$[Ca^{2+}]_i$ Imaging in PC12 Cells: Multiple Response Patterns to Receptor Activation Reveal New Aspects of Transmembrane Signaling

Fabio Grohovaz,* Daniele Zacchetti,‡ Emilio Clementi,‡ Paola Lorenzon, Jacopo Meldolesi,*‡ and Guido Fumagalli

Department of Pharmacology, Consiglio Nazionale delle Ricerche Center of Cytopharmacology, *Bruno Ceccarelli Center of Peripheral Neuropathies, and ‡S. Raffaele Institute, University of Milano, Milano, Italy

Abstract. Fura-2 imaging microscopy was used to study $[Ca^{2+}]_i$ in nerve growth factor–differentiated PC12 cells exposed to agonists (bradykinin, carbamylcholine, and ATP) binding to receptors coupled to polyphosphoinositide hydrolysis. With all the treatments employed, the response to an individual agonist was often incomplete, i.e., composed of either release from intracellular stores or influx only. In individual cells the responses were closely similar when only one and the same agonist was employed, and markedly heterogeneous, with considerable variation of the release/influx ratio, when different agonists were delivered in sequence. In a recently isolated PC12 cell clone, heterogeneity of the receptor-induced $[Ca^{2+}]_i$ responses was markedly lower than in the overall population, although the release/influx ratio was still variable. We conclude that the large response heterogeneity observed in the overall PC12 cell population is due (*a*) to the coexistence of multiple clones; and (*b*) to the variable activation of intracellular transduction mechanisms.

PC12 is a neurosecretory cell line very frequently employed as a nerve cell model since its establishment by Greene and Tischler (1976). The cells of this line, particularly after long-term treatment with nerve growth factor (NGF),[1] express a variety of properties shared by neurons, including the overall phenotype (Greene and Tischler, 1976, 1982), specific voltage-gated ion channels (e.g., the N-type Ca^{2+} channel; Plummer et al., 1989), and the sensitivity to the presynaptic stimulatory toxin, α-latrotoxin (Rosenthal and Meldolesi, 1989). During the last several years PC12 cells have been intensely investigated also for changes of the cytosolic free Ca^{2+} concentration, $[Ca^{2+}]_i$, an effect induced by numerous neurotransmitters and various other treatments, such as plasma membrane depolarization (Di Virgilio et al., 1987; Pozzan et al., 1986; Fasolato et al., 1988 and 1990; Inoue and Kenimer, 1988). Up to now, however, these studies have been conducted primarily on populations of detached cells, first loaded with a specific dye (initially quin-2, Tsien et al., 1982; more recently fura-2, Grynkiewicz et al., 1985) and then analyzed while suspended in the cuvette of a conventional fluorimeter. Clearly, the results obtained by this approach represent averages of the $[Ca^{2+}]_i$ changes occurring in the analyzed cell population, and provide no information attributable to either individual cells or cellular regions. Fura-2 studies at the single cell level have been already carried out, by applying computerized imaging ratio microscopy at two excitation wavelengths to a number of preparations, including primary cultures of neurons from both the central and peripheral nervous systems (see references within Connor et al., 1988; Tank et al., 1988; Lipscombe et al., 1988; Hockberger et al., 1989; Mayer and Miller, 1990), as well as chromaffin cells (O'Sullivan et al., 1989). However, a nerve cell line had never been investigated in detail by this technique. The present results demonstrate that, when PC12 cells are exposed to specific agonists binding to receptors coupled to polyphosphoinositide (PPI) hydrolysis, their $[Ca^{2+}]_i$ responses can be highly heterogeneous, not only in size but also in their sustaining mechanisms, i.e., intracellular release and influx across the plasma membrane. Such heterogeneity, which appears to be primarily due to the coexistence of multiple cell clones in the investigated PC12 cell population, was instrumental to the identification of specific response patterns which enabled us to reconsider various problems of receptor signaling and $[Ca^{2+}]_i$ response control.

1. *Abbreviations used in this paper*: BK, bradykinin; CCh, carbamylcholine; NGF, nerve growth factor; PPI, polyphosphoinositide.

Materials and Methods

Cell Culture

PC12 cells (Greene and Tischler, 1976) were cultured at 37°C in the RPMI medium containing 2 mM glutamine, 10% horse serum, and 5% FCS (biochemicals from Gibco Laboratories, Grand Island, NY), under a humidified atmosphere with 5% CO_2. They were plated weekly 1:3 in 10-cm Petri dishes. For experimental use, cells were cultured on 22-mm glass coverslips coated with polyornithine (Sigma Chemical Co., St. Louis, MO) which were glued to the bottom of perforated 35-mm Petri dishes. To obtain synchronization and neuron-like differentiation, one day after the final plating the cells were first serum-deprived for 24 h and then treated with 50 ng/ml mouse 2.5S NGF for one day in the same serum-free medium (Rudkin et

al., 1989), after which culture was continued for at least one week in complete RPMI medium supplemented with NGF.

PC12 Subcloning

Since initial attempts with limiting dilution and coculturing with irradiated macrophages failed to produce persistent clones, PC12 cells were transfected with the pMV7 vector containing the neo gene (generous gift of Dr. H. Bourne, University of California, San Francisco, CA) essentially as previously described by Schweitzer and Kelly (1985). 3×10^6 cells were plated on 10-cm polyornithine-coated plastic tissue culture dishes. One day later, the plasmid DNA was added to the HeBS rinsed cells (20 μg/3–10^6 cells) in the form of a calcium phosphate precipitate. After 20 min incubation at room temperature, cells were supplemented with DME containing 5% FCS, incubated at 37°C for 6 h, osmotically shocked with 25% glycerol in DME for 1 min, and then rapidly washed. The entire population of cells was grown in standard culture medium for 72 h before adding 0.8 mg/ml of G418 (Geneticin; Gibco Laboratories) to select for stable transfectants. 60 individual colonies were isolated by this procedure, and one (#15) was used for the present studies.

Fura-2 Loading

Cells were incubated for 30–45 min at 37°C with 0.5–4 μM fura-2 pentaacetoxymethylester (Calbiochem Co., San Diego, CA) in RPMI medium containing 5% FCS, and then washed and further incubated for 15–30 min in the same medium to allow deesterification of the dye. Cells were then rinsed in Krebs-Ringer solution buffered with Hepes (150 mM NaCl, 5 mM KCl, 1 mM $MgSO_4$, 2 mM $CaCl_2$, 10 mM glucose, 10 mM Hepes/NaOH, pH 7.4) and transferred to the heated stage of the microscope, where temperature was maintained at 36°C throughout the experiment. Incubations were carried out in 2 ml of either the same medium, which was then additioned with 3 mM EGTA in the course of the experiment; or in its Ca^{2+}-free version, which contained no $CaCl_2$ added but 1 mM EGTA. Reintroduction of 3 mM Ca^{2+} was made to reach the final $[Ca^{2+}]_o$ concentration of ~2 mM. Rapid addition of the various reagents was obtained by loading appropriate volumes of 100× concentrated solutions into a 2-ml syringe connected to the incubation chamber via a small tube. Aspiration to the syringe of 1 ml incubation medium followed by reintroduction of this mixture into the chamber yielded accurate and rapid (<1 s) delivery and mixing of the agents.

