Cyclic GMP regulates activation of phosphoinositidase C by bradykinin in sensory neurons

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Prior exposure of cultured neonatal rat dorsal root ganglion (DRG) neurons to bradykinin resulted in marked attenuation of bradykinin-induced activation of phosphoinositidase C (PIC). The (log concentration)–response curve for bradykinin-induced \([^{3}H]\)inositol trisphosphate (\([^{3}H]\)IP$_3$) formation was shifted to the right and the maximum response was reduced. Bradykinin increases cyclic GMP (cGMP) in DRG neurons [Burgess, Mullane, McNeill, Coote, Minhas and Wood (1989) J. Neurochem. 53, 1212–1218] and treatment of the neurons with dibutyryl cGMP (dbcGMP) had a similar, inhibitory, effect on these cells to bradykinin [9]. Evidence for such a role has also been provided by Dray et al. [12] in a sensory fibre preparation of the (log concentration)–response curve for bradykinin-induced \([^{3}H]\)IP$_3$ formation. The ability of LNNA to prevent desensitization was reversed by excess l-arginine, indicating that its actions were mediated through inhibition of nitric oxide synthase. In addition to functional desensitization, exposure to bradykinin reduced the number of cell-surface receptors detected with \([^{3}H]\)bradykinin, without affecting its $K_D$ value for the remaining sites. In contrast to bradykinin, pretreatment with dbcGMP had no effect on either the $K_D$ or $B_{max}$ for \([^{3}H]\)bradykinin binding. This implies that the inhibitory effect of dbcGMP was downstream from the binding of bradykinin to its receptor and upstream of IP$_3$ formation. The lack of effect of dbcGMP on \([^{3}H]\)bradykinin binding suggests that the decrease in receptor number induced by bradykinin was mediated by a different mechanism and was not a key factor in the rapid phase of desensitization in these cells.

INTRODUCTION

The mechanisms by which receptors that couple to phosphoinositidase C (PIC) desensitize are not as well understood as those for the ß-adrenoceptor. A role for protein kinase C in the desensitization of PIC-linked hormones has been suggested in a number of cells (e.g. [1,2]) and depletion of intracellular Ca$^{2+}$ stores, resulting in lack of feed-forward activation of PIC, has been implicated in heterologous desensitization [3]. Receptor phosphorylation by specific receptor kinases has been proposed as a mechanism for homologous desensitization for this class of receptor (for a review see [4]).

In dorsal root ganglion (DRG) neurons bradykinin, acting via $B_2$ receptors, stimulates PIC [5–7]. This leads to the activation of a protein kinase C-sensitive inward current, termed $I_{na}$ that is carried mainly by sodium ions and is likely to be at least partly responsible for depolarizing and activating the neurons in response to bradykinin [6,8]. These responses desensitize after short exposures of the neurons to bradykinin [9,10]. Bradykinin also causes a rise in cellular cyclic GMP (cGMP) in DRG neurons [9,11] and we have previously suggested that this cyclic nucleotide may play a role in regulating the responsiveness of these cells to bradykinin [9]. Evidence for such a role has also been provided by Dray et al. [12] in a sensory fibre preparation and by McGeehe et al. [10], who showed that dibutyryl cGMP (dbcGMP) could attenuate bradykinin-induced activation of $I_{na}$ in cultured DRG neurons. McGeehe et al. [10] also reported that bradykinin-induced desensitization of $I_{na}$ could be reduced by a number of l-arginine analogues that inhibit nitric oxide synthase (NOS), the Ca$^{2+}$-sensitive enzyme responsible for nitric oxide (NO) formation. In the present study, the role and mechanism of the NO/cGMP pathway in regulating the sensitivity of DRG neurons to bradykinin has been investigated at the level of binding of \([^{3}H]\)bradykinin to the $B_2$ bradykinin receptor and activation of PIC.

