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Heterologous Desensitization of Both Phosphoinositide and Ca^{2+} Signaling in SH-SY5Y Neuroblastoma Cells: A Role for Intracellular Ca^{2+} Store Depletion?

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SUMMARY

Measurement of the intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) in fura-2-loaded single cells of the human neuroblastoma line SH-SY5Y indicated coexpression of muscarinic and bradykinin receptors linked to activation of phosphoinositidase C (PIC). Both agonists elevated $[Ca^{2+}]_i$ and inositol-1,4,5-trisphosphate [Ins(1,4,5)P_3] levels in populations of adherent cells, although in cells used directly upon attainment of confluence the responses to carbachol were greater than those to bradykinin and displayed additional sustained components. This model system was used to examine heterologous interactions when a second PIC-linked agonist was added 100–300 sec after but in the continued presence of the first. Maximal (1 mM) carbachol concentrations abolished the elevation of $[Ca^{2+}]_i$ produced by bradykinin but the muscarinic antagonist atropine (10 μM) restored the response, provided that extracellular Ca^{2+} was present throughout the experiment or was added before bradykinin. Carbachol also abolished bradykinin-mediated Ins(1,4,5)P_3 elevation. In contrast, bradykinin did not influence $[Ca^{2+}]_i$ or Ins(1,4,5)P_3 responses to carbachol in the presence of extracellular Ca^{2+}. In cells maintained at confluence for 2 weeks, the rapid peak elevations of $[Ca^{2+}]_i$ and Ins(1,4,5)P_3 levels induced by carbachol and bradykinin were approximately equivalent in magnitude. In these cells carbachol again abolished bradykinin-mediated elevation of $[Ca^{2+}]_i$ but only attenuated, rather than abolished, the elevation of Ins(1,4,5)P_3 levels. The $[Ca^{2+}]_i$ and Ins(1,4,5)P_3 responses to bradykinin were fully restored 100 sec after atropine only in the presence of extracellular Ca^{2+}. Thus, depletion of an intracellular Ins(1,4,5)P_3-sensitive Ca^{2+} store may underlie the ability of carbachol to produce not only heterologous desensitization of the $[Ca^{2+}]_i$ elevation induced by bradykinin but also that of the Ins(1,4,5)P_3 response. This suggests a feed-forward activation of PIC by Ca^{2+} released from Ins(1,4,5)P_3-sensitive stores. Furthermore, studies in which Ins(1,4,5)P_3-sensitive stores were depleted with thapsigargin and cells were challenged in the presence or absence of extracellular Ca^{2+} indicated that Ca^{2+}, irrespective of its origin (intra- or extracellular), potentiated the Ins(1,4,5)P_3 response to bradykinin alone. In cells maintained at confluence for 2 weeks, bradykinin was again unable to influence either $[Ca^{2+}]_i$ or Ins(1,4,5)P_3 responses to carbachol in the presence of Ca^{2+}. This lack of heterologous desensitization may be due to the rapid, full, homologous desensitization of bradykinin receptors, compared with an incomplete homologous desensitization of muscarinic receptors.

A large number of agonists, after interaction with their cell surface receptors, increase the activity of one or more isoforms of PIC via G proteins, predominantly of the $G_{q/11}$ family (1). Activation of PIC enhances the hydrolysis of phosphoinositide-4,5-bisphosphate, producing Ins(1,4,5)P_3 and 1,2-diacylglycerol, which release Ca^{2+} from intracellular stores and activate protein kinase C, respectively (1). Ins(1,4,5)P_3-sensitive Ca^{2+} stores are rapidly depleted and, although the influx of extracellular Ca^{2+} may sustain the elevation of $[Ca^{2+}]_i$ (2), this is rarely at the level produced after the rapid release from intracellular stores. Depletion of these stores may, therefore, constitute a mechanism for the rapid attenuation or termination of this limb of the signaling pathway. There is, however, accumulating evidence that the activation of PIC itself may be subject to acute desensitization. Thus, the very early accumulation of [^{3}H]inositol phosphates and/or Ins(1,4,5)P_3 after agonist exposure in several systems displays biphasic profiles consistent with a rapid, although often only partial, desensitization (3–7). The mechanism underlying this desensitization is unclear but may include receptor phosphorylation by specific receptor kinases (5, 8) and appears to be in addition to the desensitization associated with receptor internalization and down-regulation.

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ABBREVIATIONS: PIC, phosphoinositidase C; AM, acetoxymethyl ester; $[Ca^{2+}]_e$, extracellular calcium concentration; $[Ca^{2+}]_i$, intracellular calcium concentration; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; Ins(1,4,5)P_3, inositol-1,4,5-trisphosphate; PI, phosphoinositide; SH-SY5Y_{2wc} cells, SH-SY5Y cells maintained at confluence for 2 weeks; TCA, trichloroacetic acid.

The logic for such homologous desensitization in the face of continued or repetitive stimulation by the same agonist is clear, and desensitization appears to occur in many receptor systems (2). It is not unreasonable to suspect that, in the interests of cellular regulation, activation of a signaling pathway by one agonist may also influence cellular responses to different agonists linked to the same transduction pathway. Such heterologous desensitization of PIC-linked receptors has indeed been observed, particularly at the level of $[Ca^{2+}]_i$ elevation. Depletion of Ins(1,4,5)P_3-sensitive Ca^{2+} stores has been implicated in this desensitization (9–15), although limitation of the response at a step before this in the signaling pathway has been suggested (9, 16–18).

The current study used the SH-SY5Y human neuroblastoma cell line, which expresses both muscarinic (predominantly M3) (19) and bradykinin (B2) (20, 21) receptors linked to activation of PIC, to examine potential heterologous desensitization of the rapid transient increases in both $[Ca^{2+}]_i$ and Ins(1,4,5)P_3 levels. This used experimental protocols in which the second agonist was added after, but in the continued presence of, the first agonist. We demonstrate that heterologous desensitization occurs at the level of both $[Ca^{2+}]_i$ elevation and PIC activation. These phenomena require ongoing agonist occupation of the receptor used for the primary challenge and are not present after either complete homologous desensitization or antagonism of receptor activation. Heterologous desensitization of the $[Ca^{2+}]_i$ response can be accounted for by depletion of the Ins(1,4,5)P_3-sensitive Ca^{2+} pool, which itself may play a role in the heterologous desensitization of Ins(1,4,5)P_3 responses due to a lack of Ca^{2+} feed-forward activation of PIC.