Fura-2 Videomicroscopy

The digital fluorescence-imaging microscopy system was built around a Zeiss inverted IM 35 light microscope equipped with a controlled heating stage which will be described in detail elsewhere. Briefly, excitation light beams were provided by two 150 W Xenon arc lamp sources (Oriel Co., Stratford, CT) appropriately placed over an optical bench and individually focused on two monochromators (Oriel Co.). Electronic shutters, operated by the main computer, were located at the exit of each monochromator. Optical and mechanical components were arranged in order to combine (by a semireflecting mirror) the monochromatic beams and provide Koehler epi-illumination. A Nikon 40×, 1.3 NA objective lens, and a 418-nm long pass emission filter were used. Fluorescence and bright field images were enlarged by a home-made zoom (fitted on the side port of the microscope) and collected with a low-light level ISIT camera (2400-09, Hamamatsu Photonics, Herrsching, FRG) which allowed the use of low level illumination intensities. The camera output was fed into a digital image processor (Argus 100; Hamamatsu Photonics) where video frames were digitized and integrated in real time on four 0.5 Mbyte memory boards.

In the present study, the electronic shutters were operated by the Argus 100 image processor to yield the following sequence of events: (*a*) illumination at 350 nm excitation wavelength and integration of five consecutive video images (256 × 256 × 16 bits) on part of the first memory board; (*b*) dark interval of 200 ms to eliminate persistence of the previous image on the sensitive target of the camera (<2% at the end of the lag interval); (*c*) illumination at 385 nm and integration of five consecutive video images as above on part of the second memory board; (*d*) dark interval of 200 ms. This sequence of commands was run continuously. When the first two memory boards were filled up, the next images were stored in the two remaining boards; meanwhile, the digital data present in the two filled memories were transferred at high rate (more than 1.5 Mbyte/s) via a connecting board placed on the VME bus of Argus 100 into a Motorola 68020 based host computer, and stored in two 300 Mbyte hard disks. By this procedure, which did not interfere with the activity of the Argus 100 image processor, up to 4,000 digital images (two images/s) could be stored in a single experiment. After background and calibration images were similarly acquired at the two wavelengths, the calculation of Ca^{2+} concentration was carried out pixel by pixel on pairs of corresponding 350 and 385 images according to Grynkiewicz et al. (1985). Since thresholding of cell intensity did not provide accurate definition of cell boundaries and, in addition, varied during the experiment, bright field images were acquired from time to time to be later used to prepare masks matching the cell shape. The masks were superimposed to the final Ca^{2+} images before visualization on the monitor as 16 level pseudocolor images. Mean values of the pixel intensity in the areas of interest could be calculated from the entire sequence of frames, thus, providing a quantitative temporal analysis in spatially distinct areas during the experiment.

Materials

Most of the fine chemicals used were purchased from Sigma Chemical Co., while fura-2 was from Calbiochem Behring Corp. (La Jolla, CA). Nitrendipine and Verapamil were the gift of Bayer and Knoll AG, respectively. ω-conotoxin was purchased from Peninsula Lab. (Belmont, CA), while the B_2 antagonist Arg^0[Hyp^3, $Thi^{5,8}$, D-Phe^7]BK was the gift of Dr. D. Regoli (University of Sherbrooke).

Results

PC12 Cells at Rest

Under the conditions of our experiments, the overall phenotype of the NGF-pretreated PC12 cells was variable. Many cells appeared just spherical or exhibited only one neurite, whereas other cells were endowed with a well-developed and arborized neurite tree. To minimize artifacts, and focus our study at the cellular level, most of the measurements were made on either single or small groups of cells where boundaries could be easily identified on the bright field image, as described under Materials and Methods.

In the resting cell population investigated, $[Ca^{2+}]_i$ appeared often moderately uneven (see Fig. 1; discussion in Tsien and Tsien, 1990). No consistent correlation was noticed between the areas with relatively high or low apparent $[Ca^{2+}]_i$ values and specific intracellular structures identified on the bright field image, such as nuclei. Interestingly, in many (but not all) cells low signal areas remained appreciable even after stimulation, because $[Ca^{2+}]_i$ apparently increased in parallel there and in the surrounding, higher signal areas. Moreover, no major differences of apparent resting $[Ca^{2+}]_i$ were observed among the various cell regions, i.e., body, dendrites, and varicosities (see Fig. 1). In a series of experiments the $[Ca^{2+}]_o$ of the medium bathing resting cells was varied from 2 mM down to ~10^{-9} M (addition of excess EGTA) and back. Except for a very few (<1%), clearly damaged cells, these treatments had only marginal effects on the resting $[Ca^{2+}]_i$.

Receptor Stimulation

In most of our experiments, receptor stimulants, bradykinin (BK), ATP, and carbamylcholine (CCh), were applied at concentrations inducing maximal $[Ca^{2+}]_i$ responses (0.1, 100, and 500 μM, respectively). The standard experimental protocol consisted of adding the agonist to cells bathed in a Ca^{2+}-free, EGTA-containing medium, and reintroducing Ca^{2+} to the medium, most often ~2 min later. *A* and *B* of Fig. 1 show $[Ca^{2+}]_i$ pseudocolor representations of a cell endowed with a single neurite and a large terminal varicosity, exposed to BK according to the protocol described above.