MATERIALS AND METHODS

Materials
All tissue-culture reagents were obtained from Gibco BRL Life Technologies Ltd., Paisley, U.K. myo-2-[\(^{3}H\)]inositol (specific radioactivity 10–20 Ci/mmol) and \([^{3}H]\)bradykinin (specific radioactivity 65 Ci/mmol) were obtained from Amersham International plc., Amersham, U.K. Bradykinin, was obtained from Bachem A.G., Bubendorf, Switzerland, dbcGMP was obtained from Calbiochem c/o Novabiochem, Nottingham, U.K. and the cGMP ELISA kits were supplied by the Cayman Chemical Company, Ann Arbor, MI, U.S.A. All other reagents were from BDH, Dagenham, U.K., or Sigma, Poole, U.K.

Culture of DRG neurons
DRG neurons from neonatal rats were prepared as described previously [6] and plated on to poly-d-ornithine- and laminin-coated coverslips (30000 cells/coverlip) for measurement of \([^{3}H]\)inositol trisphosphate (\([^{3}H]\)IP$_3$) and 40000 cells/coverlip for \([^{3}H]\)bradykinin binding experiments and for measurement of...
cGMP). Briefly, the cells were maintained in 500 µl of growth medium containing 50% (v/v) F-14 culture medium, 50% (v/v) conditioned medium from C6 glioma cells, 5 µg/ml nerve growth factor and 10 nM cytosine arabinoside. After 3–4 days, when more than 90% of the non-neuronal cells had been eliminated, the cytosine arabinoside was removed from the medium and the neurons were used 3–4 days later.

Measurement of [3H]IP₃ production

The DRG neurons were incubated in Minimum Essential Medium (Eagle’s medium) with Earle’s salts, without l-methionine or glutamine, containing 50 µCi/ml ³H]inositol phosphate formation was performed as described previously [6]. Briefly, the cells were exposed to drugs by placing the coverslips in 1 ml of 25 mM Hepes-buffered Dulbecco’s modified Eagle’s medium (DMEM) at 37 °C. The reactions were stopped in 4.5% perchloric acid containing 1 mg/ml phytic acid at 4 °C. Following neutralization, the [³H]inositol phosphates were separated by anion-exchange chromatography on Dowex columns (formate form). [³H]IP₃ was eluted from the columns with 0.8 M ammonium formate in 0.1 M formic acid and expressed as a percentage of cellular [³H]phosphoinositides, the majority of which (> 90%) is [³H]phosphatidylinositol (PI) in these cells [6]. The amount of radioactivity in the lipid fraction did not alter during the course of the experiments.

Measurement of [³H]bradykinin binding

Cells were pretreated with buffer, bradykinin or dbcGMP, as described in individual experiments. Following pretreatment, the cells were washed by dipping the coverslips into 25 ml of 25 mM Hepes-buffered DMEM at 37 °C for 1 min. As bradykinin remaining on the cells would have interfered with the [³H]bradykinin binding assay, the effectiveness of the wash was determined by spiking some samples with [³H]bradykinin and measuring the amount of radioactivity left on the cells. The washing procedure described above removed more than 99.9% of the [³H]bradykinin from the neurons (results not shown). The coverslips were then placed in 225 µl of binding buffer (25 mM K₂HPO₄, 240 mM sucrose, 1 mM captopril, 1 mM dl-mercaptomethyl guanidinioethyl thiopropanoic acid and 0.2% BSA, pH 6.8) containing the appropriate dilutions of [³H]bradykinin in polyornithine-coated 24-well plates and incubated for 90 min (equilibrium) at 4 °C. Non-specific binding was determined in the presence of 100 µM bradykinin. Following exposure of the cells to [³H]bradykinin, the cells were washed by dipping the coverslips into 20 ml of assay buffer containing 0.2% BSA for 45 s at 4 °C. This was followed by two 20 s washes in assay buffer without BSA. The cells were then solubilized in 200 µl of 1 M NaOH and an aliquot (25 µl) removed for protein determination. The radioactivity in the remainder of the sample was determined by liquid scintillation counting. The concentration of free radioligand during the assay was measured by counting radioactivity in 100 µl samples of the incubation buffer. Results were analysed using LIGAND [13]. Kᵦ and Bₘax values from independent sets of data were compared to assess the differences between sets of data.

