K currents in bullfrog sympathetic ganglion cells. *Proceedings of the National Academy of Sciences of the U.S.A.* **82**, 3040–3044.

QUANDT, F. N. & NARAHASHI, T. (1984). Isolation and kinetic analysis of inward currents in neuroblastoma cells. *Neuroscience* **13**, 249–262.

REGOLI, D. & BARABE, J. (1980). The pharmacology of bradykinin and related kinins. *Pharmacological Reviews* **32**, 1–46.

REGOLI, D., BARABE, J. & PARK, W. K. (1977). Receptors for bradykinin in rabbit aorta. *Canadian Journal of Pharmacology* **55**, 855–867.

REISER, G. & HAMPRECHT, B. (1982). Bradykinin induces hyperpolarizations in rat glioma cells and in neuroblastoma × glioma hybrid cells. *Brain Research* **239**, 191–199.

REISER, G. & HAMPRECHT, B. (1985). Bradykinin causes a transient rise of intracellular Ca^{2+}-activity in cultured neural cells. *Pflügers Archiv* **405**, 260–264.

SPECTOR, I. (1981). Electrophysiology of clonal nerve cell lines. In *Excitable Cells in Tissue Culture*, ed. NELSON, P. G. & LIEBERMAN, M., pp. 247–277. New York: Plenum Press.

YANO, K., HIGASHIDA, H., INOUE, R. & NOZAWA, Y. (1984). Bradykinin-induced rapid breakdown of phosphatidylinositol 4,5-bisphosphate in neuroblastoma × glioma hybrid NG108-15 cells. A possible link to agonist-induced neuronal functions. *Journal of Biological Chemistry* **259**, 10201–10207.