Second Messengers Involved in the Mechanism of Action of Bradykinin in Sensory Neurons in Culture

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Application of bradykinin to neonatal rat dorsal root ganglion neurons caused a depolarization associated with an inward current and an increase in membrane conductance that was probably due to the opening of sodium channels. No hyperpolarization or outward current was detected. In addition, bradykinin increased the rate of ⁴⁵Ca uptake into the neurons by a mechanism that was blocked by the dihydropyridine calcium channel antagonist nifedipine. Direct activation of protein kinase C (PKC) with phorbol esters mimicked the ability of bradykinin to depolarize the neurons and to increase the rate of ⁴⁵Ca uptake. Down-regulation of PKC by prolonged treatment with phorbol esters and treatment of the cells with staurosporine, which inhibits PKC, blocked both bradykinin- and phorbol ester-induced ⁴⁵Ca influx, and substantially reduced the proportion of cells that gave electrophysiological responses to either agent.

Bradykinin also activated polyphosphoinositidase C in the dorsal root ganglion neurons, elevating levels of inositol(1,4,5)-trisphosphate and 1,2-diacylglycerol, an endogenous activator of PKC. It is suggested, therefore, that PKC may mediate some of the effects of bradykinin in sensory neurons.

Bradykinin (Bk) is a potent pain-producing peptide, formed at sites of injury, that stimulates the peripheral terminals of sensory afferent fibers, including the polymodal nociceptors (Erdos, 1979). Much information about activation of nociceptors has come from studies of sensory fibers *in vivo* (e.g., Beck and Handwerker, 1974; Mense and Schmidt, 1974; Franze and Mense, 1975), but as sensory nerve terminals are small and inaccessible structures, there is little information about the biochemical events underlying their activation by chemical or other types of stimuli. Electrophysiological studies on nodose ganglion cells (Higashi et al., 1982; Weinreich, 1986) have suggested that Bk may exert an excitatory effect by reducing potassium conductance, but only in neuroblastoma–glioma hybrid cells (Higashida and Brown, 1986; Tertoolen et al., 1987; Brown and Higashida, 1988) has the biochemical basis for this type of membrane effect been studied. The use of primary cultures of sensory neurons allows both the electrophysiological and biochemical properties of these cells to be examined in detail (Dichter and Fischbach, 1977;

Baccaglini and Hogan, 1983; Miller, 1987; Burgess et al., 1988; Lindsay and Rang, 1988; Thayer et al., 1988), and we have taken advantage of this to try to elucidate the mechanism of action of Bk on rat dorsal root ganglion (DRG) neurons.

Our main aim was to determine the relationship, if any, between the biochemical events initiated by Bk in these neurons and the electrophysiological excitation. We have found, as recently reported by Thayer et al. (1988), that Bk, as in many other cells, causes an elevation of inositol(1,4,5)trisphosphate [$\text{Ins}(1,4,5)\text{P}_3$] and of 1,2-diacylglycerol (1,2-DAG). There was also an increase in arachidonic acid. The membrane response, which occurred independently of extracellular calcium, consisted of an inward (depolarizing) current associated with increased membrane conductance.

There is much evidence that protein kinase C (PKC) is important in controlling various aspects of neuronal function, modulating the activity of several ion channels, and regulating neurotransmitter release (see Kaczmarek, 1987; Miller, 1987), and it has recently been suggested that PKC might play a role in the excitation of polymodal nociceptors. Activation of PKC leads to the depolarization of the C fibers of the rat vagus (Rang and Ritchie, 1988) and excitation of the capsaicin-sensitive C-fibers in the tail of the neonatal rat spinal cord and tail preparation (Bettaney et al., 1988; Dray et al., 1988). As the Bk-mediated increase in 1,2-DAG (or arachidonic acid or both, see Nishizuka, 1988) is likely to activate PKC in the DRG neurons, the possibility that the membrane effects of Bk might be mediated by PKC was tested by comparing the effects of phorbol esters, which activate PKC directly, with those of Bk.

It was found that the electrophysiological response, as well as an associated increase in ⁴⁵Ca uptake, could be mimicked by phorbol esters and by a diacylglycerol analog, and inhibited both by down-regulation of PKC and by staurosporine, a PKC inhibitor (see Tamaoki et al., 1986). This supports the possibility that PKC activation may play a role in the process whereby Bk causes excitation of these neurons.

Materials and Methods

Culture of DRG neurons. DRG neurons from neonatal rats were prepared as described previously (Baccaglini and Hogan, 1983; Wood et al., 1988). Dissociated cells were plated on poly-D-ornithine-coated petri dishes for 1 hr. During this period, the non-neuronal cells from the ganglia became firmly attached to the dish, and the neuronal cells could be washed off to give a suspension consisting mainly of neurons. The non-neuronal cells, which remained attached to the dish, could be maintained in culture and used as controls for biochemical experiments. The enriched neuronal preparation was plated onto coverslips coated with poly-D-ornithine and laminin in Hams F-14 medium containing: NaHCO_3 , 14.0 mM; glutamine, 1.0 mM; penicillin, 100 $\mu\text{g}/\text{ml}$; streptomycin, 100 units/ml; horse serum, 10%; nerve growth factor, 5 $\mu\text{g}/\text{ml}$; and cytosine arabinoside, 10 μM . After about 3 d, when the non-

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neuronal cells represented only 5% of the cells, the cytosine arabinoside was removed and the DRG neurons grown for a further 5–7 d before use. For biochemical experiments about 90% of the cells in the cultures were neurons. Non-neuronal cells were grown in the same medium but without nerve growth factor or cytosine arabinoside.

Electrophysiology. DRG neurons were plated at low density (1000–5000 cells) on coverslips that were placed in a 0.5 ml chamber on the stage of an inverted phase-contrast microscope. The chamber was perfused with physiological salt solution containing (mM) NaCl, 154.0; NaHCO₃, 1.8; KCl, 5.6; CaCl₂, 2.0; glucose, 5.6; HEPES, 10.0 (buffered to pH 7.3 with 6.5 mM NaOH) at 2 ml/min. Low-sodium (8.3 mM) Locke was made by equimolar replacement of NaCl with *N*-methyl glucamine chloride. The temperature was maintained at 30–32°C. Recordings were made by the tight-seal, whole-cell voltage-clamp technique (Hamill et al., 1981), with fire-polished pipettes of resistance 2–7 MΩ attached to the head stage of an Axopatch recording amplifier (Axon Instruments Inc.). For most experiments, the recording pipettes were filled with a medium of the following composition (in mM): KCl, 140; MgCl₂, 1; Mg-ATP, 1; K-HEPES, 10; EGTA, 10; CaCl₂, 5.2 (calculated free calcium concentration, 100 nM), pH 7.3; variations are indicated under Results. Drugs, made up in physiological salt solution, were applied by pressure ejection from pipettes of about 5 μm diameter placed about 10–20 μm from the cell body. The cells normally had resting potentials of –50 to –70 mV and gave overshooting action potentials in response to depolarizing current pulses. In most experiments the membrane potential was clamped to –80 mV, with alternating jumps to –100 and –60 mV repeated every 3 sec; the amplitude of the associated current deflections indicated changes in membrane conductance.