Measurement of cGMP levels

The level of cGMP in the neurons was measured as described previously [9].

Data calculation

Fitting of curves, other than saturation binding curves, to individual data sets was done using the Marquardt–Levenberg non-linear least-squares curve-fitting algorithm in ORIGIN (Version 3.0), from MicroCal Software, Inc. Results were compared using the unpaired one-tailed Student’s t-test. Differences were considered to be significant if P < 0.05.

RESULTS

Desensitization of bradykinin-induced [³H]IP₃ formation

[³H]IP₃ increases rapidly in primary cultures of neonatal rat DRG neurons challenged with bradykinin [6]. In neurons that have been pretreated with bradykinin this response is significantly reduced. For example, a 1 min pretreatment of DRG neurons with 100 nM bradykinin followed by extensive washing, attenuated the increase in [³H]IP₃ formation induced by a test challenge with bradykinin (30 nM, 10 s), applied 15 min later, by 82.4 ± 6.4% (n = 4). This protocol, details of which are given in the legend of Figure 1 and in the Materials and methods section, was used for subsequent experiments in which the mechanism of desensitization was investigated. Pretreatment with bradykinin also reduced the rise in total [³H]inositol phosphates induced by bradykinin, suggesting that the decrease in [³H]IP₃ was due to reduced formation, rather than increased metabolism, of [³H]IP₃ (results not shown). Desensitization of bradykinin-induced [³H]IP₃ formation was dependent on the concentration of bradykinin applied during the desensitizing challenge. Following a 1 min exposure, half-maximal inhibition of a test challenge with 30 nM bradykinin occurred at 23.8 ± 2.7 nM bradykinin (n = 3) (Figure 1).

When cells were pretreated with 100 nM bradykinin for 1 min, using the protocol described in Figure 1, the (log-concentration)–response curve for bradykinin-induced [³H]IP₃ formation was

![Figure 1](image-url)
shifted to the right. The \(K_n\) value increased from 7.6 ± 0.6 nM to 57.2 ± 8.3 nM and the maximum response in the desensitized cells was only 44.4 ± 2% of the response in control cells (Figure 2).

\(\text{[H]}\text{[bradykinin} binding\n
The effect of pretreating DRG neurons with bradykinin on their ability to bind \(\text{[H]}\text{[bradykinin was investigated. Computer fitting by LIGAND of saturation data for the binding of}[\text{H}]\text{bradykinin in control DRG neurons revealed a single site with a }K_n\text{ of 0.35 ± 0.18 nM and a }B_{\text{max}}\text{ of 282 ± 65 fmol/mg of protein (Figure 3). In cells that had been pre-exposed to 100 nM bradykinin for 1 min, the }K_n\text{ value was 0.25 ± 0.12 nM which was not significantly different from the value in control cells (see also Table 1). There was, however, a marked loss of binding sites in the bradykinin-treated cells, the }B_{\text{max}}\text{ value falling from 282 ± 65 fmol/mg to 62 ± 18 fmol/mg of protein (}\text{P < 0.0001). A two-site model did not show a significant statistical improvement over a one-site model for the data obtained before or after treatment with bradykinin.\n
Role of cGMP in regulating the sensitivity of sensory neurons to bradykinin

Application of dbcGMP (1 mM) to the neurons for 2 min had no effect on resting levels of \(\text{[H]}\text{IP}3\) (0.29 ± 0.02% \(\text{[H]}\text{PI, compared with 0.30 ± 0.02% \(\text{[H]}\text{PI in control cells, n = 4). However, pretreatment with both dbcGMP (1 mM) and sodium nitroprusside (30 \(\mu\)M) for 2 min reduced the response to a test challenge with bradykinin (30 nM for 10 s) by 57.4 ± 10.3% and 72.8 ± 2.5% respectively. Following exposure to 1 mM dbcGMP, the \(EC_{50}\) value for bradykinin-induced \(\text{[H]}\text{IP}3\) formation was shifted from 7.6 ± 0.6 nM to 22.5 ± 8.7 nM and the size of the maximum response was reduced to 66.3 ± 3.5% of the response in control cells (see Figure 2). Increasing the time that the neurons were exposed to dbcGMP from 2 min to 5 min, or increasing the concentration of the cyclic nucleotide from 1 mM to 10 mM, caused no further inhibition of the bradykinin response (results not shown).