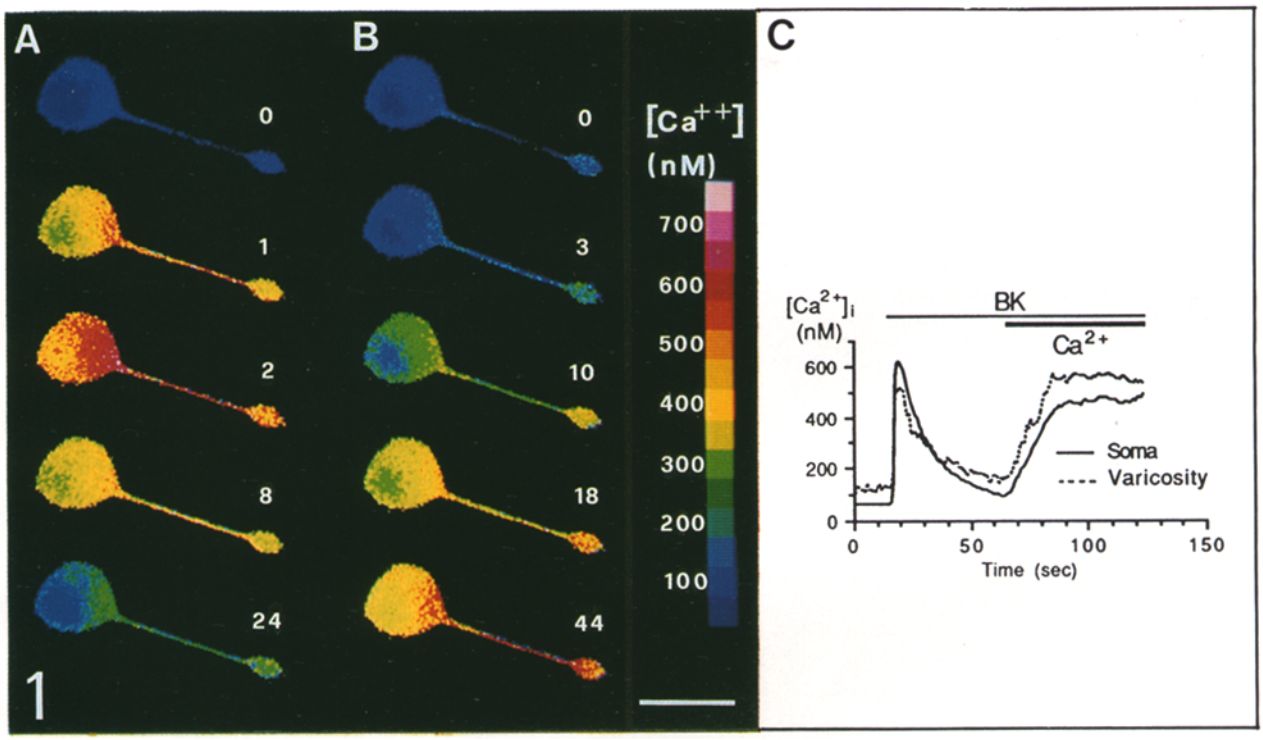

Figure 1. $[Ca^{2+}]_i$ changes induced by 100 nM BK in an NGF-differentiated PC12 cell. (*A* and *B*) Pseudocolor representation of $[Ca^{2+}]_i$. The cell shown in *A* was bathed in Ca^{2+}-free medium containing 1 mM EGTA. The numbers indicate seconds after BK addition. Notice that $[Ca^{2+}]_i$ changes occurred simultaneously and to the same extent in both the soma and the varicosity. A roundish area with apparent low $[Ca^{2+}]_i$ was present in the soma at rest and remained appreciable also during stimulation. *B* shows the same cell after addition (at time 0) of 3 mM $CaCl_2$ to the BK and EGTA-containing medium. $[Ca^{2+}]_i$ reached values similar to *A* but the kinetic was different. *C* is the temporal plot of $[Ca^{2+}]_i$ changes occurring in the soma and varicosity. In this and the other plots shown in this paper, thin bars mark the presence of the agonist and thick bars the presence of $[Ca^{2+}]_o$. Moreover, in the morphological panels the color scale gives $[Ca^{2+}]_i$ expressed in nM and the white bar corresponds to 20 μm.

The time-course plot of the $[Ca^{2+}]_i$ changes recorded in the cell body and terminal varicosity is shown in C. As can be seen, a sudden increase occurred both in the soma and in the neurite, with a short delay (~2 s) from the application of BK to the Ca^{2+}-free medium (*A* and *C*). The increase peaked at 4 s and then declined in parallel in the entire cell, to reach resting values after 1 min. The subsequent $[Ca^{2+}]_i$ increase triggered by addition of Ca^{2+} to the incubation medium (Fig. 1, *B* and *C*) resembled the Ca^{2+}-free response both in size and intracellular distribution. However, the time-course was different, with a slower rise leading in ~25 s to a plateau which persisted almost unchanged as long as BK was maintained in contact with the cell.

Additional series of experiments were carried out to validate the standard experimental protocol by the use of receptor blockers, i.e., Arg[0][Hyp[3], Thi[5,8], D-Phe[7]]BK (10 μM) for the B_2 receptor (Regoli et al., 1990) and atropine (1 μM), for the muscarinic receptor. These blockers were administered to the cells either before or at various times after BK or CCh. In the first case, in agreement with the previous fluorimetric results (Pozzan et al., 1986; Fasolato et al., 1988), the blockers completely prevented the $[Ca^{2+}]_i$ increase responses induced by their corresponding receptor agonists (not shown). Fig. 2 illustrates results obtained with atropine, administered after Ca^{2+} reintroduction into the medium bathing five cells, three responsive and two unresponsive to CCh. As can be seen, the blocker dissipated quickly the $[Ca^{2+}]_i$ increase sustained by influx in the responsive cells, with return to values close to those preceding the stimulation. Likewise, atropine dissipated the $[Ca^{2+}]_i$ increase sustained by intracellular release when administered shortly after CCh. Results similar with these with CCh and atropine were obtained with BK and the B_2 blocker (not shown).

We next investigated the possibility that voltage-gated Ca^{2+} channels (of L and N type), known to be expressed in PC12 cells (Plummer et al., 1989; Sher et al., 1988), participate in the influx phase of the receptor-triggered $[Ca^{2+}]_i$ responses. To this end, blockers (nitrendipine and verapamil, 1 μM, for L type; ω-conotoxin, 1.2 μM, for N type channels) were applied to the cells, individually or in sequence, after reintroduction of Ca^{2+} into the medium. In no case was any effect of these substances observed on the receptor-triggered responses, while parallel responses triggered by high K^+ (via depolarization-induced activation of voltage-gated Ca^{2+} channels) were markedly inhibited by nitrendipine and verapamil (not shown).

The results of Figs. 1 and 2, where responsive cells exhibit both release and influx, do not represent the rule in the analyzed cell population. Indeed, a large heterogeneity both in the intracellular distribution (to be described elsewhere) and in the size and type (see below) of the responses was ob-