Experimental Procedures

Cell culture. Cultures of the human neuroblastoma cell line SH-SY5Y (originally given by Dr. J. Biedler, Sloan-Kettering Institute, New York, NY) were maintained in minimum essential medium supplemented with 50 IU/ml penicillin, 50 μg/ml streptomycin, 2.5 μg/ml amphotericin B, 2 mM L-glutamine, and 10% (v/v) newborn calf serum. Stock cultures were maintained at 37° in 5% CO_2/humidified air, passaged weekly at 1:4–6, and fed or re-fed alternately two or three times each week. Experiments were performed on cells of passages 70–90.

Population $[Ca^{2+}]_i$ measurements. These measurements were performed upon adherent confluent populations of cells grown on glass coverslips as described elsewhere (21). Briefly, cells were loaded with fura-2/AM (3.3 μM, 40 min at room temperature) after determination of autofluorescence. Unloaded fura-2/AM was removed by washing and cells were left to stand at 37° for 5 min before use. When agonist challenges were to be at reduced $[Ca^{2+}]_e$, cuvettes were washed with low-Ca^{2+} buffer and left to stand for an additional 5 min at 37°. Agonists were added in buffer (with or without Ca^{2+}) in 50-μl aliquots. With emission set at 509 nm, the 340-nm/380-nm excitation ratio was recorded every 3.8 sec at 37°, as an index of $[Ca^{2+}]_i$. Buffer (Krebs/HEPES) composition was 10 mM HEPES, 4.2 mM $NaHCO_3$, 10 mM glucose, 1.2 mM $MgSO_4$, 4.7 mM KCl, 118 mM NaCl, and 2 mM $CaCl_2$. The $[Ca^{2+}]_e$ concentration was approximately 2 μM in nominally Ca^{2+}-free buffer or was set at 50–100 nM in low-Ca^{2+} buffer by the addition of EGTA. The Ca^{2+} concentration was determined using fura-2 (22). An *in situ* calibration of cellular fura-2 fluorescence was performed in separate studies deriving R_{max} with either ionomycin or Br-A23187 (6–33 μM) and $[Ca^{2+}]_e$ of up to 10 mM, followed by EGTA addition for R_{min}. These values were used to convert population 340/380 ratios to $[Ca^{2+}]_i$ values (22).

Single-cell $[Ca^{2+}]_i$ measurements. Cells were harvested from confluent cultures using 10 mM HEPES, 154 mM NaCl, 0.54 mM EDTA, pH 7.4, and were reseeded in 3 ml of medium, at low density (cells from an area of the confluent flask equivalent to 1/20 of coverslip size added), on sterile, glass, 11- × 22-mm coverslips in eight-well multidishes. The following day medium was replaced with 2 ml of Krebs/HEPES buffer with 5 μM fura-2/AM. The buffer adjacent to the coverslip was stirred with a magnetic stirring bar, and the cells were left at room temperature for 40 min. The coverslip was then washed with 5 ml of buffer, broken into small fragments, and left in buffer at room temperature until use (<1 hr). $[Ca^{2+}]_i$ measurements were made on single cells by standard epifluorescence microscopy, using a Photon Technology International Deltascan system. With emission recorded at 510 nm, excitation was chopped between 340 nm and 380 nm to provide a ratio every 1 sec. Cells were optically isolated by manual *x/y* shutters fitted to the photomultiplier housing. On each day, background fluorescence was determined on 10 individual cells and averaged (coefficient of variation, <5%) for automatic subtraction. Cells were superfused at 1 ml/min with Krebs/HEPES buffer at 37°. Agonists were added directly to the cells at the required concentration via a gravity-driven multiline perfusion pipette lying approximately 10 diameters from the cell. No calibration of the fluorescence ratio was performed.

Measurement of Ins(1,4,5)P_3 mass. This was done by a previously described (21) modification of a radioreceptor method characterized for stereospecificity and positional specificity (23). Briefly, confluent cultures of cells in 24-well multidishes were washed and preincubated in Krebs/HEPES buffer at 37° for 30 min before use. Buffer was aspirated from wells individually, and 100 μl of buffer with agonist were added immediately. In studies in which agonist stimulations were performed in low-Ca^{2+} buffer, standard buffer was removed and the cells were washed once with 1 ml of the low-Ca^{2+} buffer before agonist challenge. The Ca^{2+} concentration of this buffer was set to 50–100 nM by the addition of EGTA and was determined using fura-2 (22). In experiments examining potential heterologous interactions, after the initial exposure of cells the buffer, with or without agonist, was removed and replaced immediately with 100 μl of buffer alone or buffer containing the required agonist or combination of agonists. When muscarinic receptor activation was inhibited with atropine, 100 μl of buffer containing the antagonist were added to the stimulating buffer at the stated time. After the required length of exposure, the 200 μl of buffer were then removed and 100 μl of buffer containing the required reagents or combination of reagents were added immediately. In all instances appropriate manipulations were made to negate any potential nonspecific effects. Reactions were stopped with 100 μl of ice-cold 1 M TCA. For time 0 values, TCA was added before agonist addition. Extraction and assay of Ins(1,4,5)P_3 were exactly as described previously (21).

Sources of reagents. Reagents of analytical grade and highly purified Elgastat water were used throughout. Minimum essential medium, penicillin/streptomycin, amphotericin-8, glutamine, newborn calf serum, and tissue culture flasks were from GIBCO. Multidishes (eight and 24 well) were from Nunc. Fura-2/AM, fura-2 free acid, ionomycin free acid, and 4-Br-A23187 were from Calbiochem. HEPES, atropine, carbachol, EGTA, EDTA, and bradykinin were from Sigma. TCA and all other reagents were from Fisons except as previously reported (21).

Statistical analysis and data presentation. Data are generally expressed as mean ± standard error of n determinations. Comparison of these data was by unpaired Student's t test, with significance accepted at $p < 0.05$. All data in the text are presented in this format. Where multiple comparisons were required, analysis was performed by one-way analysis of variance and, for $p < 0.05$, the significance of difference between individual group means was determined with Duncan's multiple-range test at $p < 0.05$ and $p < 0.01$. These data are presented in Table 1 and Figs. 6, 7, and 9 as means ± standard deviations.