Treatment of the DRG neurons with dbcGMP, at a concentration (1 mM) that reduced bradykinin-mediated \(\text{[H]}\text{IP}3\) formation, had no significant effect on either the \(K_n\) value or the \(B_{\text{max}}\) for \(\text{[H]}\text{bradykinin binding (Table 1). A two-site model did not show a significant statistical improvement over a one-site model for data obtained after treatment with dbcGMP.\n
Effects of N\textsuperscript{5}-nitro-L-arginine (LNNA) and L-arginine

LNNA inhibits NOS and the generation of cGMP in a number of tissues (e.g. [11] and see [14] for a review). In the cultured DRG neurons LNNA was able to prevent bradykinin-induced cGMP formation. The basal level of cGMP was 1.4 ± 0.6 pmol/mg of protein (n = 3) and this increased to 5.4 ± 1.7 pmol/mg of protein after a 30 s exposure to 30 nM bradykinin in control cells, compared with 1.54 ± 0.4 pmol/mg of protein (n = 3) in cells pretreated for 30 min with 300 \(\mu\)M LNNA. This meant that LNNA could be used to test the possibility that cGMP generated by bradykinin was involved in regulating bradykinin-mediated \(\text{[H]}\text{IP}3\) formation.

A 30 min preincubation of the DRG neurons with LNNA (300 \(\mu\)M) had no effect on either resting levels of \(\text{[H]}\text{IP}3\) or

<table>
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<th>Pretreatment</th>
<th>(K_n) (nM)</th>
<th>(B_{\text{max}}) (fmol/mg of protein)</th>
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<tbody>
<tr>
<td>Control</td>
<td>0.35 ± 0.18</td>
<td>282 ± 65</td>
</tr>
<tr>
<td>100 nM Bradykinin</td>
<td>0.25 ± 0.12</td>
<td>62 ± 18*</td>
</tr>
<tr>
<td>1 mM DbcGMP</td>
<td>0.59 ± 0.17</td>
<td>215 ± 56</td>
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Formation of \[^{3}H\]IP\(_3\) in response to a test challenge with bradykinin (30 nM, 10 s) was measured after a wash and a 15 min recovery period in the presence of buffer, LNNA or LNNA plus \(\ell\)-arginine (\(\ell\)-Arg) as appropriate. An asterisk indicates that the value is significantly different from the response to bradykinin in control cells exposed to a desensitizing challenge with bradykinin (\(P < 0.001\)). There was no significant difference (\(P > 0.05\)) between the response to bradykinin (30 nM, 10 s) in desensitized cells and desensitized cells preincubated with a combination of LNNA plus \(\ell\)-arginine.

**DISCUSSION**

In the present study we have investigated the role of cGMP in regulating bradykinin-induced activation of PIC in sensory neurons. Exposure of DRG neurons to bradykinin led to functional uncoupling of the B\(_2\) receptor from PIC that was manifested by a rightward shift of the (log concentration)–response curve for bradykinin-induced \[^{3}H\]IP\(_3\) formation and a substantial reduction in the size of the maximum response.

In addition to functional uncoupling, brief (60 s) exposure to bradykinin resulted in a decrease in the number of receptors accessible to \[^{3}H\]bradykinin. This loss occurred with no change in the affinity of the remaining sites for \[^{3}H\]bradykinin and could represent either sequestration or internalization of the B\(_2\) receptors. Similar effects on \[^{3}H\]bradykinin binding have been described in other cell types [20–23], although usually after longer exposures to bradykinin.