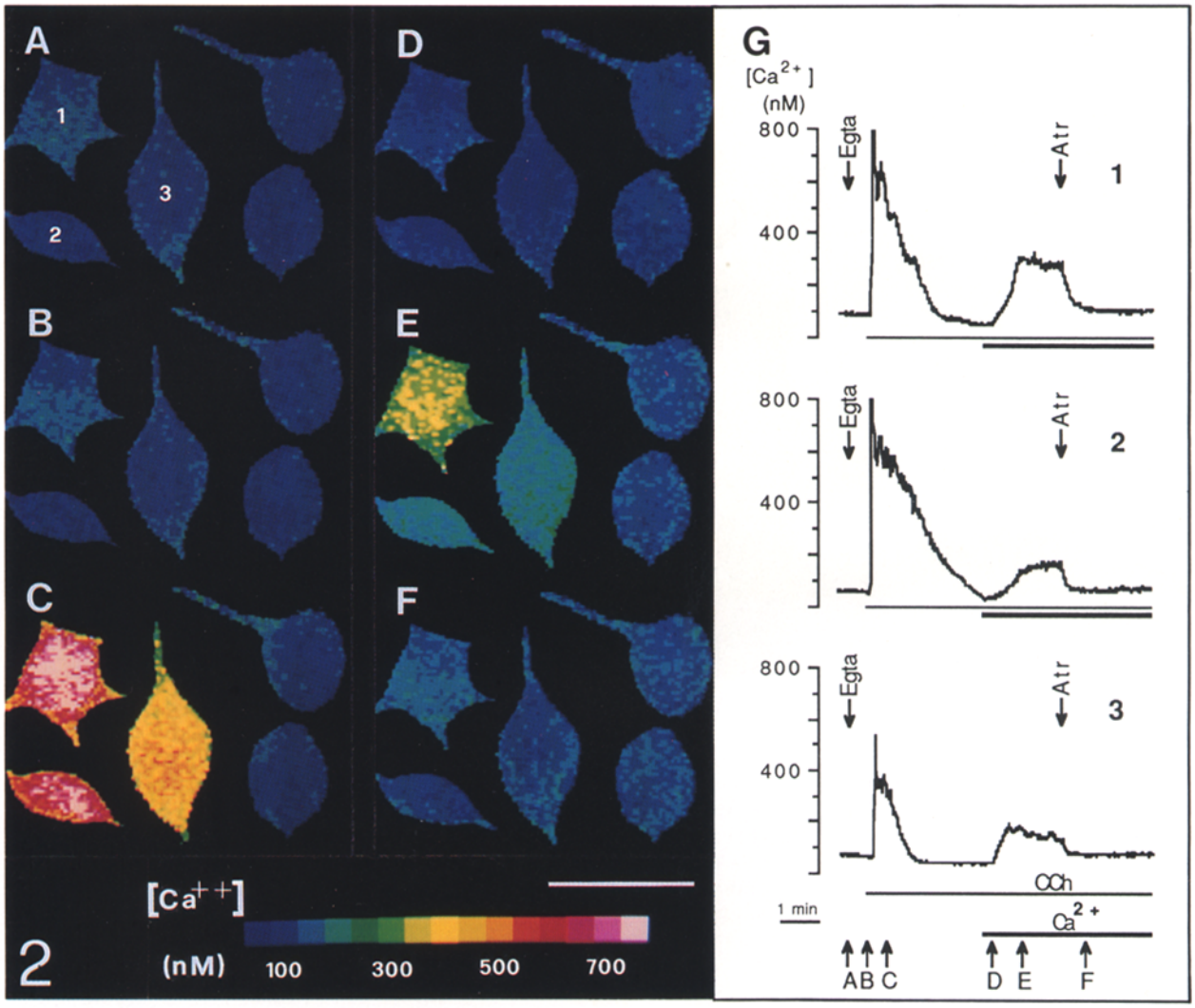

Figure 2. $[Ca^{2+}]_i$ effects induced by 500 μM CCh and 1 μM atropine in a group of five PC12 cells. The six (*A–F*) morphological panels illustrate $[Ca^{2+}]_i$ images of the cells incubated in the Ca^{2+}-containing medium (*A*); after addition of excess EGTA (*B*); at the peak of the intracellular release response induced by CCh (*C*); at the end of the latter response (*D*); at the top of the $[Ca^{2+}]_i$ increase sustained by influx, after reintroduction of Ca^{2+} into the medium (*E*); and after application of atropine (*F*). Notice that two cells were unresponsive while three exhibited both release and influx responses to CCh. The latter response was dissipated by atropine addition. The temporal plots of the $[Ca^{2+}]_i$ changes in these three cells (labeled *1–3*, see *A*), with indication of the times of addition of EGTA, CCh, and atropine, are shown to the right.

served. An example of the various response patterns to BK is given in Fig. 3. Of the six cells present in the field, three were unresponsive to BK. Of the others, cell 3 showed release only, cell 2 influx only, and cell 1 both processes. A similar heterogeneity was seen using ATP or CCh. The results obtained with the three agonists in the overall PC12 cell population are summarized in Table I. The percentage of responsive cells varied depending on the agonist, from ~80% with ATP to <50% with CCh. Also, the nature of the responses varied. The complete response, (i.e., both release and influx) was observed in only ~1/3 of the population when treated with any of the agonists. Of the remaining cells, ~40% exhibited appreciable release with no detectable influx stimulation, with the remaining group exhibiting influx but no release. A more detailed analysis of the results with the three agonists is provided in Fig. 4 *A*. Notice that, even in the cells exhibiting both release and influx, the ratio between the two components varied considerably.

On the other hand, in individual cells the response patterns to a single agonist, and thus the release vs. influx ratio

Table I. $[Ca^{2+}]_i$ Response Patterns in PC12 Cells

Agent	Analyzed cells	Responsive cells	Cells exhibiting release	Cells exhibiting influx	Cells exhibiting release and influx
			%	%	%
100 nM BK	320	219	43.0	23.4	33.6
500 μM CCh	177	78	38.8	28.8	32.4
100 μM ATP	105	82	41.2	24.1	34.7

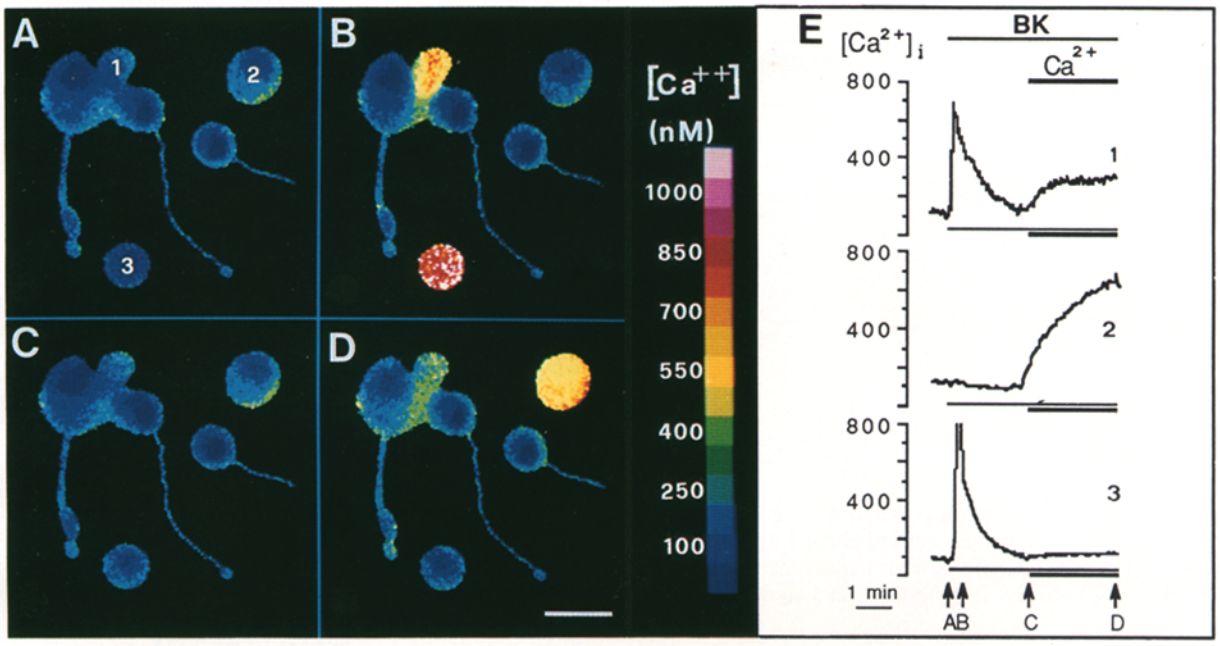

Figure 3. Heterogeneous responses to 100 nM BK in a group of PC12 cells. BK applied in Ca^{2+}-free medium activated $[Ca^{2+}]_i$ release from intracellular stores in cells 1 and 3 (compare *B* and *A*); after Ca^{2+} readdition, Ca^{2+} influx responses occurred in cells 1 and 2 but not in cell 3 (compare *D* and *C*). Three unresponsive cells were also present in the field. *E* shows the temporal plots of $[Ca^{2+}]_i$ changes measured in the three responsive cells. Arrows with letters indicate the timing of the images shown in *A–D*.