Measurement of inositol phosphate formation. DRG neurons (30,000 cells/coverslip) were incubated for 20 hr in Eagle's medium with Earle's salts that contained *myo*-2-[³H]-inositol (50 μCi/ml). At the end of this period, the labeled medium was removed, and the cells were washed and placed in nonradioactive HEPES-buffered Dulbecco's modified Eagle's medium (DMEM) for the duration of the experiment. Cells were exposed to drugs by dipping the coverslips into 10 ml of control or drug-containing DMEM at 37°C for the times indicated in individual experiments. The reactions were terminated by plunging the coverslips into 2 ml of ice-cold 10% trichloroacetic acid (TCA). The ³H-inositol phosphates were extracted for 20 min, and the TCA was removed by extracting 5 times in 5 vol of diethyl ether. The samples were then neutralized with NH₄OH.

The ³H-inositol phosphates were separated either by anion-exchange chromatography on Dowex columns (formate form) as described previously (Berridge, 1983) or, in some cases, by high-pressure liquid chromatography (HPLC). Where Dowex columns were used, the fraction referred to as inositol triphosphate (InsP₃) was eluted with 0.75 M ammonium formate in 0.1 M formic acid.

Separations by HPLC were done on a Whatman Partisil 10 SAX anion-exchange column with modifications of ammonium formate/phosphate (pH 3.7) gradients as described by Burgess et al. (1985) and Heslop et al. (1985). All the inositol phosphates except inositol tetrakisphosphate (InsP₄) were eluted by a nonlinear gradient (convex, no. 4 on a Waters model 660 gradient programmer) at 1.2 ml/min, increasing from 0 to 1 M ammonium formate in 40 min. The 1 M buffer was run for a further 5 min, and then the concentration was further increased over 5 min via a linear gradient to 1.7 M. This final solution was run for a further 20 min to elute InsP₄. ³H-Ins(1,4,5)P₃ from DRG neurons was identified by comparison with either ³²P-Ins(1,4,5)P₃ (prepared as described by Downes et al., 1982) or with a ³H-Ins(1,4,5)P₃ standard from Amersham. ³H-InsP₄ was identified by comparison with a ³H-Ins(1,3,4,5)P₄ standard from Amersham, although the material from the DRG neurons was not necessarily this isomer. A peak eluting immediately prior to Ins(1,4,5)P₃ was tentatively identified as ³H-Ins(1,3,4)P₃ on the basis that material obtained from several other tissues that elutes in this position has been chemically determined to be Ins(1,3,4)P₃ (Irvine et al., 1984b; Burgess et al., 1985). For each experiment the radioactivity in the inositol phosphates (corrected for losses during extraction and separation) was expressed as a percentage of the total lipid radioactivity, which was almost all associated with phosphatidylinositol.

Measurement of diacylglycerol formation and arachidonic acid release. DRG neurons, plated at 50,000/coverslip, were loaded with ¹⁴C-arachidonic acid (2 μCi/ml) for 90 min at 37°C in bicarbonate buffered DMEM. After this loading period, the cells were washed 5 times with HEPES-

buffered DMEM to remove unincorporated radioactive material. Cells were exposed to drugs by dipping the coverslips into 10 ml of control or drug-containing HEPES-buffered DMEM (37°C) for the times indicated in individual experiments. The incubation was terminated by plunging the coverslips into 1 ml ice-cold chloroform/methanol (1:2). Lipids were then extracted for 15 min, after which 0.4 ml chloroform and 0.4 ml H₂O were added to each tube. The tubes were vortexed and extracted for a further 10 min, after which they were centrifuged for 10 min and the organic layer removed. The aqueous phase was reextracted with 0.8 ml chloroform and the second organic phase was combined with the first and dried under nitrogen. The samples were then redissolved and spotted onto thin-layer chromatography (TLC) plates along with authentic 1-stearyl-2-[¹⁴C]-arachidonyl-glycerol and ¹⁴C-arachidonic acid. Lipids and free fatty acids were separated by developing the plates in one dimension in benzene/diethyl ether/ethyl acetate/acetic acid (80:10:10:0.2 by volume) plus 0.01% butylated hydroxytoluene until the solvent was 16 cm from origin (Storry and Tuckley, 1967). ¹⁴C-labeled lipids were visualized by autoradiography following exposure of the TLC plates to Kodak X-OMAT film for 14 d. Quantitation of the radioactivity in individual spots was achieved by scraping the relevant regions of the TLC plates and counting in a liquid scintillation counter. ¹⁴C-1,2-DAG and ¹⁴C-arachidonic acid from DRG neurons was expressed as a percentage of the total radioactivity in each sample.

Measurement of the initial ⁴⁵Ca uptake rate. DRG neurons plated at 50,000/coverslip were incubated with or without drug for 30 sec at 37°C in HEPES-buffered DMEM without added calcium. The cells were then incubated with the same medium but containing 2 μCi/ml ⁴⁵Ca for either 5, 10, 15, or 20 sec. (The calcium concentration of the medium was determined by atomic absorption spectroscopy to be about 10 μM.) The coverslips were then immediately dipped into 3 successive 10 ml washes of ice-cold DMEM containing 1.8 mM CaCl₂ and 100 μM LaCl₃ and placed in a scintillation vial containing 225 μl of 1 M NaOH. After 4 hr, a sample was removed for protein determination and the radioactivity associated with the cells determined. Coverslips without cells were treated in an identical fashion and the radioactivity in these blanks (about 10% of the radioactivity in the presence of cells) was subtracted from the cell samples. The initial rate of uptake of the ⁴⁵Ca was expressed as pmol ⁴⁵Ca/min/mg protein.

PKC-associated immunoreactivity. PKC immunoreactivity was measured using MC5, a monoclonal antibody that recognizes the α and β forms of PKC, following the protocol described by Amersham.

Values given in the Results section represent means ± SEM.

Materials. All radiochemicals were obtained from Amersham. Tissue culture materials were obtained from Gibco, except for collagenase and trypsin (Worthington), DNase and trypsin inhibitor (Sigma), and nerve growth factor, which was a gift from Dr. R. Lindsay. MC5 used in initial experiments was a generous gift from Dr. P. Parker and was later obtained from Amersham. Other sources were as follows: Fluka, staurosporine; Sigma, nifedipine, mepacrine, and all phorbol esters; Avanti Polar Lipids, oleoyl-acetyl-glycerol (OAG); Porton Down, U.K., pertussis toxin; Wellcome Research Laboratories, BW7755C. Bk was obtained from Sandoz, Basle, and other peptides were obtained from Nova Biochem.

Results

Electrophysiological response to Bk

The majority of the neurons in culture were of a similar morphological type, with phase-bright, roughly spherical cell bodies 15–30 μm in diameter and an extensive neurite tree. These cells gave action potentials of varying duration in the range of 2–10 msec, usually with an obvious hump on the falling phase. The remaining cells were larger (up to 70 μm), and their action potentials were short (usually 1–2 msec), with no hump on the falling phase. Previous studies of DRG and nodose ganglia from various species have demonstrated the existence of 2 major neuronal subtypes, namely, A-cells, which have myelinated, fast-conducting axons and short action potential durations, and C-cells, which have nonmyelinated, slowly conducting axons and action potentials with a distinct shoulder on the falling phase (Gallego and Eyzaguirre, 1978; Yoshida et al., 1978; Gorke and Pierau, 1980; Harper and Lawson, 1985a, b; Ikeda et al., 1986).