**Role of cGMP**

Previous studies have provided circumstantial evidence to suggest that cGMP may be involved in regulating the sensitivity of sensory neurons to bradykinin [9, 10, 12] and a recent report suggests that it may also play a role in desensitization of bradykinin-mediated relaxation of canine coronary arteries [24]. The results of the present study demonstrate that the functional uncoupling induced by dbcGMP was qualitatively similar to bradykinin-induced desensitization in terms of the rightward shift of the (log concentration)–response curve and reduction of the maximal response to bradykinin. The fact that it was not quite as effective as desensitization could be because local intracellular concentrations of cGMP are higher after receptor

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**Table 2** Heterologous desensitization

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<tr>
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<th>Formation of [^{3}H]IP(_3) (% of total [^{3}H]PI)</th>
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<tbody>
<tr>
<td></td>
<td>Basal</td>
</tr>
<tr>
<td>Naive</td>
<td>0.24 ± 0.05</td>
</tr>
<tr>
<td>Bradykinin pretreatment</td>
<td>0.19 ± 0.04</td>
</tr>
<tr>
<td>NPY pretreatment</td>
<td>0.25 ± 0.03</td>
</tr>
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**Figure 4** Effects of LNNA and \(\ell\)-arginine

(A) Cells were preincubated with buffer alone (–), 300 \(\mu\)M LNNA or 300 \(\mu\)M LNNA plus 3 mM \(\ell\)-arginine for 30 min before and during a desensitizing challenge with bradykinin (100 nM, 1 min). The response to a test challenge of bradykinin (30 nM, 10 s) was measured after a wash and a 15 min recovery period in the presence of buffer, LNNA or LNNA plus \(\ell\)-arginine (\(\ell\)-Arg) as appropriate. An asterisk indicates that the value is significantly greater (\(P < 0.001\)) than the response of control cells to a test challenge with 30 nM bradykinin (3.2 ± 0.7% of \[^{3}H\]IP\(_3\) in control cells, compared with 2.5 ± 0.8% of \[^{3}H\]IP\(_3\) in cells treated with \(\ell\)-arginine for 30 min).

In contrast to its ability to reverse bradykinin-mediated desensitization, LNNA had no effect on dbcGMP-mediated desensitization of the response to bradykinin (\(P > 0.1\)) (Figure 4B).

**Heterologous desensitization**

Autoradiography [15] and electrophysiological studies [16] have demonstrated that receptors for neuropeptide Y (NPY) and bradykinin are co-localized on cultured DRG neurons. As NPY has been reported to activate PIC in DRG neurons [17], the possibility that cross-desensitization could occur was investigated. Preliminary experiments confirmed that NPY increased \[^{3}H\]IP\(_3\) in the DRG neurons. The response to 100 nM NPY was abolished if the cells were pre-exposed to 1 \(\mu\)M NPY for 1 min (Table 2). Table 2 also shows that a 1 min pretreatment with 1 \(\mu\)M NPY reduced the response to a test challenge with bradykinin (30 nM, 10 s) by 48%, and that pretreatment of the cells with 100 nM bradykinin for 1 min reduced the response to NPY (100 nM, 10 s) by 56%.
activation than following exogenous application of the cGMP analogue. Alternatively, a degree of receptor occupancy may be required for efficient uncoupling of the functional response from the receptor. Receptor occupancy appears to be required for heterologous desensitization of responses to bradykinin and carbachol in MDCK cells [1] and a role for receptor occupancy in the regulation of α2-adrenoceptors by phosphorylation has been proposed [2]. Indeed, in a study in sensory neurons [10], minimal B3 receptor occupancy was required for inhibition of bradykinin-mediated activation of I\textsubscript{max} by cGMP analogues. Receptor-regulated factors that could be involved include protein kinase C, which has been implicated in the regulation of the B3 receptor in other cells [21,25,26], or a receptor-specific kinase [4].