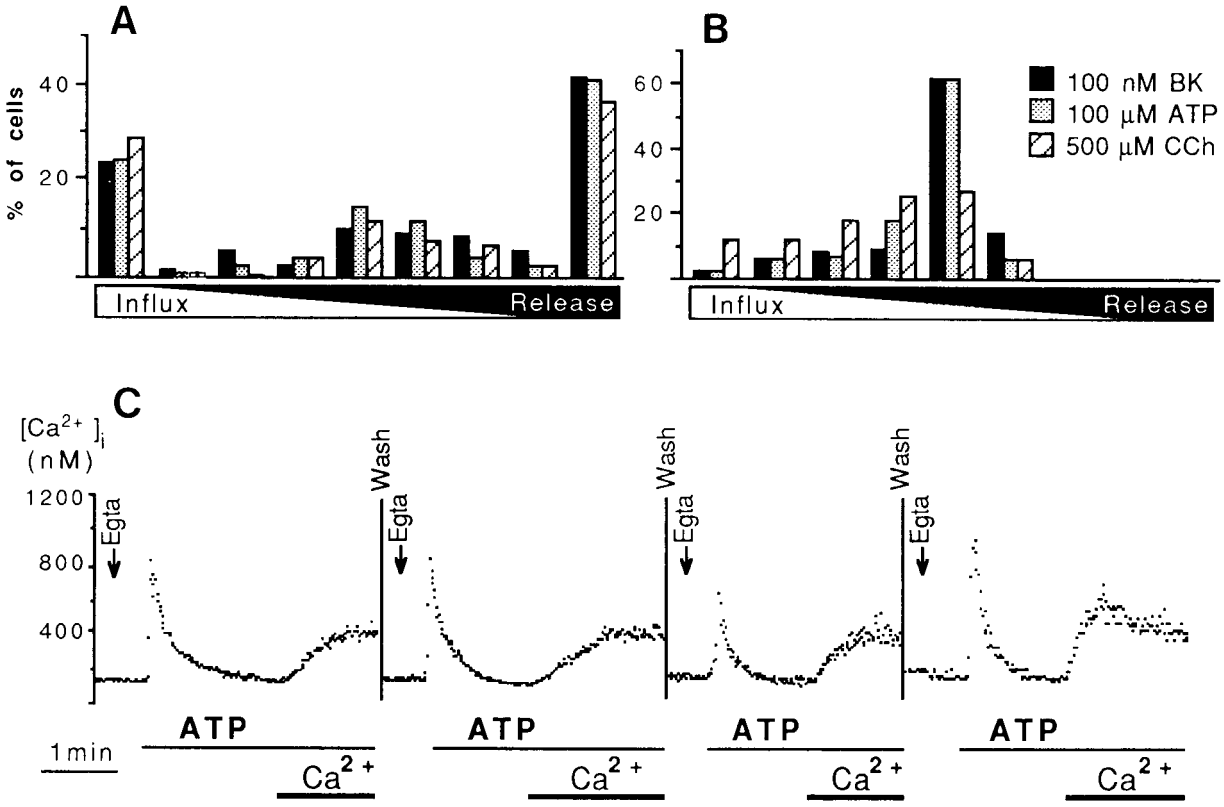

Figure 4. Frequency distribution of the release/influx ratios in PC 12 cells treated with BK, ATP, and CCh according to the $[Ca^{2+}]_o$-free/$[Ca^{2+}]_o$ reintroduction protocol (*A* and *B*) and $[Ca^{2+}]_i$ temporal plot in a single cell exposed to four subsequent pulses of ATP (100 μM) delivered according to the above protocol (*C*). Release/influx ratios were calculated by dividing the maximal $[Ca^{2+}]_i$ values measured after agonist treatment in Ca^{2+}-free medium with those measured after Ca^{2+} readdition. (*A*) Overall PC12 cell population; number of cells analyzed: BK = 219, ATP = 82, and CCh = 78. (*B*) Cells of the clone #15; number of cells for each agonist is 52. (*C*) Single cell of the overall population. Washes were for 20 min in the Ca^{2+}-containing incubation medium.

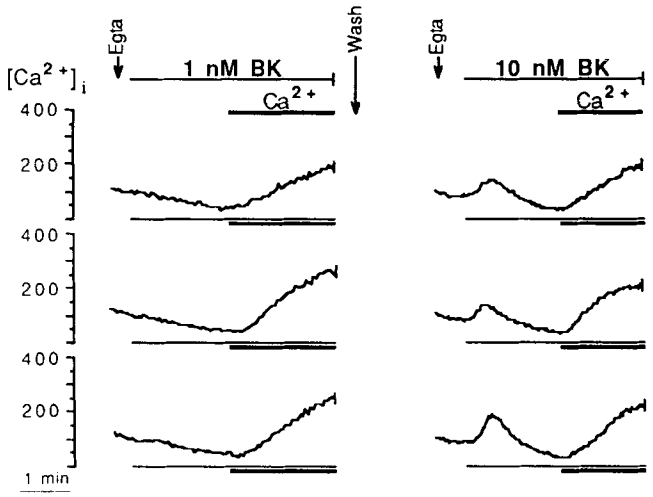

Figure 5. $[Ca^{2+}]_i$ changes induced by low concentrations of BK. In all of the three cells shown, influx was already elicited by BK at concentrations as low as 1 nM (*left panels*), while intracellular release was detectable only at 10 nM BK (*right panels*) and above.

values, were reproducible. When, in fact, a treatment (consisting of a 7-min $[Ca^{2+}]_o$ free/$[Ca^{2+}]_o$ readdition pulse followed by a 20-min washing in the Ca^{2+}-containing medium) was administered repeatedly (up to five times), the responses were found to vary only marginally. An example of these results (with ATP) is shown in Fig. 4 *C*. Results of this kind were obtained also with agonist concentrations higher (up to 100-fold) than those commonly employed. With lower concentrations some quantitative and qualitative changes were observed, which were investigated in detail with BK. In cells responding with both release and influx at 100 nM BK, only the second component of the response was appreciated with 1 nM BK (Fig. 5, *left plots*). Both components were elicited at agonist concentration of 10 nM; however, the $[Ca^{2+}]_i$ rise occurring during the release phase of the experiment was usually slower and smoother than that usually observed with higher BK concentrations (compare the right plots in Fig. 5 with those in Figs. 1 *C* and 3 *E*).