Figure 1. Membrane response of DRG neurons to Bk recorded by whole-cell voltage clamp. The records show membrane current, inward current downward. The holding potential was -80 mV in all records, with 400 msec excursions alternatively to -60 and -100 mV, resulting in upward and downward deflections of the current trace. (In *C*, the excursions were from -80 to -40 mV.) *A*, Consecutive responses to Bk ($1 \mu\text{M}$) applied for 1 sec at bar. The interval between the responses was 10 min. This cell was unusual in showing little or no desensitization. *B*, As in *A*, in a different cell from the same culture. The solution was changed to one containing 8.3 mM Na^+ 3 min after the first response. *C*, Response to Bk ($1 \mu\text{M}$ for 10 sec at bar) in a solution containing no added Ca^{2+} . *D*, As *C*, in normal Locke; pipette solution contained $140 \text{ mM K-aspartate}$ in place of KCl.

Bk had no effect on A cells. Application of Bk ($1 \mu\text{M}$ for 1–2 sec) produced, in 32% (107/335) of the C cells tested, an inward current at the holding potential of -80 mV, associated with an increase in membrane conductance (Fig. 1). In the responding cells, the mean amplitude of this inward current at -80 mV was $0.54 \pm 0.06 \text{ nA}$ ($n = 107$). The inward current response developed within a few seconds, reaching a peak at $17 \pm 2 \text{ sec}$ ($n = 107$), and declined within 30 sec of the end of the puff.

In a few cultures the response to Bk showed different patterns; namely, a much slower inward current, again associated with an increase in membrane conductance, lasting up to 15 min, or

Table 1. Effect of intracellular free Ca^{2+} and tetrodotoxin on the electrophysiological response to Bk

$[\text{Ca}^{2+}]_i$ (nM)	TTX (μM)	Cells responding/ cells tested	Mean response (nA)
0	0	21/50	0.31 ± 0.09 (21)
0	1	15/40	0.44 ± 0.16 (15)
100	0	107/335	0.54 ± 0.06 (107)

The intracellular solution contained 10 mM EGTA and either no added calcium or 5.2 mM CaCl_2 to give an estimated free $[\text{Ca}^{2+}]$ of 100 nM . The inward current responses to $1 \mu\text{M}$ Bk were measured at -80 mV holding potential.

Figure 2. Effect of Bk on the rate of uptake of ^{45}Ca into DRG neurons. Time course of ^{45}Ca uptake in absence and presence of Bk (100 nM). DRG neurons were incubated with or without Bk for 30 sec, then transferred to the same medium containing ^{45}Ca for the times indicated. The ^{45}Ca content of the cells has been expressed as pmol ^{45}Ca /mg protein. Bars indicate SEM of at least 4 experiments.

a burst of fast inward currents (presumably action potentials originating in unclamped neurites), without any maintained inward current or conductance change recorded at the soma. These have not been included in the analysis.

Bk did not produce an outward current response, even when the membrane potential was held at -40 mV ; this is in contrast to studies on NG108-15 cells, in which a major component of the response to Bk is an increase in a Ca^{2+} -activated K^+ conductance (Brown and Higashida, 1988).

The inward current response to Bk showed marked desensitization, and a second application, up to 30 min later, usually evoked little or no response. This made it difficult to study the pharmacology or the ionic dependence of the response in detail, as controls on the same cell were seldom possible. Occasionally, cells were encountered that showed little desensitization (see Fig. 1*A*, in which an interval of 10 min separated the first and second responses), allowing paired observations to be made.

The ionic dependence of the fast inward current response has been examined, and the following results obtained:

1. The response was abolished, or greatly reduced, when external sodium was reduced to 8.3 mM . In 3 experiments, 10/19 cells responded to Bk in normal Locke (mean current amplitude, $0.18 \pm 0.03 \text{ nA}$) compared with 0/16 in low sodium. In one experiment, shown in Figure 1, in a culture showing less than the normal degree of desensitization, a second application of Bk to the same cell, 10 min after the first, gave a mean response that was $60 \pm 18\%$ ($n = 8$) of the first (Fig. 1*A*). If low-sodium solution was applied before the second test, the second response was only $4 \pm 2\%$ ($n = 3$) of the first (Fig. 1*B*), suggesting that most of the inward current was carried by Na^+ . Addition of tetrodotoxin (TTX, $1 \mu\text{M}$) to the medium did not affect the response to Bk, even though the action potential was blocked or greatly attenuated (Table 1).

2. Omission of extracellular calcium did not prevent or ob-

Figure 3. Effect of nifedipine and high K^+ on the Bk-induced increase in the rate of ^{45}Ca uptake into DRG neurons. Protocol as in Figure 2; the cells were incubated in the ^{45}Ca for 20 sec. *Left*, Incubation of DRG neurons with nifedipine ($1\ \mu\text{M}$, *Nif*) for 2 min prior to and during measurement of ^{45}Ca uptake, blocked the effect of $300\ \text{nM}$ Bk. *Right*, Depolarization with $50\ \text{mM}$ K^+ (replacing Na^+) also increased the rate of uptake of ^{45}Ca into the DRG neurons. In the presence of $50\ \text{mM}$ K^+ Bk ($300\ \text{nM}$) no longer increased ^{45}Ca uptake. Bars indicate SEM of 3 experiments.

viously reduce the response to Bk (Fig. 1C). Omission of calcium from the pipette solution, which reduced $[\text{Ca}^{2+}]_i$ from $100\ \text{nM}$ to zero, slightly reduced the response (Table 1).

3. Replacement of KCl in the pipette with K-aspartate did not prevent the Bk response (Fig. 1D), suggesting that Cl^- was not a major charge carrier for the inward current.

Effect of Bk on the rate of uptake of ^{45}Ca

Bk caused an increase in the rate of ^{45}Ca uptake into DRG neurons (Fig. 2). Addition of Bk ($100\ \text{nM}$) 30 sec before and during exposure of the cells to ^{45}Ca approximately doubled the initial rate of uptake of ^{45}Ca . The concentration–effect relationship for this increase ($\text{EC}_{50} = 6\ \text{nM}$) is shown in Figure 5. The increase was prevented by nifedipine (0.3 or $1\ \mu\text{M}$; Fig. 3, left) and by cadmium ($100\ \mu\text{M}$). Depolarization of the cell with K^+ ($50\ \text{mM}$) also increased the rate of ^{45}Ca uptake (Fig. 3, right). The effects of maximal concentrations of Bk ($300\ \text{nM}$) and K^+ ($50\ \text{mM}$) were not additive (Fig. 3B). This finding, together with inhibitory effects of nifedipine and cadmium, suggests that the increased ^{45}Ca uptake may be secondary to the Bk-induced depolarization.

Effect of Bk on phosphoinositide metabolism

^3H -Inositol phosphate formation

The Bk receptor is linked to polyphosphoinositidase C (PIC) in several tissues (see Yano et al., 1984, 1985; Derian and Moskowitz, 1986; Jackson et al., 1987), including DRG neurons (Thayer et al., 1988). In agreement with their study, we found that $100\ \text{nM}$ Bk caused a substantial increase in the radioactivity associated with various inositol polyphosphates, ^3H -Ins($1,4,5$) P_3 , rising by a factor of 5 within 2 sec (Fig. 4).