Direct evidence for the involvement of cGMP in desensitization of bradykinin-mediated activation of PIC was obtained with the t-arginine analogue, LNNA, which prevented desensitization of bradykinin-induced [\textsuperscript{3}H]IP\textsubscript{3} formation. LNNA did not increase the response to bradykinin in naive cells nor did it prevent dbcGMP-induced inhibition of bradykinin-induced [\textsuperscript{3}H]IP\textsubscript{3} formation, suggesting that it was not augmenting bradykinin responses in a non-specific manner. As the effects of LNNA were reversed with excess t-arginine and LNNA abolished bradykinin-induced GMP formation it appears most likely that its effect was mediated through inhibition of NO formation and the subsequent induced cGMP formation, suggesting that it was not augmenting bradykinin receptor mediated inward current I\textsubscript{max} [10]. The demonstration that LNNA can reverse desensitization of bradykinin-induced IP\textsubscript{3} formation suggests that the site of action of cGMP is upstream of IP\textsubscript{3} formation.

There is little information about the action of cGMP-dependent kinase on PIC signalling, although it has been suggested that it might phosphorylate one or more components of this signal transduction pathway [32]. A number of receptors linked to PIC, including the pancreatic cholecystokinin receptors [27] and M3-muscarinic receptors [28], are phosphorylated during desensitization. Indeed, sequence data for both rat and human B3 receptors indicate that they have consensus sequences for serine and threonine phosphorylation [29,30] and studies in a human cell line suggest that the B3 receptor can become phosphorylated [31], although this has not been correlated with desensitization. However, although dbcGMP mimicked the effects of desensitization on the functional responses, it had no effect on the binding of [\textsuperscript{3}H]bradykinin. In light of this, one possibility that should be considered is that the B3 receptors on the neurons can be phosphorylated by cGMP-dependent kinase in a manner that interferes with coupling between the receptor and its G-protein, rather than the interaction between agonist and receptor. It is also possible that cGMP kinase acts directly either on the G-protein or on PIC. In Chinese hamster ovary (CHO) cells transfected with cGMP-dependent kinase, activation of the kinase resulted in a reduction of thrombin-induced Ca\textsuperscript{2+} transients by interfering with IP\textsubscript{3} formation [33], and Hirata et al. [34] showed that pretreatment of bovine aortic smooth-muscle membranes with cGMP was able to inhibit vasopressin-induced [\textsuperscript{3}H]IP\textsubscript{3} formation. These latter authors proposed that this effect was mediated downstream of the receptor, as cGMP also inhibited guanosine 5'-[\gamma-thio]triphosphate-induced [\textsuperscript{3}H]IP\textsubscript{3} formation.

The lack of effect of dbcGMP on the binding parameters for [\textsuperscript{3}H]bradykinin contrasted with the marked loss of accessible cell-surface [\textsuperscript{3}H]bradykinin sites that occurred after exposure to bradykinin. These findings suggest, first, that the decrease in B\textsubscript{max} induced by bradykinin is not necessarily involved in the rapid reduction in functional responsiveness, and secondly that it must be mediated by a mechanism other than cGMP, such as a receptor specific kinase [4]. A reduction in the number of binding sites for [\textsuperscript{3}H]bradykinin would not necessarily result in reduced sensitivity to bradykinin if there were spare receptors on the neurons.

**Heterologous desensitization**

The ability of cGMP to regulate the sensitivity of sensory neurons to bradykinin implied that other receptors that activate PIC and elevate cGMP might be able to down-regulate responses to bradykinin. The lack of complete cross-desensitization between NPY and bradykinin, which are co-localized on the same neurons [15,16], may be due partly to compartmentalization of the second messengers formed after receptor activation (e.g. [2]), or could reflect a requirement for receptor occupancy for effective desensitization [1,10]. This would provide a mechanism, other than a receptor selective kinase, for allowing selective desensitization of specific receptors to occur.

The data in this study provide direct evidence for a major role for cGMP in functional desensitization of bradykinin in DRG neurons, and implies that its site of action is downstream of receptor binding and upstream of IP\textsubscript{3} formation. cGMP does not appear to be involved in the loss of cell-surface-accessible B3 receptor, observed during desensitization, suggesting that the B3 bradykinin receptor can be regulated by several mechanisms in these cells.

**REFERENCES**

18. Reference deleted.
19. Reference deleted.

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