Single Cell Responses to Different Agonists

A final series of experiments carried out with the overall population was aimed to further characterize the specificity of the response patterns as a function of the agonists employed. To this end, a total of 29 cells were exposed in sequence to CCh, BK, and ATP as described in Fig. 6 legend. Of these cells, 7 were found to respond to three, 13 to two (BK and ATP), and the remaining 9 to only one of the agonists. Examples of the response pattern in these 29 cells are given in Fig. 6. In none of the five cells shown was the release vs. influx ratio strictly the same with the three agonists. In particular, note that in cell 3 CCh elicited a negligible release and a large influx, while with BK and ATP the release response was predominant. Similar discrepancies in the response pattern are also evident in cells 4 and 5. Here, BK induced sustained changes of $[Ca^{2+}]_i$ during both the release and the influx phases of the experiment, while ATP induced predominantly influx in cell 4 and release in cell 5.

Although the population used for this part of the study is

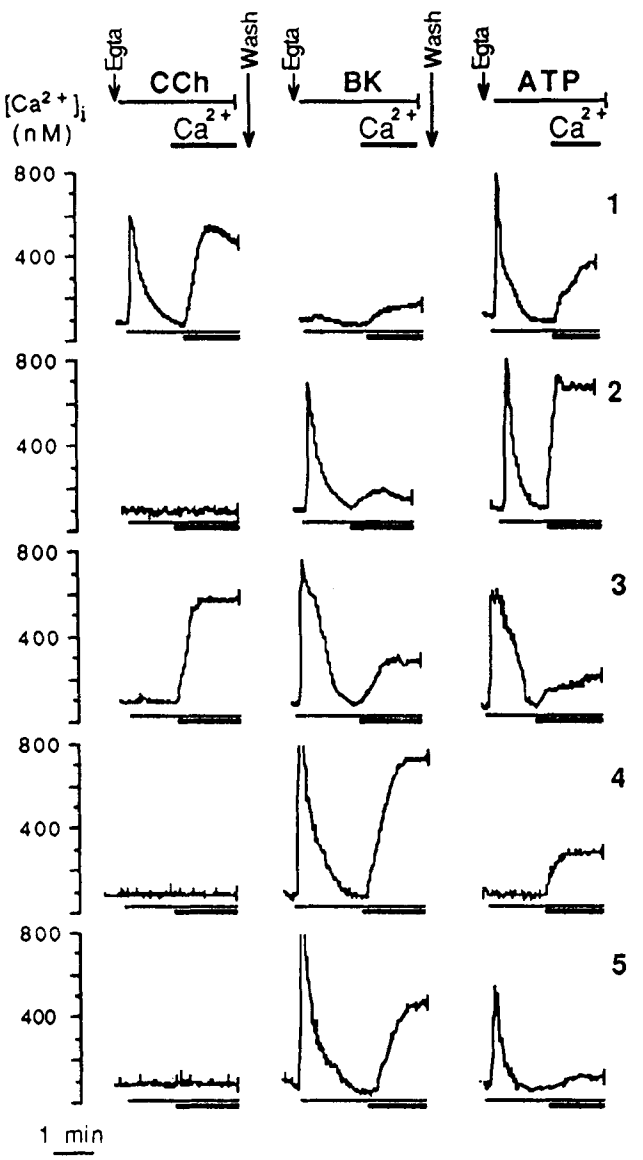

Figure 6. Temporal variations of $[Ca^{2+}]_i$ induced in single cells by subsequent treatment with 500 μM CCh, 100 nM BK, and 100 μM ATP applied according to the $[Ca^{2+}]_o$-free/$[Ca^{2+}]_o$ reintroduction protocol. After challenge with one agonist, the cells were extensively washed with complete incubation medium for 20–30 min. The five cells shown are examples of the response heterogeneity to the three agonists.

too small to draw detailed conclusions, it is clear from the results that the patterns induced in individual cells by different agonists were often markedly different, at variance with the similarity observed when multiple pulses of the same agonists were delivered (see above).

PC12 Cell Clone #15

The marked $[Ca^{2+}]_i$ response heterogeneity observed in the experiments reported so far could be due to the coexistence in the overall population investigated of multiple, heterogeneous PC12 cell clones. To investigate this possibility, the population was subcloned; one of the clones obtained (#15) was selected because of its good responsiveness to the three