Results

Carbachol- and Bradykinin-Mediated Elevation of $[Ca^{2+}]_i$ in Single Cells

Of 20 single cells tested, 18 responded to 1 μM bradykinin, producing a peak but little or no evidence of a sustained phase of elevated $[Ca^{2+}]_i$. The mean change in 340/380 ratio was 1.82 ± 0.31, from a basal level of 0.99 ± 0.07. After removal of bradykinin, all cells responded to 1 mM carbachol with both a peak and a sustained phase of elevated $[Ca^{2+}]_i$. At the peak, the mean ratio change was 5.18 ± 1.42. Peak responses to bradykinin were far more heterogeneous (coefficient of variation of ratio, 75%) than were those to carbachol (coefficient of variation, 27%). The change in 340/380 ratio in response to bradykinin varied from 0 to 100% of the carbachol peak response, with a median value of 36%.

Studies in SH-SY5Y Cells Used Directly upon Attainment of Confluence

Carbachol- and bradykinin-induced elevations of $[Ca^{2+}]_i$ and $Ins(1,4,5)P_3$ levels in populations of adherent SH-SY5Y cells. Carbachol produced biphasic elevations of both $[Ca^{2+}]_i$ and $Ins(1,4,5)P_3$, consisting of rapid (approximately 10-sec) peaks followed by lower but sustained phases. Bradykinin, however, produced rapid peaks of $[Ca^{2+}]_i$ and $Ins(1,4,5)P_3$ but little evidence of sustained phases, at least at the same proportion of the peak as that produced by carbachol (see Figs. 1 and 5).

Interaction of carbachol and bradykinin in the regulation of $[Ca^{2+}]_i$. In the presence of extracellular Ca^{2+}, 1 μM bradykinin followed 130 sec later by 1 mM carbachol did not result in any detectable reduction in the peak or sustained $[Ca^{2+}]_i$ responses to carbachol, compared with the addition of carbachol alone (Fig. 1). We showed previously that these agonist concentrations produce maximal elevations of $[Ca^{2+}]_i$ (21). After calibration, the bradykinin response was 28 ± 7% (n = 8) of the subsequent carbachol response (changes of 60 ± 12 nM, n = 8, and 341 ± 104 nM, n = 8, respectively). In the absence of bradykinin, carbachol produced a change in $[Ca^{2+}]_i$ of 410 ± 107 nM (n = 6). Under nominally Ca^{2+}-free conditions there was not a significant reduction in the original bradykinin response. However, the peak $[Ca^{2+}]_i$ response to subsequent carbachol addition was markedly attenuated (Fig. 2). Under these conditions the bradykinin response was 120 ± 17% (54 ± 11 nM, n = 3) of the subsequent carbachol response (45 ± 4 nM, n = 3), whereas this carbachol response was only 20% of that seen under nominally Ca^{2+}-free conditions with no prior bradykinin challenge (Fig. 2). Readdition of Ca^{2+} (2 mM) to cells challenged in this way resulted in a rapid and sustainded elevation of $[Ca^{2+}]_i$ (120 ± 15 nM, n = 3) (Fig. 2). Addition of bradykinin at 130 sec after carbachol challenge, in the presence (Fig. 1) or absence (data not shown) of external Ca^{2+}, did not evoke any $[Ca^{2+}]_i$ response. Reductions in the concentration of carbachol to 1 μM and 0.1 μM, when used as the initial agonist in the presence of external Ca^{2+}, resulted in a reappearance and progressive increase of the bradykinin response to 50 ± 8 nM (n = 3) and 105 ± 9 nM (n = 3), respectively (Fig. 3). In the presence of external Ca^{2+}, addition of 10 μM atropine at 130 sec after carbachol challenge returned $[Ca^{2+}]_i$ to prestimulation levels and allowed a full response to 1 μM bradykinin (136 ± 37 nM, n = 5) at 130 sec after atropine addition (Fig. 4a). However, under nominally Ca^{2+}-free conditions, the addition of atropine after carbachol did not restore the $[Ca^{2+}]_i$ response to bradykinin (14 ± 3 nM, n = 3) (Fig. 4b) unless Ca^{2+} (2 mM) was added back after atropine (Fig. 4c). This Ca^{2+} addition produced an immediate elevation of $[Ca^{2+}]_i$ (39 ± 7 nM, n = 3) and the bradykinin response was re-established (170 ± 75 nM, n = 3) (Fig. 4c).

Interaction of carbachol and bradykinin in the regulation of $Ins(1,4,5)P_3$ levels. Addition of 10 μM bradykinin and its continued presence did not affect the subsequent $Ins(1,4,5)P_3$ response to 1 mM carbachol added 130 sec later (Fig. 5). However, addition of 1 mM carbachol and its continued presence abolished the subsequent response to 10 μM bradykinin (Fig. 5). We have previously demonstrated that these agonist concentrations produce maximal elevations of $Ins(1,4,5)P_3$ at 10 sec (21).

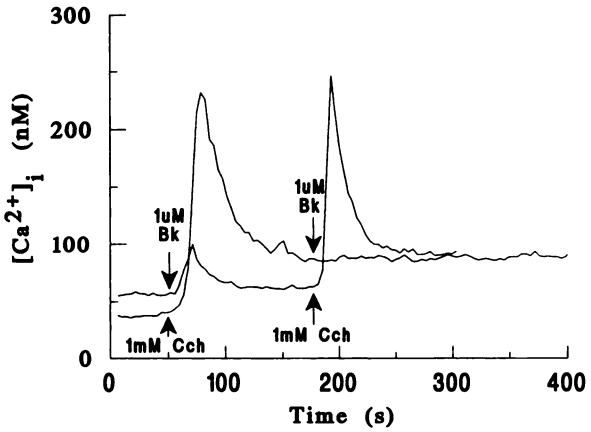


Fig. 1. Traces demonstrating that, in SH-SY5Y cells, stimulation with and the continued presence of a maximal concentration of carbachol (*Cch*) resulted in abolition of the $[Ca^{2+}]_i$ response to the subsequent addition of a maximal concentration of bradykinin (*Bk*). Bradykinin was, however, unable to influence the $[Ca^{2+}]_i$ response to carbachol. Traces are representative of a total of at least eight.