The concentration–effect relationship for the increase in ^3H -Ins P_3 ($\text{EC}_{50} = 6\ \text{nM}$) is shown in Figure 5. ^3H -Ins P_4 also rose; because of the rapidity of the responses, it was not possible to resolve a delay between the increase in ^3H -Ins($1,4,5$) P_3 and of ^3H -Ins P_4 , which is formed by phosphorylation of Ins($1,4,5$) P_3 (Irvine et al., 1986). Bk also increased the radioactivity associated with a fraction likely to be ^3H -Ins($1,3,4$) P_3 (see Materials

Figure 4. Time course of Bk-induced formation of ^3H -inositol phosphates and ^{14}C -1,2-DAG in DRG neurons. DRG neurons were incubated with $100\ \text{nM}$ Bk for the times indicated. In *A*, the inositol phosphates were separated by HPLC, and in *B*, by Dowex anion-exchange chromatography. For *A* and *B*, the radioactivity in each fraction has been expressed as a percentage of the radioactivity in phosphatidylinositol (^3H)-PtdIns, and the bars indicate SEM values for 5 experiments. For *C*, the radioactivity in the fraction corresponding to 1,2-DAG was expressed as a percentage of the total radioactivity in each sample. Bars represent SEM values for 5 experiments. In some cases, the error bars lie within the symbols.

and Methods), but in this case, there was a delay of at least 2 sec, reflecting the fact that production of Ins($1,3,4$) P_3 occurs downstream of both Ins($1,4,5$) P_3 and Ins($1,3,4,5$) P_4 (Batty et al., 1985; Irvine et al., 1986). At most, the radioactivity in this fraction comprised about 24% of the total ^3H -Ins P_3 .

^3H -inositol bisphosphate (Ins P_2) and ^3H -inositol phosphate (InsP) also rose, though more slowly, and remained elevated for at least 5 min during exposure of the cells to $100\ \text{nM}$ Bk.

The cultures of DRG neurons contained only about 10% of non-neuronal cells; nevertheless, in order to confirm that the increase in inositol phosphates was occurring in the neurons, some experiments were done on ^3H -inositol-labeled non-neuronal cell cultures derived from rat DRG. When these cells were exposed to Bk (up to $10\ \mu\text{M}$), no increase in ^3H -inositol phosphates was detected (^3H -Ins P_3 was $104.2 \pm 4.2\%$ of the basal value 20 sec after treatment with $300\ \text{nM}$ Bk, triplicate determinations from 3 experiments).

Figure 5. Concentration-effect curves for the Bk-induced formation of ^3H -InsP $_3$ and increase in the ^{45}Ca influx in DRG neurons. Protocols as in Figures 3 and 4. For the InsP $_3$ curve, the increase is expressed as a percentage of the control for each experiment. The increase in the rate of ^{45}Ca uptake is expressed as a percentage of the maximum response for each individual experiment. Bars represent SEM values of between 4 and 5 experiments.

Exposure of DRG neurons to Bk results in desensitization of the electrophysiological response (see above). Desensitization of the ^3H -InsP $_3$ response was also observed. Exposure of DRG neurons for 60 sec to either 30 or 100 nM Bk resulted in, respectively, a 50 or 80% inhibition of the response to a 20 sec challenge with 100 nM Bk (Table 2). To test the possibility that PKC might be involved in the desensitization, the effect of phorbol 12,13 dibutyrate (PDBu), which activates PKC directly, on the Bk-induced increase in ^3H -InsP $_3$ was measured. Table 2 shows that preincubation with PDBu (1 μM for 60 sec) reduced the Bk response, whereas phorbol 12,13 didecanoate (PDDec, 10 μM), an α -phorbol ester that does not activate PKC, had no effect.

^{14}C -1,2-Diacylglycerol formation

Figure 4 also shows that concomitant with the elevation in ^3H -inositol phosphates, Bk increased the radioactivity associated with 1,2,DAG in DRG neurons that had been labeled with ^{14}C -arachidonic acid. ^{14}C -1,2,DAG rose within 5 sec of exposure to 100 nM Bk (the earliest time at which a measurement was made), peaking between 5 and 20 sec. The radioactivity in 1,2,DAG then fell, rising for a second time between 30 and 60 sec and was still 1.5–2 times the basal value at 5 min. This secondary rise was also observed by Thayer et al. (1988).

Table 2. Desensitization of Bk-induced ^3H -InsP $_3$ formation

Treatment	Response to 100 nM Bk (% control response)
None	100
Bk 30 nM	52.2 $\pm$ 4.6
100 nM	18 $\pm$ 3.9
PDBu, 1 μM	56.3 $\pm$ 6.4
PDDec, 10 μM	114 $\pm$ 13

DRG neurons equilibrated with ^3H -inositol were pretreated with the solutions shown, washed, and then challenged 15 min later with 100 nM Bk. Inositol phosphates were separated by Dowex anion-exchange chromatography. The test responses are expressed as a percentage of the response of cells pretreated with control solution. Mean and SEM values of 3–5 are shown.

Effect of Bk on cellular ^{14}C -arachidonic acid

Application of Bk (100 nM) to DRG neurons also caused a small elevation in intracellular ^{14}C -arachidonic acid, which rose to $140 \pm 10\%$ ($n = 4$) of the control value 30 sec after administration of Bk and to $160 \pm 9\%$ at 5 min.

Possible role of Ca^{2+} in the Bk-induced activation of PIC

As PIC can be modulated by Ca^{2+} (Irvine et al., 1984a), it was possible that PIC activation and the resulting generation of second messengers was secondary to a Bk-mediated increase in $[\text{Ca}^{2+}]_i$ rather than a direct consequence of receptor activation. This was tested by measuring the effect of Ca^{2+} -free medium (buffered with 200 μM EGTA) on the ability of Bk to increase

Table 3. Possible role of Ca^{2+} , eicosanoids, and DRG neuron depolarization in the Bk-induced activation of PIC

Incubation conditions	^3H -InsP $_3$ (% [^3H]-PtdIns)	
	Control	Bk
A		
1.8 mM CaCl_2	0.20 $\pm$ 0.03	0.94 $\pm$ 0.2
Ca^{2+} -free medium	0.11 $\pm$ 0.035	0.64 $\pm$ 0.17
Nifedipine, 10 μM	0.21 $\pm$ 0.028	0.87 $\pm$ 0.21
Mepacrine, 20 μM	0.19 $\pm$ 0.024	1.2 $\pm$ 0.11
BW755C, 20 μM	0.23 $\pm$ 0.05	1.12 $\pm$ 0.08
K^+ , 50 mM	0.37 $\pm$ 0.068	
Ionomycin (1 μM)	0.24 $\pm$ 0.08	
B		
1.8 mM CaCl_2	0.70 $\pm$ 0.3	2.2 $\pm$ 0.17
Ca^{2+} -free medium	0.53 $\pm$ 0.28	1.7 $\pm$ 0.35

DRG neurons, equilibrated with ^3H -inositol or ^{14}C -arachidonic acid, were preincubated for 2 min in the test solutions; normal medium (1.8 mM CaCl_2); normal medium containing either 10 μM nifedipine, 20 μM mepacrine, or 20 μM BW755C; Ca^{2+} -free medium containing 200 μM EGTA, and then transferred to an aliquot of the same medium with or without Bk (300 nM) for 20 sec. ^3H -InsP $_3$ was measured as described in Table 2. Ionomycin and K^+ were applied for 20 sec in normal medium. Mean and SEM values of between 3 and 5 experiments are given.

Figure 6. Membrane responses to Bk and PDBu in DRG neurons. Cells were monitored by whole-cell voltage-clamp recording. The holding potential was -80 mV, with alternating 300 msec steps to -60 and -100 mV giving rise to the upward and downward deflections on the current trace. Inward current is represented as a downward deflection. Both Bk ($1 \mu\text{M}$ for 10 sec) and PDBu ($1 \mu\text{M}$ for 5 sec) elicited inward current responses associated with increased membrane conductance. The 2 records were obtained on different cells.