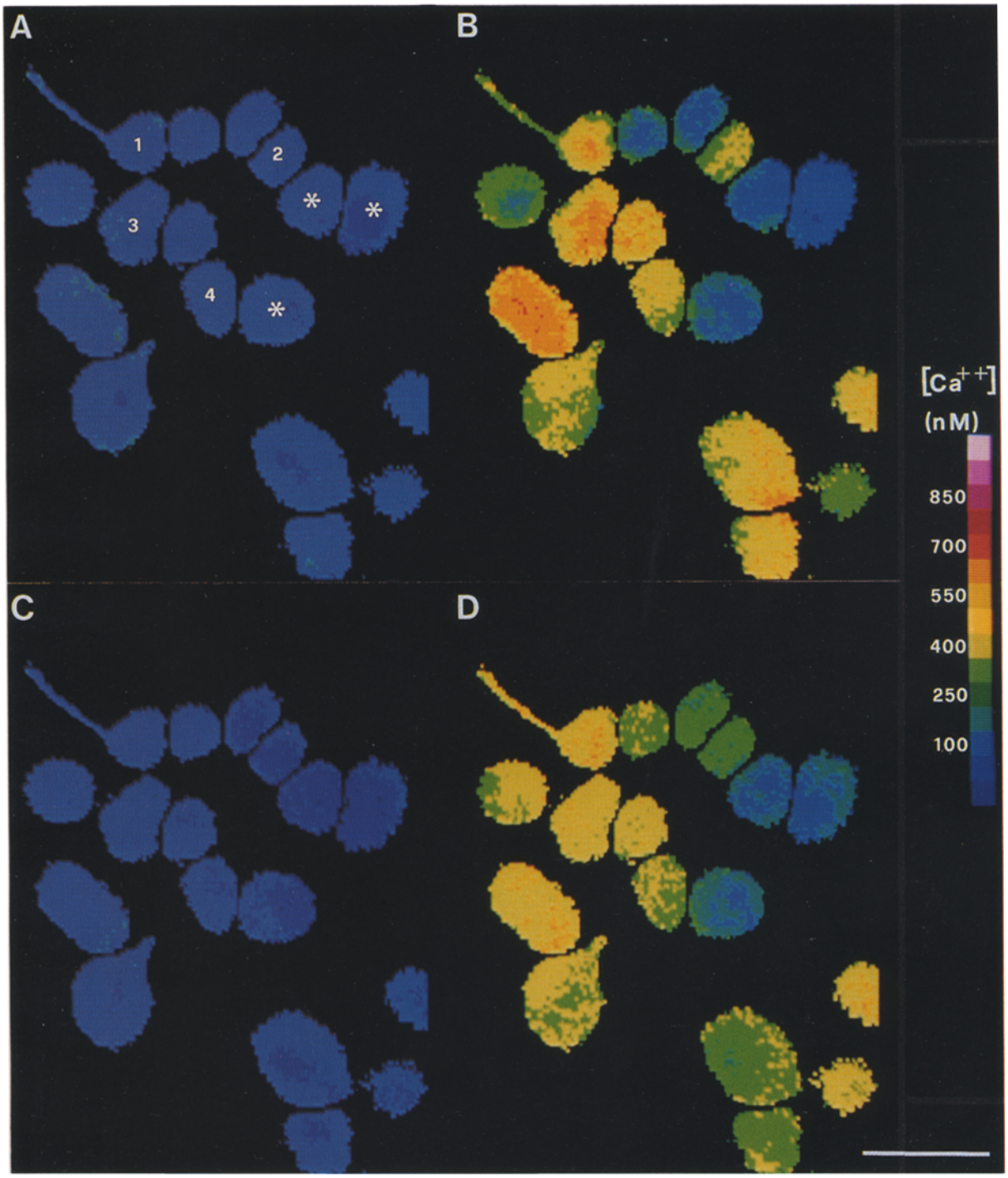

Figure 7. $[Ca^{2+}]_i$ responses induced by 100 nM BK in the cells of the PC12 clone #15. The pictures were taken before (*A*), at the peak (*B*) and at the end (*C*) of the response to 100 nM BK administered in the Ca^{2+}-free medium, and at the peak after Ca^{2+} readdition (*D*). Notice that $[Ca^{2+}]_i$ increases occurred in all cells of this field during both the release and the influx phases of the experiment, although to different extents (*asterisks* mark three hyporesponsive cells). Numbers indicate the cells whose responses to CCh, BK and ATP are plotted in Fig. 8.

agonists employed (measured in the fluorimeter cuvette; Grynkiewicz et al., 1985) and investigated at an early stage of its life (eighth passage). Indeed, the degree of heterogeneity was much smaller in the clone than in the overall population. Almost all the cells investigated (52 out of 54) were responsive to the three agonists and exhibited both release and influx. However, the intensity of the responses to a given agonist still varied among cells (see Fig. 7 for BK), and the release vs. influx ratio in individual cells was not strictly the same with the three agonists (Fig. 8). In general, the $[Ca^{2+}]_i$ changes occurring during the release and the influx phases of the experiment tended to be similar when cells were chal-

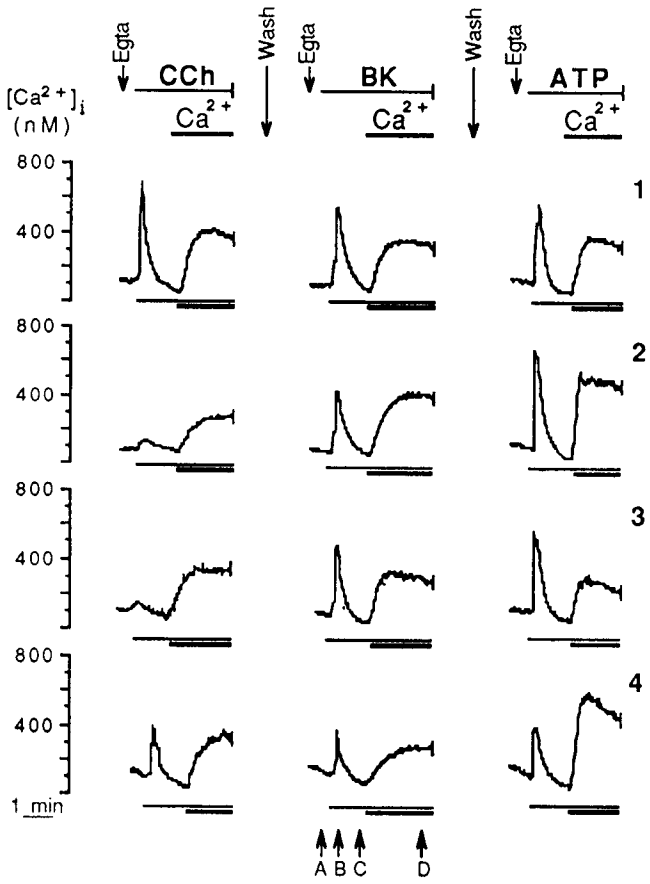

Figure 8. PC12 clone #15. Temporal plots of $[Ca^{2+}]_i$ changes induced by 500 μM CCh, 100 nM BK, and 100 μM ATP. The four cells illustrated here are those numbered in *A* of Fig. 7. Note that in cells 1, 2, and 3 the responses to BK and ATP were nearly identical in both shape and intensity, whereas in cell 4 they differed, although moderately. Responses to CCh were variable, with predominance of influx in cells 2 and 3.

lenged with either BK or ATP, while influx predominated with CCh. This is shown in the frequency distribution histogram in Fig. 4 *B*. With BK and ATP the plot revealed a tendency to unimodal distribution, with >60% of the cell exhibiting release vs. influx ratios close to 1, while with CCh the plot was flatter, and a relative dominance of influx appeared.

Discussion

The PC12 cells investigated in the present studies had been synchronized first by culture without serum (Rudkin et al., 1989), and then treated for a week with NGF. Together with the development of the neuron-like phenotype, we expected from this procedure a marked decrease of mitoses, with consequent decrease of the heterogeneity depending on cell cycling. This strategy proved however to be insignificant. Although heterogeneity of PC12 cells was previously reported, and several specialized clones isolated, (Bothwell et al., 1980; Hatanaka, 1981; Greene and Tischler, 1982; Green et al., 1986; Hempstead et al., 1989; Chijiwa et al., 1990), the extent observed in the $[Ca^{2+}]_i$ responses elicited in the overall population was unexpected. The much lower degree of heterogeneity observed in the isolated PC12 clone we have investigated, strongly suggests that the overall population available in our laboratory (and, presumably, in others), consists in a mixture of numerous clones, characterized by peculiar patterns of $[Ca^{2+}]_i$ response. Such heterogeneity proved useful because it offered the opportunity of investigating various steps of transmembrane signaling by studying the $[Ca^{2+}]_i$ responses elicited in individual cells of the population. Interesting results were obtained by the use of agonists binding to various PC12 receptors coupled to the hydrolysis of PPI: BK (B_2 receptor; Fasolato et al., 1988), CCh (an atypical M_3 receptor; Michel et al., 1989), and ATP (P_{2y} receptor; Fasolato et al., 1990). Based on previous results in bovine chromaffin cells (O'Sullivan et al., 1989), the possibility (and indeed observed, see Table I) of a nonuniform expression of the three receptors in the cell population was expected. This mechanism, however, cannot account entirely for the heterogeneity we have observed.