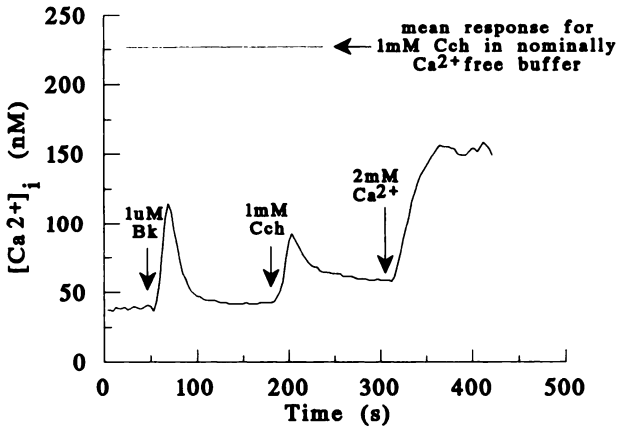


Fig. 2. Trace demonstrating that, in SH-SY5Y cells, when the buffer was nominally Ca^{2+} free (~2 μM Ca^{2+}), stimulation with and the continued presence of a maximal concentration of bradykinin (*Bk*) was able to markedly attenuate the subsequent response to a maximal concentration of carbachol (*Cch*). The trace is representative of at least three.

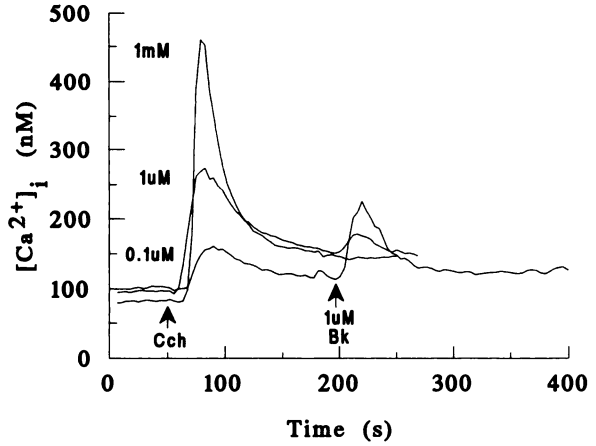


Fig. 3. Traces demonstrating the dose-dependent reduction of the subsequent $[Ca^{2+}]_i$ response to a maximal concentration of bradykinin (*Bk*) after and in the continued presence of a range of carbachol (*Cch*) concentrations in SH-SY5Y cells. Traces are representative of at least three.

Studies in SH-SY5Y$_{2wc}$ Cells, in which Peak Ins(1,4,5)P$_3$ and $[Ca^{2+}]_i$ Responses to Carbachol and Bradykinin Were Approximately Equivalent

Characterization of carbachol-and bradykinin-mediated Ins(1,4,5)P$_3$ and $[Ca^{2+}]_i$ elevations in SH-SY5Y$_{2wc}$ cells. In the experiments described above, both the Ins(1,4,5)P$_3$ and $[Ca^{2+}]_i$ responses to bradykinin were considerably smaller than those to carbachol. There were, therefore, concerns that the inability of bradykinin to influence carbachol responses and, indeed, the apparent full heterologous desensitization by carbachol of Ins(1,4,5)P$_3$ or $[Ca^{2+}]_i$ responses to bradykinin were related to the relatively small bradykinin responses. Fortunately, ongoing work within this laboratory has established that the maintenance of these cells at confluence for 2 weeks, with refeeding every second day (yielding SH-SY5Y$_{2wc}$ cells), substantially increases peak Ins(1,4,5)P$_3$ and $[Ca^{2+}]_i$ responses to bradykinin and brings forward the peak Ins(1,4,5)P$_3$ response to 5 sec, by an as yet undefined mechanism (24). In these cells, peak elevations of Ins(1,4,5)P$_3$ and $[Ca^{2+}]_i$ in response to bradykinin were similar to those of carbachol (Figs. 6 and 7; Table 1). Sustained phases of both Ins(1,4,5)P$_3$ and $[Ca^{2+}]_i$ elevation were again present in response to carbachol (Fig. 7; Table 1). There was little or no evidence for a sustained Ins(1,4,5)P$_3$ elevation in response to bradykinin (Fig. 6), although there was some evidence of a slight sustained elevation of $[Ca^{2+}]_i$ (Table 1). After 5-min exposure of SH-SY5Y$_{2wc}$ cells to 10 μM bradykinin, buffer removal and replacement with fresh buffer containing the equivalent concentration of bradykinin did not result in an elevation of Ins(1,4,5)P$_3$ (data not shown). In addition, after exposure of these cells to bradykinin, removal of the stimulating buffer and its addition to previously unstimulated cells resulted in a rapid elevation of Ins(1,4,5)P$_3$ (data not shown). Thus, the desensitization of the bradykinin response was not due to metabolism of the agonist.

Interaction of carbachol and bradykinin in the regulation of $[Ca^{2+}]_i$ and Ins(1,4,5)P$_3$ levels in SH-SY5Y$_{2wc}$ cells. Under conditions of normal $[Ca^{2+}]_e$, the presence of bradykinin did not significantly affect the Ins(1,4,5)P$_3$ (Fig. 6) or $[Ca^{2+}]_i$ (Table 1) responses to carbachol. Despite the