Figure 7. Effect of PDBu on the rate of ^{45}Ca uptake into DRG neurons. *Left*, Time course of ^{45}Ca uptake into DRG neurons in the presence and absence of PDBu ($1 \mu\text{M}$) measured as described in Figure 2. *Right*, Concentration-effect curve. The increase in the rate of uptake was expressed as a percentage of the maximum response for each experiment. The EC_{50} was about 300 nM. Bars represent SEM values for 3 experiments.

^3H -InsP $_3$ and ^{14}C -1,2,DAG. Under these conditions the elevation of ^3H -InsP $_3$ (Table 3A) and the early rise in ^{14}C -1,2,DAG (Table 3B) were not significantly different from the responses obtained in 1.8 mM CaCl_2 . Similarly, incubation of DRG neurons with nifedipine ($1 \mu\text{M}$), which blocked the Bk-evoked increase in ^{45}Ca uptake (see Fig. 3), did not alter the ability of Bk to increase the level of ^3H -InsP $_3$ (Table 3A).

Ionomycin ($1 \mu\text{M}$), a Ca^{2+} -selective ionophore, which caused a substantial elevation of $[\text{Ca}^{2+}]_i$ in DRG neurons (not shown), and 50 mM K^+ , which (as seen in Fig. 3) increased the rate of ^{45}Ca entry, did not alter the basal level of ^3H -InsP $_3$ (Table 3A). These results show that activation of PIC was not secondary to an increase in $[\text{Ca}^{2+}]_i$ or to depolarization.

Possible role of arachidonic acid and its metabolites in Bk-induced PIC activation

Metabolites of arachidonic acid, such as thromboxane A $_2$, can act as positive-feedback regulators of phosphoinositide metabolism (Sha'afi and Naccache, 1985). In view of this, and because Bk increased intracellular ^{14}C -arachidonic acid in DRG neurons, it was also necessary to determine whether PIC activation was secondary to increased eicosanoid production. Preincubation of DRG neurons with either mepacrine ($20 \mu\text{M}$), an inhibitor of phospholipase A $_2$ (PLA $_2$), or BW755C ($20 \mu\text{M}$), a dual inhibitor of cyclooxygenase and lipoxygenase, had no effect on the Bk-induced increase in ^3H -InsP $_3$ (Table 3A).

Pharmacology of the Bk-induced responses in DRG neurons

The EC_{50} value for both the Bk-induced increase in ^{45}Ca influx and in ^3H -InsP $_3$ formation was 6 nM (Fig. 5). It has been suggested (Regoli et al., 1986) that there are two main Bk receptor subtypes, B1 and B2, which differ markedly in their sensitivity to *des*-Arginine 9 -bradykinin (*des*-Arg 9 -Bk) analogs. *Des*-Arg 9 -Bk ($1 \mu\text{M}$), a putative B1 agonist, did not evoke an inward current response in cells that were subsequently shown to be responsive to Bk ($1 \mu\text{M}$), nor did it increase ^3H -InsP $_3$ formation or ^{45}Ca uptake at concentrations up to $30 \mu\text{M}$ (triplicate determinations from 3 experiments in each case). Another Bk analog, D-Arginine-4-hydroxyproline 2 -(β -[2-thienyl]-L-alanine) 5 -D-phenylalanine 7 -bradykinin (D-Arg-Hyp 2 -Thi 5 -D-Phe 7 -Bk), reported as a potent Bk receptor antagonist (Beierwaltes et al., 1987; Stewart

and Vavrek, 1987; Braas et al., 1988), inhibited the increase in ^3H -InsP $_3$ evoked by 5 nM Bk with an IC_{50} value of $0.2 \pm 0.1 \mu\text{M}$ (triplicate determinations from 4 experiments). It had a similar IC_{50} against Bk-induced ^{45}Ca uptake ($0.4 \mu\text{M}$). At $10 \mu\text{M}$ it inhibited the inward current induced by $0.3 \mu\text{M}$ Bk by 60% (data from 10 cells).

Effect of pertussis toxin on Bk-induced responses in DRG neurons

Pertussis toxin causes ADP-ribosylation of certain G-proteins (G_i and G_o) in neonatal rat DRG neurons (Scott and Dolphin, 1987), and in a few cell types it appears to inhibit the G protein that links receptors to PIC (see Dolphin, 1987). Incubation of DRG neurons for 20 hr with pertussis toxin (200 ng/ml) had no effect on the ability of Bk to increase ^3H -inositol phosphates or to stimulate ^{45}Ca entry (Table 4). Additionally, in 3 electrophysiological experiments pertussis toxin treatment had no effect on either the proportion of cells responding to Bk (6/20 cells in pertussis toxin-treated cultures, compared with 6/17 in controls) or on the mean response amplitude.

Table 4. Effect of pertussis toxin on ^3H -InsP $_3$ formation and ^{45}Ca uptake in DRG neurons

Treatment	^3H -InsP $_3$ (% [^3H]-PtdIns)	
	Control	Bk
Control	0.34 ± 0.05	1.2 ± 0.16
Pertussis toxin (200 ng/ml)	0.40 ± 0.07	1.4 ± 0.2
^{45}Ca uptake rate (pmol/min/mg protein)		
Control	75.5 ± 10	169 ± 16
Pertussis toxin (200 ng/ml)	69.0 ± 12	177 ± 21

DRG neurons were incubated overnight with or without pertussis toxin (200 ng/ml). ^3H -InsP $_3$ formation and ^{45}Ca uptake induced by 300 nM Bk for 20 sec were measured as described in Table 2 and Figure 2. Mean and SEM values of 4 experiments are given.

Table 5. Effect of phorbol esters and OAG on the rate of ^{45}Ca uptake in DRG neurons

Treatment	^{45}Ca uptake rate (pmol/min/mg protein)		
Control	70.2 ± 5.0		
Bk, 300 nM	171.0 ± 21.8		
PDBu (1 μM)	183.7 ± 26.3		
PMA (1 μM)	155.0 ± 16.5		
PDDec (10 μM)	75.4 ± 7.7		
OAG (1 μM)	157.9 ± 30		
K ⁺ , 50 mM	273.4 ± 22.4		
PDBu (1 μM) + Bk (300 nM)	160.3 ± 18.8		
OAG (1 μM) + Bk (300 nM)	167.5 ± 16.7		
PDBu (1 μM) + K ⁺ (50 mM)	259.0 ± 27.8		
		Normal	Nifedipine (1 μM)
Control		87.6 ± 6	73.0 ± 4.7
PDBu (1 μM)		159.6 ± 18	78.8 ± 8.2

The rate of ^{45}Ca entry was measured as described for Figure 2. DRG neurons were incubated with either control or drug-containing solutions (Bk, PDBu, PMA, PDDec, OAG, high K⁺, or a combination of these) for 30 sec and then transferred to the same solution but containing ^{45}Ca for 20 sec. Nifedipine was applied 2 min prior to and during the application of the agonists. Mean and SEM values of between 3 and 4 experiments are shown.

Possible role of PKC in the responses of DRG neurons to Bk

Effects of phorbol esters

Since Bk was found to increase the formation of ^{14}C -1,2-DAG, an endogenous activator of PKC, in DRG neurons (see Fig. 4), the possibility that this pathway could be involved in the excitatory effects of Bk was tested by comparing the effects of PKC-activation by phorbol esters with those of Bk.