The Ca^{2+}-free, Ca^{2+} Reintroduction Protocol

The $[Ca^{2+}]_i$ increase induced by receptor activation is known to consist of at least two components: release from intracellular Ca^{2+} stores, triggered by Ins-P_3; and influx across the plasma membrane (Pozzan et al., 1986; Fasolato et al., 1988; Berridge and Irvine, 1989). To investigate separately these two components, our experiments were most often carried out according to a two-step protocol, largely and successfully used in the past by us and others in conventional fura-2 cuvette experiments: application of an agonist to cells bathed in a Ca^{2+}-free, EGTA-containing medium followed (~2 min later, i.e., after the end of the transient Ca^{2+} release response) by the reestablishment of the physiological $[Ca^{2+}]_o$, via simple addition of the cation to the medium.

In view of its key role in our studies, it was important to establish whether this protocol can be appropriately employed even with single attached PC12 cells. Our results demonstrate that the two-step protocol does not affect unspecifically the responsiveness of individual PC12 cells, and that, therefore, the results obtained reflect the events generated after receptor activation. In fact, (*a*) without stimulation cells could be repeatedly switched from the high to the low $[Ca^{2+}]_o$ media, and vice versa, with only marginal changes of $[Ca^{2+}]_i$; (*b*) the receptor-triggered $[Ca^{2+}]_i$ responses were prevented or largely dissipated when specific antagonists were administered either before or after the corresponding agonists (BK or CCh); and (*c*) cells exposed according to the protocol to multiple cycles of stimulation with a single agonist yielded reproducible responses.

Mechanisms of Ca^{2+} Influx Stimulation

Of the two phases of the receptor-induced $[Ca^{2+}]_i$ responses, one, the Ins-P_3–induced release, appears now well characterized (see Berridge and Irvine, 1989; Meldolesi et al., 1990). In contrast, the mechanisms and regulation of the stimulated influx are not yet clear. In previous fura-2 cuvette experiments, the possible involvement of voltage-gated Ca^{2+} channels of the L and N type (both expressed in PC12 cells; Di Virgilio et al., 1986; Sher et al., 1988; Plummer et al., 1989) had been excluded based on the inefficacy of specific

blockers and on the observation that membrane potential increases (via the activation of Ca^{2+}-dependent K^+ channels) rather than decreases, after treatment with either BK or CCh (Pozzan et al., 1986; Fasolato et al., 1988). The lack of inhibition observed now with L and N-type channel blockers (nitrendipine, verapamil, and ω-conotoxin), administered during the influx phase, confirms this conclusion also in single, attached PC12 cells. Other channels that might be considered to account for influx are those participating directly in the receptor structure. Of these, however, the nicotinic receptor has been specifically investigated and found not to be expressed in the PC12 cells employed in this work (see Pozzan et al., 1986), at variance with the original cell line (Greene and Tischler, 1976) and numerous preparations originated therefrom. In contrast, the ATP-activatable P_{2w} receptor is expressed. However, as demonstrated by Fasolato et al. (1990), this receptor desensitizes rapidly and therefore is not expected to contribute significantly to the influx phase initiated by Ca^{2+} reintroduction ~2 min after application of the nucleotide trisphosphate. In contrast, the involvement of a third group of channels, designated as second messenger operated (Meldolesi and Pozzan, 1987; Berridge and Irvine, 1989; Tsien and Tsien, 1990), appears consistent with our atropine and B_2 receptor blocker results. At the moment, however, these channels are still poorly understood.

Various second messengers have been proposed to be responsible for their operation: Ins-P_3, alone (Kuno and Gardner, 1987) or together with its phosphorylation product, Ins-P_4 (see Berridge and Irvine, 1989); and increased $[Ca^{2+}]_i$ (Von Tscharner et al., 1986), but none have been identified with certainty. Recently, evidence has been provided suggesting the multiplicity of these channels and possibly also of their regulation mechanisms (Sage et al., 1989, 1990; Rink, 1990). At least one of these channels has been suggested to become indirectly activated when the Ins-P_3–sensitive stores are depleted of Ca^{2+} (Takemura et al., 1989; Hallam et al., 1989; Taylor, 1990). Whatever their activation mechanisms, these channels are known to remain open for considerable periods of time (>10 min) after the application of the agonists to PC12 cells. This explains why their contribution was revealed in the two-step protocol we have employed.

Release-Influx Dissociation

The most important result obtained in the present study is the dissociation of the two receptor-induced $[Ca^{2+}]_i$ response components, release and influx, observed in over half of the overall cell population investigated. Moreover, the response pattern, dissociated or not, of a cell could vary completely when individual cells were exposed in sequence to the three receptor agonists, at variance with the reproducible patterns observed when multiple pulses of a single agonist were applied. Occurrence of intracellular release without appreciable stimulation of Ca^{2+} influx indicates expression of receptors, generation of Ins-P_3 and stimulation of intracellular Ca^{2+} stores together with the lack of functioning of the second messenger–operated channels, which however may be recruited after the activation of another receptor. The opposite finding (lack of appreciable intracellular release with stimulation of influx) is also interesting. In these cells receptors are in fact activated, however, Ins-P_3 appears to be generated to a subthreshold level. The alternative explanation, i.e., that intracellular Ca^{2+} stores are insensitive to the second messenger, can be ruled out at least in those cells in which administration of another agonist caused intracellular Ca^{2+} to be released. It should be noted that, to our knowledge, neither of these two possibilities, lack of enough Ins-P_3 generation and insensitivity of the Ca^{2+} stores, in otherwise responsive cells had ever been even considered.

Together with the different concentration dependence of Ca^{2+} release and influx, observed with BK, the dissociation results discussed so far provide information on the regulation of the influx process. Based on our data, we can in fact exclude that influx is triggered only by increases of either Ins-P_3 or $[Ca^{2+}]_i$ (two mechanisms considered in other cell types, Von Tscharner et al., 1986; Kuno and Gardner, 1987). Moreover, a persistence of empty stores cannot account for influx stimulation in Ca^{2+} release–negative cells. In fact, if this were the case, influx in these cells would be stimulated also independently of receptor activation, and receptor blockers would be inactive, two possibilities excluded by our experimental results. A regulation mechanism apparently compatible with the observed high degree of independence between release and influx could be based on the involvement not of bona fide second messengers but of multiple G proteins mediating the direct interaction of receptors with various effectors, in particular phospholipase C and multiple channels. Although well established for the modulation of their voltage-gated counterparts (see Birnbaumer et al., 1990), a model of this kind has been considered only on theoretical grounds for the second messenger–operated channels (Fasolato et al., 1988; Rink, 1990). Specific experimental results are therefore still needed.

Conclusion

The large heterogeneity, up to the complete release-influx dissociation, of the $[Ca^{2+}]_i$ responses induced in individual PC12 cells, together with the variable patterns revealed after stimulation of different receptors, document an unexpected complexity of transmembrane signaling in this and, presumably, other types of nerve cells. Our imaging results open multiple problems, particularly in the field of second messenger–operated channels, which however cannot be solved by the single experimental approach employed so far. In this respect, the results already obtained with the recently isolated clone #15 are particularly encouraging. Many additional PC12 clones are in fact already available and they are now being characterized for receptor-induced $[Ca^{2+}]_i$ responses. When available, a full panel of appropriately different cell clones will, in fact, enable us to pursue our studies at the biochemical and molecular level, in order to identify the mechanisms responsible for the observed cell biological events.

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