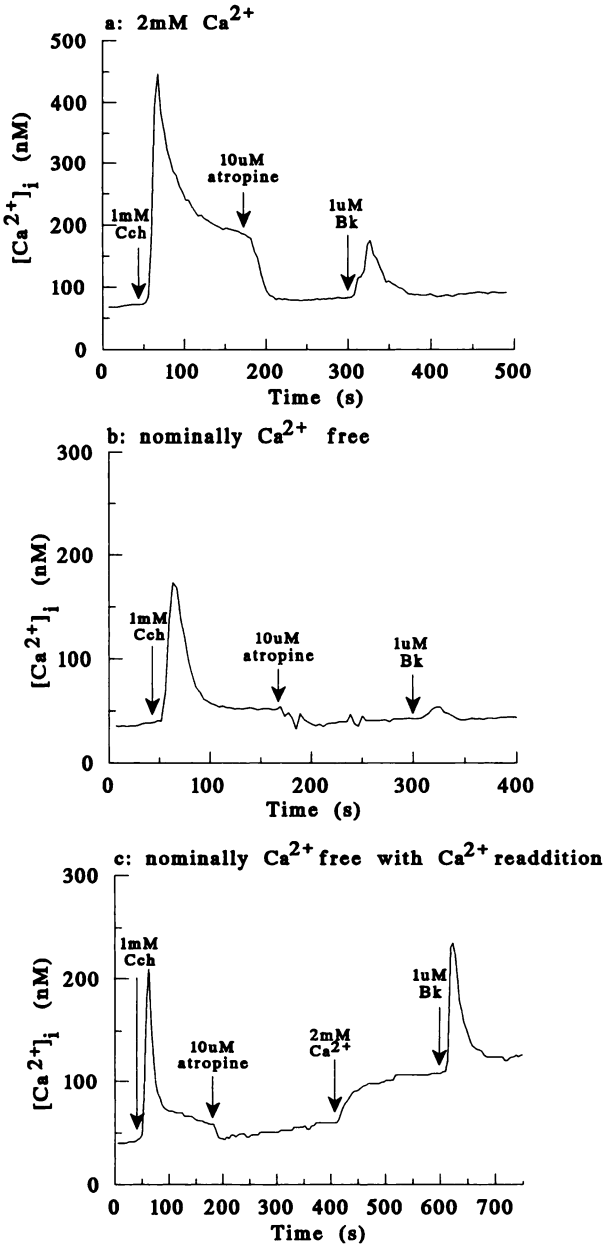


Fig. 4. Traces (representative of at least three) demonstrating that in SH-SY5Y cells the $[Ca^{2+}]_i$ response to a maximal concentration of bradykinin (*Bk*) was restored by the addition of atropine to antagonize the effect of a maximal concentration of carbachol (*Cch*) used as the initial stimulus (a), atropine was not able to restore the bradykinin response using the same protocol as above but under nominally Ca^{2+}-free conditions (b), and the readdition of Ca^{2+} to nominally Ca^{2+}-free buffer after carbachol and atropine was able to restore the bradykinin response (c).

similar magnitudes of peak $[Ca^{2+}]_i$ responses for the two agonists and a slightly longer period between agonist additions, $[Ca^{2+}]_i$ responses to bradykinin were still entirely absent after and in the continued presence of carbachol in SH-SY5Y$_{2wc}$ cells (Table 1). Under these conditions bradykinin was, however, able to cause a small but significant elevation of Ins(1,4,5)P$_3$ that was significantly reduced, compared with the response in the absence of carbachol (Fig. 7i). The time course of this response was unaffected (data not shown). As in normal SH-SY5Y cells, the addition of atropine after carbachol provided a marked restoration of the $[Ca^{2+}]_i$

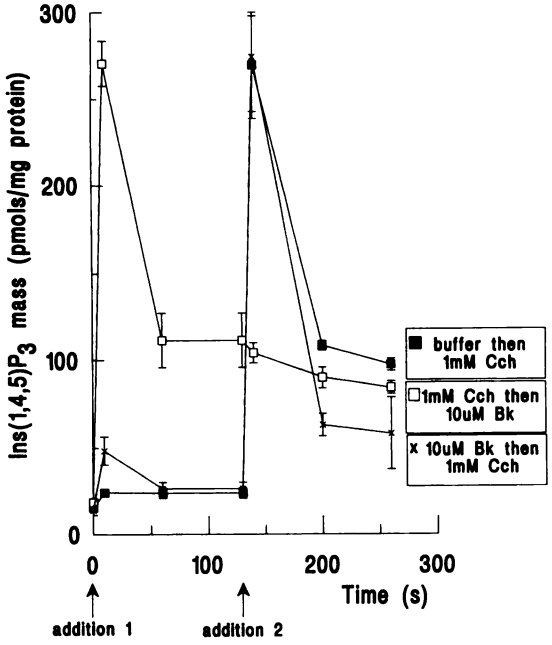


Fig. 5. Agonist-mediated changes in Ins(1,4,5)P_3 mass in SH-SY5Y cells, demonstrating that stimulation with and the continued presence of a maximal concentration of carbachol (*Cch*) resulted in abolition of the Ins(1,4,5)P_3 response to the subsequent addition of a maximal concentration of bradykinin (*Bk*). Bradykinin was, however, not able to influence the Ins(1,4,5)P_3 response to carbachol. Data are mean ± standard error (three experiments).

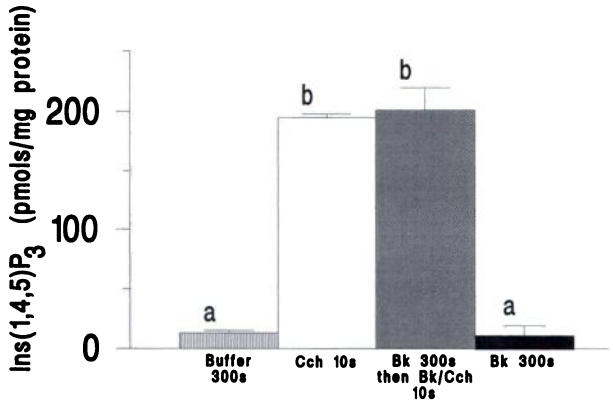
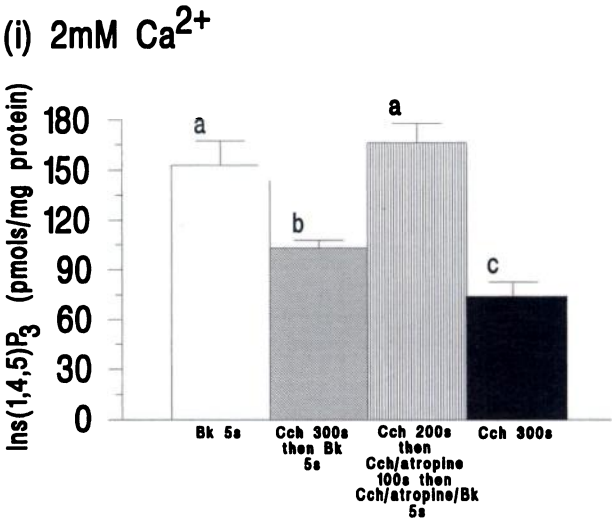