Electrophysiological responses to phorbol esters. As shown in Figure 6, PDBu (1 μM applied for 5 sec) gave a response that was very similar to that produced by Bk, consisting of an inward current (mean amplitude, 0.36 ± 0.07 nA at -80 mV; $n = 90$) associated with increased membrane conductance. The response to PDBu was generally delayed in onset compared with the Bk response, reaching a peak at 29 ± 2 sec ($n = 90$) and was somewhat longer-lasting (2–5 min). As with Bk, PDBu occasionally caused a much slower inward current response (also associated with an increase in membrane conductance), lasting for up to 15 min, or evoked unclamped neurite action potentials with no response at the soma membrane; these have not been included in the analysis. A second application of PDBu produced no effect, or sometimes a greatly reduced response. PDDec (10 μM) did not produce a membrane response.

The proportion of cells that responded to PDBu with an inward current was 37% (90/241), similar to the proportion responding to Bk. If PKC activation is involved in the mechanism whereby Bk causes an inward current, then PDBu-insensitive cells, which presumably lack the relevant PKC or PKC-activated ion channel, would not be expected to respond to Bk. In fact, a small proportion of the PDBu-insensitive cells (19%, 22/112) gave an inward current response to Bk, which argues against PKC being the only mechanism involved in excitation by Bk.

In most cells, the inward current response to PDBu was abolished in low (8.3 mM) sodium medium. Thus, in 3 experiments

in which the solution was switched between normal and low sodium (8.3 mM) Locke, 10/19 cells responded to PDBu in normal Locke solution (mean current amplitude, 0.32 ± 0.07 nA) compared with 2/14 responding to PDBu in low- Na^+ solution (current amplitudes, <0.05 nA). In contrast, the very slow inward current response to PDBu is unaffected by removal of Na^+ (Lindsay and Rang, 1988). The PDBu response also resembled the fast Bk response in that it could be obtained in Ca^{2+} -free Locke solution. In Ca^{2+} -free Locke 13/32 cells responded to PDBu (40.6%) compared with 37% (see above) in normal Locke.

^{45}Ca uptake induced by phorbol esters. PDBu (1 μM) increased the rate of entry of ^{45}Ca into DRG neurons (Fig. 7). Maximal concentrations of Bk and PDBu produced approximately similar increases in ^{45}Ca influx. Phorbol myristate acetate (PMA), another active phorbol ester, also stimulated ^{45}Ca uptake, as did the synthetic diacylglycerol, oleoyl-acetyl-glycerol (OAG, 1 μM), whereas PDDec had no effect (Table 5). The responses to maximal concentrations of Bk and PDBu were not additive, implying that a similar mechanism could be operating for both agonists (Table 5). Additionally, the response to PDBu (1 μM), like that of Bk, was not additive with the response to a maximally effective concentration (50 mM) of K⁺, and could be blocked by nifedipine (Table 5).

Effect of down-regulation and inhibition of PKC on Bk-induced responses

If PKC mediates some of the actions of Bk in DRG neurons, then preventing its activation should interfere with these responses. PKC activation can be prevented in 2 ways: (1) by prolonged incubation of cells with phorbol esters, which leads to down-regulation of PKC (Young et al., 1987), and (b) by the use of inhibitors such as staurosporine (Tamaoki et al., 1986).

Effect of PKC down-regulation on responses to Bk and PDBu

Incubation of DRG neurons for 18 hr with 300 nM PDBu, a protocol that causes loss of PKC from rat glioma cells (Young et al., 1987), resulted in marked loss of PKC-associated immunoreactivity as measured with an antibody (MC5) that recognizes the α - and β -forms of PKC (see also Ewald et al., 1988a). This treatment prevented the increase in ^{45}Ca uptake induced by both Bk and PDBu (Table 6) and greatly reduced the number of cells that showed electrophysiological responses to both agents. Thus, in 5 experiments, in which control cultures from the same batch of cells were compared with cultures incubated overnight with PDBu, the proportion of Bk-responsive cells was reduced from 42 to 13% and the proportion of PDBu-responsive cells was reduced from 62 to 7% (see Table 6). Table 6 also shows that the down-regulation protocol did not affect the ability of Bk to activate PIC, as the Bk-induced increase in ^3H -InsP₃ formation remained unchanged after overnight treatment with PDBu.

Effect of staurosporine on the responses to Bk and PDBu

Staurosporine inhibited the increase in ^{45}Ca uptake induced by Bk and PDBu (Fig. 8). The IC_{50} value for inhibition of Bk-induced ^{45}Ca uptake was about 20 nM; it had no effect on the increased ^{45}Ca uptake induced by 50 mM K⁺. Staurosporine also abolished the electrophysiological response to PDBu, reducing the percentage of responsive cells from 55% (16/29) to 0% (0/19). The effect of staurosporine on the electrophysiological response to Bk was less clear cut, the percentage of responsive

cells being reduced from 47.5% (19/40) to 30% (8/26). The amplitudes of the inward currents in those cells that responded were not significantly different from the controls. The reason for the discrepancy between the effect of PKC down-regulation and inhibition of PKC with staurosporine on the proportion of cells that responded to Bk with an inward current is not clear.

Discussion

The aim of the experiments reported here was to examine the role of second messengers in excitation of sensory neurons by Bk. The approach taken was to determine whether exposure of DRG neurons to Bk resulted in the formation of any intracellular messengers and then to determine if there was any correlation between these changes and the ability of Bk to cause an inward, depolarizing current and to increase Ca^{2+} influx, responses likely to be important for, respectively, neuronal excitation and neurotransmitter release.

Bradykinin activates PIC in sensory neurons

The results of these experiments provide clear evidence that Bk activates PIC in sensory neurons from rat DRG, producing a rapid increase in $\text{Ins}(1,4,5)\text{P}_3$ and 1,2,DAG. Bk does not activate PIC in non-neuronal cells from rat DRG, though it does cause an increase in cAMP in these cells (Burgess et al., in press). The biphasic nature of the 1,2,DAG response in the neurons may mean that some 1,2,DAG is formed by another mechanism, for example, the hydrolysis of either phosphatidylinositol (see Imai and Gershengorn, 1986; Griending et al., 1987) or phosphatidylcholine (see Besterman et al., 1986; Irving and Exton, 1987). In 2 experiments we found that removal of extracellular Ca^{2+} prevented the secondary increase in 1,2,DAG, leaving the initial rise unaltered, and this supports the hypothesis that 2 mechanisms are involved.

Bk also increased the cellular level of ^{14}C -arachidonic acid. Whether this occurred as a consequence of PIC activation or whether, as has been described in fibroblasts (Burch and Axelrod, 1987), the Bk receptor is linked directly to PLA2 in DRG neurons has not been examined.

Is PIC activation related to sensory neuron excitation by Bk?

Both inositol phosphates and 1,2,DAG have important second-messenger functions in many tissues (see Berridge, 1987), and arachidonic acid products can also act as intracellular messengers, regulating the activity of plasma membrane ion channels

Figure 8. Effect of staurosporine on the Bk- and PDBu-induced increase in the rate of uptake of ^{45}Ca into DRG neurons. DRG neurons were preincubated for 2 min with the PKC inhibitor staurosporine at the concentrations indicated. The neurons were then exposed to solutions containing 100 nM Bk (●) or 1 μM PDBu (■) and the indicated concentrations of staurosporine, and the increase in the rate of ^{45}Ca uptake was measured as described for Figure 3. The increase in the rate of ^{45}Ca uptake was expressed as a percentage of the response to 100 nM Bk in the absence of staurosporine. Bars represent SEM values for 3 experiments.

