**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.

**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% CO₂. 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
PC12 Subcloning

Since initial attempts with limiting dilution and coculturing with irradiated
cells (20 µg/3 x 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; Gibeo Laboratories) to select for stable transfectants.

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.
A-conotoxin was purchased from Peninsula Lab. (Belmont, CA), while the
B2 antagonist Arg°[Hyp 3, Thi 5~, D-Phe7]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 bound-
aries could be easily identified on the bright field image, as
described under Materials and Methods.

In the resting cell population investigated, [Ca^{2+}] appeared often moderately uneven (see Fig. 1; discussion in
Tsien and Tsien, 1990). No consistent correlation was no-
ticed between the areas with relatively high or low apparent
[Ca^{2+}] 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 apprecia-
tive even after stimulation, because [Ca^{2+}] apparently in-
creased in parallel there and in the surrounding, higher sig-
nal areas. Moreover, no major differences of apparent resting
[Ca^{2+}] were observed among the various cell regions, i.e.,
body, dendrites, and varicosities (see Fig. 1). In a series of
experiments the [Ca^{2+}] of the medium bathing resting cells
was varied from 2 mM down to ~10^{-9} M (addition of ex-
cess EGTA) and back. Except for a very few (<1%), clearly
damaged cells, these treatments had only marginal effects on
the resting [Ca^{2+}].

Receptor Stimulation

In most of our experiments, receptor stimulants, bradykinin
(BK), ATP, and carbamylcholine (CCh), were applied at
concentrations inducing maximal [Ca^{2+}] 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+}] pseudocolor representations of a cell
endowed with a single neurite and a large terminal varicosity,
exposed to BK according to the protocol described above.

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Figure 1. 

The time-course plot of the [Ca\textsuperscript{2+}] 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\textsuperscript{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\textsuperscript{2+}] increase triggered by addition of Ca\textsuperscript{2+} to the incubation medium (Fig. 1, B and C) resembled the Ca\textsuperscript{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\textsuperscript{°}[Hyp\textsuperscript{3}, Thi\textsuperscript{S}, D-Phe\textsuperscript{3}]BK (10 μM) for the B\textsubscript{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\textsuperscript{2+}] increase responses induced by their corresponding receptor agonists (not shown). Fig. 2 illustrates results obtained with atropine, administered after Ca\textsuperscript{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\textsuperscript{2+}] increase sustained by influx in the responsive cells, with return to values close to those preceding the stimulation. Likewise, atropine dissipated the [Ca\textsuperscript{2+}] 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\textsubscript{2} blocker (not shown).

We next investigated the possibility that voltage-gated Ca\textsuperscript{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\textsuperscript{2+}] 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\textsuperscript{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\textsuperscript{+} (via depolarization-induced activation of voltage-gated Ca\textsuperscript{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 \(\mu\)M CCh and 1 \(\mu\)M 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.

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 \(\sim80\%\) 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 \(\sim1/3\) of the population when treated with any of the agonists. Of the remaining cells, \(\sim40\%\) 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 | Release % | Influx % | Release and Influx % |
|-------|----------------|-----------------|-----------|----------|---------------------|
| 100 nM BK | 320            | 219             | 43.0      | 23.4     | 33.6                |
| 500 \(\mu\)M CCh | 177            | 78              | 38.8      | 28.8     | 32.4                |
| 100 \(\mu\)M 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 Ca²⁺-free medium activated [Ca²⁺]ₐ release from intracellular stores in cells 1 and 3 (compare B and A); after Ca²⁺ readdition, Ca²⁺ 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 [Ca²⁺]ₐ 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 PC12 cells treated with BK, ATP, and CCh according to the [Ca²⁺]ₐ-free/[Ca²⁺]ₐ reintroduction protocol (A and B) and [Ca²⁺]ₐ 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 [Ca²⁺]ₐ values measured after agonist treatment in Ca²⁺-free medium with those measured after Ca²⁺ 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 Ca²⁺-containing incubation medium.
values, were reproducible. When, in fact, a treatment (consisting of a 7-min [Ca\textsuperscript{2+}]\textsubscript{o} free/[Ca\textsuperscript{2+}]\textsubscript{i} readdition pulse followed by a 20-min washing in the Ca\textsuperscript{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\textsuperscript{2+}]\textsubscript{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\textsuperscript{2+}] 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 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\textsuperscript{2+}] 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

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**Figure 5.** [Ca\textsuperscript{2+}]\textsubscript{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.

**Figure 6.** Temporal variations of [Ca\textsuperscript{2+}]\textsubscript{i} induced in single cells by subsequent treatment with 500 µM CCh, 100 nM BK, and 100 µM ATP applied according to the [Ca\textsuperscript{2+}]\textsubscript{o} free/[Ca\textsuperscript{2+}]\textsubscript{i} 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.
Figure 7. [Ca^{2+}] 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+}] 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+}] changes occurring during the release and the influx phases of the experiment tended to be similar when cells were chal-
though heterogeneity of PC12 cells was previously reported, the development of the neuron-like phenotype, we expected 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, the extent observed in the [Ca\(^{2+}\)]\(_i\) responses elicited in the over-all 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\(^{2+}\)]\(_i\) responses. 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\(_\gamma\) 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\(^{2+}\)]\(_i\), 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 un especifically 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\(^{2+}\)]\(_i\), media, and vice versa, with only marginal changes of [Ca\(^{2+}\)]; (b) the receptor-triggered [Ca\(^{2+}\)] 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\textsuperscript{2+}-dependent K\textsuperscript{+} 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 \(\omega\)-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\textsubscript{2} 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\textsuperscript{2+} reintroduction \(\sim 2\) 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\textsubscript{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\textsubscript{3}, alone (Kuno and Gardner, 1987) or together with its phosphorylation product, Ins-P\textsubscript{4} (see Berridge and Irvine, 1989); and increased [Ca\textsuperscript{2+}]. (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\textsubscript{3}-sensitive stores are depleted of Ca\textsuperscript{2+} (Takeamura 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\textsuperscript{2+}], 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\textsuperscript{2+} influx indicates expression of receptors, generation of Ins-P\textsubscript{3}, and stimulation of intracellular Ca\textsuperscript{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\textsubscript{3} appears to be generated to a subthreshold level. The alternative explanation, i.e., that intracellular Ca\textsuperscript{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\textsuperscript{2+} to be released. It should be noted that, to our knowledge, neither of these two possibilities, lack of enough Ins-P\textsubscript{3}, generation and insensitivity of the Ca\textsuperscript{2+} stores, in otherwise responsive cells had ever been even considered.

Together with the different concentration dependence of Ca\textsuperscript{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 conclude that influx is triggered only by increases of either Ins-P\textsubscript{3} or [Ca\textsuperscript{2+}] (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\textsuperscript{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\textsuperscript{2+}], 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\textsuperscript{2+}], 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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