Fig. 6. Agonist-induced changes in Ins(1,4,5)P_3 mass in SH-SY5Y_{2wc} cells, demonstrating that stimulation with and the continued presence of a maximal concentration of bradykinin (*Bk*) (10 μM) did not result in a sustained phase of Ins(1,4,5)P_3 generation and did not influence the subsequent response to a maximal concentration of carbachol (*Cch*) (1 mM). ▥, Buffer alone at 300 sec; □, carbachol response at 10 sec; ■, bradykinin response at 300 sec; ■, peak carbachol response at 10 sec in the presence of bradykinin, which was present for 300 sec before carbachol addition. All data are mean ± standard deviation (three experiments). For *a* versus *b*, $p < 0.01$.

response to bradykinin when tested 100 sec later (change of 165 ± 21 nM, $n = 3$, compared with 264 ± 24 nM, $n = 3$, in the absence of carbachol) (Fig. 8). However, atropine did not restore the bradykinin response in the absence of extracellular Ca^{2+} [changes of 15 ± 3 nM, $n = 3$, in nominally Ca^{2+}-free buffer (data not shown) and 8 ± 1 nM in EGTA-containing buffer (Fig. 8)]. This addition of atropine also fully restored the Ins(1,4,5)P_3 response to bradykinin (Fig. 7i). However, in



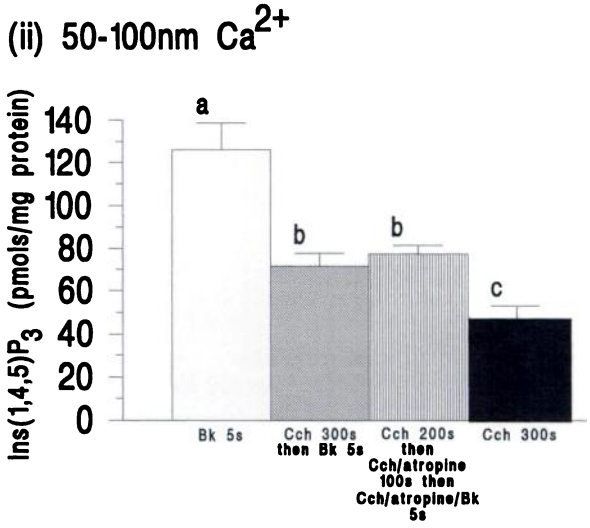


Fig. 7. Agonist-induced changes in Ins(1,4,5)P_3 mass in SH-SY5Y_{2wc} cells. i, In the presence of extracellular Ca^{2+} the addition of atropine after a maximal concentration of carbachol (*Cch*) was able to fully restore the peak response to a maximal concentration of bradykinin (*Bk*). All data are mean ± standard deviation (three experiments). For *a* versus *b* and *c*, $p < 0.01$; for *b* versus *c*, $p < 0.05$. The basal level was 20.9 ± 4.3 pmol/mg of protein. ii, Under conditions where the $[Ca^{2+}]_e$ was buffered to 50–100 nM with EGTA, the addition of atropine after a maximal concentration of carbachol was not able to restore the peak response to a maximal concentration of bradykinin. All data are mean ± standard deviation (six experiments). For *a* versus *b* and *c* and *b* versus *c*, $p < 0.01$. The basal level was 14.3 ± 2.3 pmol/mg of protein. □, Bradykinin peak response at 5 sec; ■, sustained carbachol response at 300 sec; ▧, bradykinin peak response at 5 sec in the presence of carbachol, which was present for 300 sec before bradykinin addition; ▥, bradykinin peak response at 5 sec in the presence of carbachol, which was present for 300 sec before bradykinin addition, and with atropine added 100 sec before bradykinin. Bradykinin was used at 10 μM, carbachol at 1 mM, and atropine at 10 μM.

the absence of extracellular Ca^{2+}, bradykinin addition after carbachol caused a small but significant increase in Ins(1,4,5)P_3 levels, which was unchanged by the addition of atropine and which was significantly reduced, compared with the response to bradykinin alone (Fig. 7ii).

TABLE 1

Interaction of carbachol and bradykinin in the stimulation of $[Ca^{2+}]_i$ elevation in SH-SY5Y$_{2wc}$ cells

$[Ca^{2+}]_i$ was monitored in fura-2-loaded populations of adherent SH-SY5Y$_{2wc}$ cells as described in Experimental Procedures. Basal and plateau values were determined from the average for the preceding 30–50 sec. Data are means ± standard deviations (seven or eight experiments).

$[Ca^{2+}]_i$				
			In the presence of 1 μM bradykinin	
Basal	1 μM Bradykinin peak	1 μM Bradykinin plateau (300 sec)	1 mM Carbachol peak	1 mM Carbachol plateau (300 sec)
		nM		
50 ± 5	424 ± 175	88 ± 14	422 ± 76	116 ± 12
$[Ca^{2+}]_i$				
			In the presence of 1 mM carbachol	
Basal	1 mM Carbachol peak	1 mM Carbachol plateau (300 sec)	1 μM Bradykinin peak	1 μM Bradykinin plateau (300 sec)
		nM		
48 ± 11	573 ± 179	129 ± 33	0	112 ± 8

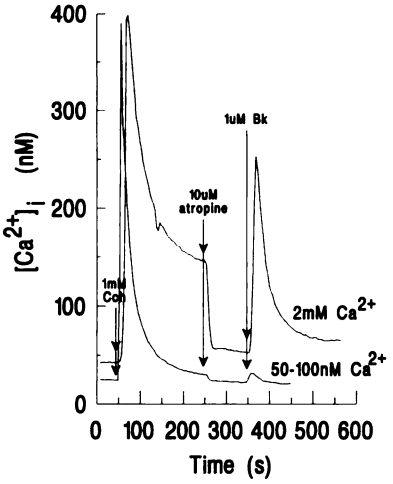


Fig. 8. Traces demonstrating that, in SH-SY5Y$_{2wc}$ cells, the addition of atropine after a maximal concentration of carbachol (*Cch*) restored the $[Ca^{2+}]_i$ elevation produced by a maximal concentration of bradykinin (*Bk*) only in the presence of extracellular Ca^{2+}. Traces are representative of at least three.