(Piomelli et al., 1987). These molecules could therefore be considered as mediators of the Bk-induced excitation or increase in ^{45}Ca influx, provided certain criteria are fulfilled. In particular, the time course of the biochemical response must be fast enough to account for the electrophysiological events, and the pharmacology should correspond. The possibility that the biochemical response could be occurring as a consequence of the membrane responses must also be considered. These questions have been examined using ^3H - InsP_3 formation as an index of PIC activation. No detailed examination of arachidonic acid products as possible mediators of the membrane events has been made, beyond showing that an increase in arachidonic acid and its metabolites is not required for Bk-induced activation of PIC.

Pharmacology of Bk responses

Both the biochemical and electrophysiological responses of DRG neurons appeared to be mediated by the subtype of Bk receptor classified by Regoli et al. (1986) as B2. The responses could not be elicited by *des*-Arg⁹-Bk (reported to be selective for B1 re-

Table 6. Effect of PKC down-regulation on the Bk-induced increase in ^{45}Ca uptake, inward current response, and formation of ^3H - InsP_3 in DRG neurons

Assay	Treatment	Control	PKC down-regulated
^{45}Ca uptake rate (pmol/min/mg protein)	None	79.5 ± 8.6	64.5 ± 11.5
	Bk, 300 nM	125.3 ± 7.7	73.9 ± 6.9
	PDBu, 1 μM	171.9 ± 30.0	74.5 ± 16.6
Inward current (cells responding/cells tested)	Bk, 1 μM	19/45	6/47
	PDBu, 1 μM	22/35	3/42
^3H - InsP_3 formation (% basal value)	Bk, 100 nM	408 ± 55	531 ± 64

DRG neurons were incubated for 18 hr in the presence or absence of 300 nM PDBu, then washed and allowed to recover for 90 min. The rate of ^{45}Ca uptake was measured as described in Figure 3, the formation of ^3H - InsP_3 was measured as described in Table 2, and the inward current response was measured as described for Figures 1 and 6. Mean ± SEM values for 3 experiments are given.

ceptors), and they were inhibited by the Bk receptor antagonist D-Arg-Hyp²-Thi^{5,8}-D-Phe⁷-Bk. Nociceptor excitation by Bk is also believed to involve B2 receptors (Whalley et al., 1987; Steranka et al., 1988).

None of the Bk-mediated responses tested were blocked by pretreatment of the cells with pertussis toxin, implying that the Bk receptor involved was not linked to either G_i or G_o. Although the pertussis toxin sensitivity of the 1,2,DAG response was not examined, the fact that Bk-induced formation of InsP₃ was not affected by pertussis toxin means that Bk does increase 1,2,DAG in pertussis toxin-treated cells. Pertussis toxin has been shown to block the actions of a variety of neurotransmitters and peptides that inhibit Ca²⁺ currents in DRG neurons (see Holz et al., 1986; Ewald et al., 1988b), implying that a G_i or G_o-like protein may couple inhibitory receptors to Ca²⁺ channels in sensory neurons. It seems that the Bk receptor, which mediates excitation of DRG neurons, is coupled to PIC, and possibly to other effectors, by a pertussis toxin-insensitive G-protein.

Time course of the responses

The formation of Ins(1,4,5)P₃ and 1,2,DAG both occur rapidly in response to Bk, ³H-Ins(1,4,5)P₃ (and ³H-InsP₃) reaching a peak in 2–5 sec, and ¹⁴C-1,2,DAG in about 20 sec. This is rapid enough to account for both the increase in ⁴⁵Ca uptake and the inward current.

Could PIC activation be secondary to the membrane response?

Manipulations designed to mimic or prevent the Bk-induced increase in Ca²⁺ entry, to prevent the formation and metabolism of arachidonic acid or to mimic the Bk-induced depolarization had no effect on ³H-InsP₃ formation. This suggests that activation of PIC was a direct consequence of Bk receptor activation and not secondary to other Bk-induced changes in the DRG neurons.

Involvement of PKC

The evidence discussed so far is consistent with the hypothesis that PIC activation is responsible for the excitatory effects of Bk on sensory neurons but does not exclude other possibilities, for example, that Bk receptors are coupled to an ion channel either directly or via a G-protein.

There is much evidence that the main role of the inositol phosphates, particularly Ins(1,4,5)P₃, is to raise [Ca²⁺], (see Berridge, 1987), and in sensory neurons, as in many other cell types, this has the effect of opening K⁺ and Cl⁻ channels (Gorke and Pierau, 1980; Kameyama, 1983; Mayer, 1985), which exerts an inhibitory rather than an excitatory effect. 1,2,DAG, which is an endogenous activator of PKC (see Nishizuka, 1988) appeared, therefore, to be a stronger candidate for a second messenger than the inositol phosphates. Not only is PKC particularly abundant in neuronal tissue (Girard et al., 1986; Worley et al., 1986), but it has been implicated as a key regulator of neuronal function (see Kaczmarek, 1987; Miller, 1987); furthermore, it has recently been implicated as a mechanism in C-fiber depolarization (Bettaney et al., 1988; Dray et al., 1988; Rang and Ritchie, 1988). The depolarizing phase of the complex response of NG108-15 cells to Bk has also been linked to PKC activation (Brown and Higashida, 1988). It seemed possible, therefore, that PKC might also be the link between the Bk receptor and DRG neuron excitation.

In support of this, it was found that PDBu caused membrane responses that closely resembled those produced by Bk. The

most common type of response elicited by both Bk and PDBu was an inward current associated with an increase in membrane conductance. For both agents, this inward current response could be evoked in the absence of Ca²⁺ but was blocked in the absence of Na⁺. This suggests that Na⁺ is the main charge carrier, but on the basis of its insensitivity to TTX and relative lack of voltage dependence, the channel involved does not correspond to the fast voltage-gated Na⁺ channel. The ionic selectivity and gating characteristics of this channel need more detailed study to establish whether Bk and PDBu activate the same ion channel. Bk and PDBu sometimes produced very slow inward current responses, and preliminary results suggested that the slow response to PDBu was due to activation of chloride conductance (see Lindsay and Rang, 1988). It is unclear why the pattern of response shows this variation, but the fact that very similar results were obtained with Bk and PDBu supports the hypothesis that PKC may be involved in the electrophysiological effects of Bk. A direct effect of PKC activation in causing the opening of ion channels, as distinct from a modulatory effect on voltage- or Ca²⁺-activated channels, has not hitherto been described, except in C-fiber axons (Rang and Ritchie, 1987, 1988) and may be a mechanism unique to nociceptive neurons.

It had previously been shown (Perney et al., 1986) that substance P release from DRG neurons could be blocked by dihydropyridine Ca²⁺ antagonists. It seemed likely, therefore, that activators of DRG neurons would stimulate Ca²⁺ entry, but this had not been demonstrated experimentally. Here, it has been shown that Bk increases ⁴⁵Ca uptake into DRG neurons by a nifedipine-sensitive mechanism and that this effect could be mimicked by PKC activators such as PDBu, PMA, and OAG. Neither Bk nor PDBu caused a further increase in Ca²⁺ uptake in K⁺-depolarized neurons, suggesting that neither acts directly on Ca²⁺ permeability.