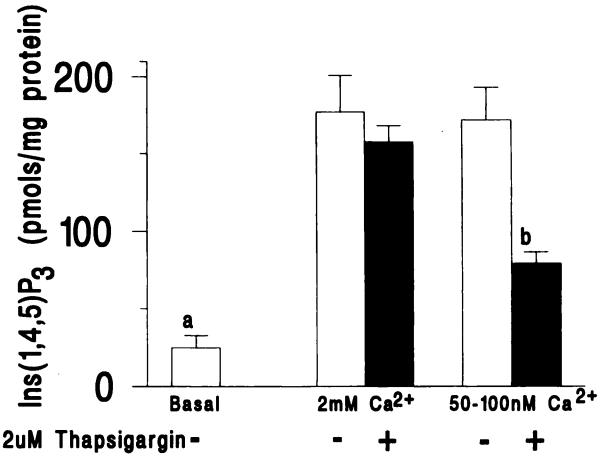


Fig. 9. Peak agonist-induced changes in Ins(1,4,5)P_3 mass in SH-SY5Y$_{2wc}$ cells at 5 sec after bradykinin addition, demonstrating that, under conditions where $[Ca^{2+}]_i$ is effectively clamped at basal levels (5-min pretreatment with thapsigargin followed by stimulation at 50–100 nM $[Ca^{2+}]_e$), the Ins(1,4,5)P_3 response to a maximal concentration of bradykinin is markedly attenuated. All data are mean ± standard deviation (four experiments). For *a* versus *b*, $p < 0.05$; for *a* and *b* versus all other groups, $p < 0.01$.

Evidence that inhibition of $[Ca^{2+}]_i$ elevation in response to bradykinin also attenuates the Ins(1,4,5)P_3 response in SH-SY5Y$_{2wc}$ cells

When SH-SY5Y$_{2wc}$ cells were treated with 2 μM thapsigargin, to deplete the intracellular Ins(1,4,5)P_3-sensitive Ca^{2+} pool, and were challenged with bradykinin at 50–100 nM $[Ca^{2+}]_e$, there was no elevation of $[Ca^{2+}]_i$ (data not shown). In addition, there was a significant attenuation of the Ins(1,4,5)P_3 response, compared with cells challenged under conditions in which $[Ca^{2+}]_i$ either was already elevated (thapsigargin at 2 mM $[Ca^{2+}]_e$) or was elevated in response to bradykinin (2 mM or 50–100 nM $[Ca^{2+}]_e$ but no thapsigargin) (Fig. 9).

Discussion

The current study demonstrates that SH-SY5Y human neuroblastoma cells coexpress muscarinic and bradykinin receptors linked to PIC activation, and it reveals a rapid heterologous desensitization of bradykinin-mediated $[Ca^{2+}]_i$ and Ins(1,4,5)P_3 responses in the face of persistent muscarinic receptor activation. This allowed analysis of the mechanisms of these interactions and provided additional evidence for the complexity of regulation of PI signaling.

In agreement with previous studies from this laboratory (4, 21, 25), the transient nature of the rapid maximal $[Ca^{2+}]_i$ and Ins(1,4,5)P_3 elevations in these cells in response to either carbachol or bradykinin suggests homologous desensitization at both of these levels of receptor signaling. A lower but sustained elevation of $[Ca^{2+}]_i$ and Ins(1,4,5)P_3 levels in response to carbachol indicated a partial desensitization of muscarinic receptors, whereas their rapid return to basal levels suggested that desensitization of bradykinin receptors was complete or nearly complete. We have argued that the rapid reductions in Ins(1,4,5)P_3 levels reflect reduced PIC activity through receptor desensitization, possibly as a consequence of phosphorylation by receptor-specific kinases (5, 8).

In addition to these homologous desensitizations, the full inhibition of the $[Ca^{2+}]_i$ response to bradykinin produced by

a maximal concentration of carbachol in both SH-SY5Y cells and SH-SY5Y_{2wc} cells [which displayed comparable $[Ca^{2+}]_i$ and Ins(1,4,5)P_3 peak responses to both agonists] (24) suggested that heterologous desensitization is also a feature of this limb of the signal transduction pathway. Such cross-desensitization emphasized that receptors for both agonists are coexpressed on individual cells. The inability of bradykinin to provoke a $[Ca^{2+}]_i$ elevation was dependent upon continued muscarinic receptor activation, because the addition of atropine before bradykinin restored the response in SH-SY5Y and SH-SY5Y_{2wc} cells. Such a phenomenon has been reported previously for the inhibition by carbachol of $[Ca^{2+}]_i$ responses to histamine in 1321 astrocytoma cells (11), to cholecystokinin octapeptide in isolated gastric glands (26), and to substance P in parotid acinar cells (13).

The reappearance and progressive increase of the $[Ca^{2+}]_i$ response to bradykinin after reductions in the concentration of the initial carbachol challenge in SH-SY5Y cells suggested a concentration-dependent inhibition or depletion of some common factor within the PI signaling pathway. Furthermore, the inability of atropine to restore the bradykinin response in SH-SY5Y and SH-SY5Y_{2wc} cells at reduced $[Ca^{2+}]_e$ suggested that depletion of the Ins(1,4,5)P_3-sensitive Ca^{2+} pool is responsible for the heterologous desensitization. The abolition by carbachol of bradykinin-mediated $[Ca^{2+}]_i$ elevation is likely to result, therefore, from the sustained generation of Ins(1,4,5)P_3, maintaining the Ca^{2+} pool in a depleted state and thereby preventing subsequent release by bradykinin. Antagonism of muscarinic receptor activation by atropine would permit Ca^{2+} store refilling, but only if extracellular Ca^{2+} is present. This store refilling must be rapid, given both the rapid return of $[Ca^{2+}]_i$ to basal levels with atropine, indicating no prolonged capacitative Ca^{2+} entry (2), and the appearance of almost full bradykinin-induced $[Ca^{2+}]_i$ responses in SH-SY5Y and SH-SY5Y_{2wc} cells within 2 min of atropine addition. Depletion of the Ins(1,4,5)P_3-sensitive Ca^{2+} pool has also been reported as the mechanism for carbachol inhibition of responses to endothelin-1, bradykinin, and ATP in single LAN-1 human neuroblastoma cells (14). The current data also indicate that Ca^{2+} released from intracellular stores is not resequestered for subsequent release, at least at low $[Ca^{2+}]_e$, and that carbachol and bradykinin share a common Ins(1,4,5)P_3-sensitive intracellular Ca^{2+} pool. The fact that the pool is shared by the two agonists is supported by the lack of additivity of the $[Ca^{2+}]_i$ elevations in either SH-SY5Y or SH-SY5Y_{2wc} cells (data not shown) and the ability of bradykinin to markedly attenuate the subsequent $[Ca^{2+}]_i$ response to carbachol in the absence of extracellular Ca^{2+} (shown in SH-SY5Y cells), when Ca^{2+} store refilling could not occur.

The ability of carbachol to desensitize the $[Ca^{2+}]_i$ response to bradykinin is dependent upon persistent muscarinic receptor activation. Thus, despite the rapid, partial, homologous desensitization of the responses to carbachol, this level of muscarinic receptor activation in response to a maximal concentration of carbachol is able to abolish the $[Ca^{2+}]_i$ response to bradykinin. In contrast to that of the muscarinic receptor, the extent of bradykinin receptor desensitization appears to be complete or nearly complete. This may, therefore, account for the inability of bradykinin to influence the $[Ca^{2+}]_i$ response to carbachol, because the Ca^{2+} stores are able to refill immediately. A lack of sustained receptor activation has been previously suggested to account for the inability of PIC-linked agonists to mediate heterologous desensitization of $[Ca^{2+}]_i$ responses (13, 14).

Heterologous desensitization of PIC-linked responses among a variety of agonists has been demonstrated in isolated cells (12, 13), tissues (26), and established cell lines (9–11, 15–17). Most of these studies have, however, examined desensitization only at the level of the $[Ca^{2+}]_i$ response, and several have implied that Ca^{2+} pool depletion is fully or partially responsible for the desensitization (9–13, 15). Although desensitization by neurotensin of a bradykinin-mediated [^{3}H]inositol trisphosphate response has been reported (17), our knowledge of heterologous desensitization at stages before Ca^{2+} signaling, particularly at the level of the rapid transient changes in Ins(1,4,5)P_3, is very limited. In the present study, the inability of bradykinin to increase the level of Ins(1,4,5)P_3 in SH-SY5Y cells in the presence of carbachol clearly suggests desensitization at this level. Given that agonists are able to elevate $[Ca^{2+}]_i$ more potently than Ins(1,4,5)P_3 levels (21, 27) and that, as a consequence, Ca^{2+} mobilization may occur without a measurable increase in Ins(1,4,5)P_3 levels, these data do not necessarily indicate a full inhibition of PIC activity. In SH-SY5Y_{2wc} cells heterologous desensitization is indeed incomplete, as demonstrated by the substantially reduced, but still apparent, Ins(1,4,5)P_3 response to bradykinin in the presence of carbachol. These data also support, therefore, the full depletion of the Ins(1,4,5)P_3-sensitive Ca^{2+} pool and emphasize that examination of Ca^{2+} signaling will not enable identification of heterologous desensitization events at stages before Ca^{2+} mobilization. Such desensitization may have consequences for diacylglycerol formation and therefore protein kinase C activation (1).

Resensitization of the Ins(1,4,5)P_3 response to bradykinin after atropine addition to carbachol-challenged SH-SY5Y_{2wc} cells was critically dependent upon extracellular Ca^{2+}. Thus, in the absence of a $[Ca^{2+}]_i$ elevation in response to bradykinin the Ins(1,4,5)$_3$ response was markedly attenuated. These data are consistent with both the reported Ca^{2+} sensitivity of some isoforms of PIC (28) and the enhancement of agonist-mediated Ins(1,4,5)P_3 formation with increasing $[Ca^{2+}]$, over a physiologically relevant range, in permeabilized SH-SY5Y cells (6, 29). In addition, in SH-SY5Y cells we recently demonstrated a feed-forward activation of PIC by Ca^{2+} released from Ins(1,4,5)P_3-sensitive stores in response to carbachol (6). Using an identical protocol of depleting nonmitochondrial intracellular Ca^{2+} stores with thapsigargin and stimulating the cells at low (50–100 nM) $[Ca^{2+}]_e$, the current study demonstrated that, in the absence of a $[Ca^{2+}]_i$ elevation, the Ins(1,4,5)P_3 response to bradykinin was also markedly attenuated. These data suggest, therefore, that Ca^{2+} released from internal stores would normally activate PIC in a feed-forward manner. Because the Ca^{2+} stores are depleted in the presence of carbachol in the present study (see above) and subsequent addition of bradykinin does not, therefore, provoke an elevation of $[Ca^{2+}]_i$, this feed-forward Ca^{2+} activation of PIC does not occur, which may account for the undetectable (SH-SY5Y) or reduced (SH-SY5Y_{2wc}) Ins(1,4,5)P_3 generation. Although the data are consistent with the Ca^{2+} dependence of the restoration of this response lying at this level, it is also possible that this or additional Ca^{2+} dependence could reside at other levels. This could, for example,

take the form of the need for activation of a Ca^{2+}-dependent enzyme involved in the resynthesis of PIC substrates or in receptor dephosphorylation and therefore resensitization. The time course of any of these processes must, however, be similar to that of Ca^{2+} pool refilling.

In conclusion, the current study demonstrates that after and in the continued presence of carbachol there was a heterologous desensitization of both $Ins(1,4,5)P_3$ and $[Ca^{2+}]_i$ responses to bradykinin in the human neuroblastoma SH-SY5Y cell line. The depletion of intracellular $Ins(1,4,5)P_3$-sensitive Ca^{2+} stores is the most likely cause of the heterologous desensitization of the $[Ca^{2+}]_i$ response to bradykinin. Indeed, this may also account for the desensitization of the $Ins(1,4,5)P_3$ response via lack of a Ca^{2+} feed-forward activation of PIC. Bradykinin was unable to influence the responses to carbachol, probably due to a rapid, complete or nearly complete, homologous desensitization. These studies again highlight the fact that PIC signaling displays rapid regulatory features that differ among individual receptors. Thus, whereas bradykinin (this study) and substance P receptors (3, 13) demonstrate almost complete homologous desensitization at the level of both $[Ca^{2+}]_i$ elevation and PIC activation, muscarinic m3 (6) and bombesin (3) receptors are only partially regulated in this way. The present studies reveal that these aspects of homologous desensitization are also crucial for the ability of PIC-linked receptors to mediate heterologous regulation of PI and Ca^{2+} signaling.

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