Effects of inhibition and down-regulation of PKC

The strongest evidence implicating PKC in the excitation of DRG neurons by Bk comes from experiments in which PKC activation was prevented. Thus, down-regulation of PKC by prolonged exposure to PDBu, which led to marked loss of PKC-associated immunoreactivity (see also Young et al., 1987; Ewald et al., 1988a), completely blocked the ability of Bk and PDBu to stimulate Ca²⁺ influx, and the proportion of cells responding to Bk with an inward current was much reduced. The ability of Bk to increase ³H-InsP₃ formation was, however, unaltered, implying that PIC activation was not secondary to activation of PKC and that the treatment had not compromised the Bk receptor or any part of the signal-transduction pathway through to the generation of inositol phosphates. Additionally, the ability of both PDBu and Bk, but not K⁺, to stimulate Ca²⁺ influx was blocked by staurosporine, a potent inhibitor of PKC *in vitro* (Tamaoki et al., 1986). Staurosporine is not specific for PKC, but inhibits a wide variety of protein kinases, including cAMP- and cGMP-dependent protein kinases (see Tamaoki et al., 1986). Bk does not, however, increase cAMP in DRG neurons, and although it does increase cGMP, this is unlikely to be responsible for the ⁴⁵Ca uptake, as neither dibutyl-³-cGMP or sodium nitroprusside (which activates guanylate cyclase directly) alters the rate of ⁴⁵Ca uptake (Burgess et al., in press). The lack of effect of staurosporine on the K⁺-induced ⁴⁵Ca uptake suggests that it was not blocking Ca²⁺ channels directly, so it is likely that its action on Bk- and PDBu-mediated Ca²⁺ influx is at the level of PKC, although effects on other kinases cannot be ruled out.

The effect of staurosporine on the electrophysiological responses to Bk in DRG neurons was not so clear cut. Staurosporine reduced the proportion of cells that responded to Bk, but was less effective in this regard than down-regulation of PKC; the reason for this discrepancy is not known. The observations that some cells were able to respond to Bk, both in the presence of staurosporine and when PKC had been down-regulated and that a small number of PDBu-insensitive cells responded to Bk, combine to suggest that PKC activation is not the only mechanism by which DRG neurons can be activated by Bk. On the other hand, excitation of C-fibers in the tail of the neonatal rat by both Bk and PDBu is strongly blocked by staurosporine (Bettaney et al., 1988; Dray et al., 1988), suggesting that PKC activation may be important *in vivo*.

How does PKC activation control Ca^{2+} entry?

The increase in Ca^{2+} uptake induced by either Bk or PDBu was not additive with that induced by K^+ , and the effect of all 3 agents was blocked by nifedipine. This suggests that Bk and PDBu both act by causing depolarization rather than by opening Ca^{2+} channels directly.

There is evidence from several tissues that PKC can regulate the activity of voltage-activated Ca^{2+} channels. In *Aplysia* neurons (DeRiemer et al., 1985) and NG108-15 cells (Osugi et al., 1986), PKC activation facilitates Ca^{2+} currents, and in $A_{7.75}$ smooth muscle cells phorbol esters cause an increase in the rate of dihydropyridine-sensitive Ca^{2+} influx (Sperti and Colucci, 1987). On the other hand, PKC activation attenuates Ca^{2+} currents in PC12 cells (Di Virgilio et al., 1986), aortic muscle cells (Galizzi et al., 1987), and, notably, chick DRG neurons (Rane and Dunlap, 1986). Inhibition of Ca^{2+} currents by neuropeptide Y in rat DRG neurons has also been attributed to PKC activation (Ewald et al., 1988a). Neither Bk nor PDBu affected the increase in Ca^{2+} uptake in response to K^+ , which argues against a strong facilitatory or inhibitory effect of PKC on the nifedipine-sensitive Ca^{2+} channels of these cells. The studies of Rane and Dunlap (1986) and Ewald et al. (1988a) suggest that PKC activation inhibits L-type Ca^{2+} currents, and we plan to test whether Bk has a similar effect in rat DRG neurons.

Comparison with NG108-15 cells

The NG108-15 cell line, which possesses PIC-linked Bk receptors (Yano et al., 1984, 1985; Higashida et al., 1986; Brown and Higashida, 1988), has been used as a model to characterize the roles of the second messengers of the phosphoinositide system in the response of neuronal cells to Bk. Though there are superficial similarities between DRG neurones and NG108-15 cells, in that both show a depolarization and an increase in dihydropyridine-sensitive Ca^{2+} uptake in response to Bk and to phorbol esters, the mechanism for the depolarizing response seems to be dissimilar. Thus, in NG108-15 cells the PKC-mediated depolarization is accompanied by a decrease, rather than an increase in membrane conductance, which is associated with inhibition of a voltage-sensitive K^+ current (see Brown and Higashida, 1988).

Another difference between the cells is that NG108-15 cells respond to Bk with an initial hyperpolarization, associated with an $Ins(1,4,5)P_3$ -mediated increase in $[Ca^{2+}]_i$, and the opening of Ca^{2+} -activated K^+ channels (Brown and Higashida, 1988). This hyperpolarizing response is not seen in DRG neurons, despite a substantial increase in $Ins(1,4,5)P_3$. This lack of a Ca^{2+} -mediated hyperpolarization in DRG neurons is surprising since

these cells are known to possess Ca^{2+} -gated K^+ channels (Gorke and Pierau, 1980; Kameyama, 1983). Although Thayer et al. (1988) have recently reported that 100 nM Bk increases $[Ca^{2+}]_i$ in the soma of DRG neurons, the peak $[Ca^{2+}]_i$ observed was only about 200 nM, which may be insufficient to activate Ca^{2+} -gated K^+ channels.

Effect of PKC activation on Bk-induced formation of $InsP_3$

An important role of the DAG/PKC pathway in many tissues is down-regulation of receptor-activated changes in phosphoinositide metabolism and Ca^{2+} signaling (e.g., Leeb-Lundberg et al., 1985; Vincentini et al., 1985; Watson and Lapetina, 1985; Liles et al., 1986; Francel et al., 1987; Osugi et al., 1987). Desensitization of the membrane response to Bk and the increase in 3H - $InsP_3$ was very marked in DRG neurons. Pretreatment of the cells with PDBu transiently reduced the Bk-evoked increase in 3H - $InsP_3$. This suggests that prior exposure to Bk or PDBu modulates either the receptor, PIC, or the G-protein that links them. The site(s) at which PKC exerts its effect and its possible involvement in desensitization are under investigation.

Conclusions

Our results suggest the following sequence of events when Bk is applied to DRG neurons. First, PIC is activated, leading to an increase in the cellular level of $Ins(1,4,5)P_3$ and 1,2-DAG. The increase in 1,2-DAG activates PKC, which in turn causes an inward current by opening Na^+ channels and hence depolarizes the neurons. It is likely, however, that other mechanisms, such as channels linked directly to the Bk receptor via a G-protein or operated by arachidonic acid or an inositol phosphate, contribute to the depolarizing effect of Bk in a proportion of cells. The depolarization, by opening voltage-regulated Ca^{2+} channels, is responsible for the Bk-induced increase in Ca^{2+} influx.

These results provide more evidence for the suggestion (see Bettaney et al., 1988; Dray et al., 1988) that activation of PKC may be important in the chemical excitation of nociceptive neurons. Several subtypes of PKC exist that have different patterns of expression in different tissues (Coussens et al., 1986; Parker et al., 1986; Housey et al., 1987; see also Worley et al., 1986). It will be important to determine which subtype(s) is involved in the transmission of nociceptive information for Bk.

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