Ca^{2+} Waves in PC12 Neurites: A Bidirectional, Receptor-oriented Form of Ca^{2+} Signaling

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Abstract. Spatial and temporal aspects of Ca^{2+} signaling were investigated in PC12 cells differentiated with nerve growth factor, the well known nerve cell model. Activation of receptors coupled to polyphosphoinositide hydrolysis gave rise in a high proportion of the cells to Ca^{2+} waves propagating non decrementally and at constant speed (2–4 μm/s at 18°C and ~10-fold faster at 37°C) along the neurites. These waves relied entirely on the release of Ca^{2+} from intracellular stores since they could be generated even when the cells were incubated in Ca^{2+}-free medium. In contrast, when the cells were depolarized with high K^+ in Ca^{2+}-containing medium, increases of cytosolic Ca^{2+} occurred in the neurites but failed to evolve into waves. Depending on the receptor agonist employed (bradykinin and carbachol versus ATP) the orientation of the waves could be opposite, from the neurite tip to the cell body or vice versa, suggesting different and specific distribution of the responsible surface receptors. Cytosolic Ca^{2+} imaging results, together with studies of inositol 1,4,5-trisphosphate generation in intact cells and inositol 1,4,5-trisphosphate-induced Ca^{2+} release from microsomes, revealed the sustaining process of the waves to be discharge of Ca^{2+} from the inositol 1,4,5-trisphosphate- (and not the ryanodine-) sensitive stores distributed along the neurites. The activation of the cognate receptor appears to result from the coordinate action of the second messenger and Ca^{2+}. Because of their properties and orientation, the waves could participate in the control of not only conventional cell activities, but also excitability and differential processing of inputs, and thus of electrochemical computation in nerve cells.

VIDEO microscopy studies carried out on individual cells during the last several years have revealed that the changes of cytosolic Ca^{2+} concentration ($[Ca^{2+}]_i$) in response to various stimuli do not develop always as a whole but can exhibit peculiar spatiotemporal aspects. In some cases, in fact, these responses have been shown to promote steep Ca^{2+} gradients between a discrete cytosolic area and the rest of the cell (Müller and Connor, 1991; Guthrie et al., 1991; Silver et al., 1990); in others, to be composed by series of rhythmic $[Ca^{2+}]_i$ spikes, the oscillations (Berridge and Galione, 1988; Jacob, 1990*b*; Tsien and Tsien, 1990; Meyer and Stryer, 1991; Berridge, 1993), that are now believed to originate from an intracellular pacemaker or "trigger zone" (Rooney et al., 1990; D'Andrea et al., 1993; Kasai et al., 1993); in others, to consist of Ca^{2+} waves (Meyer, 1991; Amundson and Clapham, 1993; Berridge, 1993). Because of these processes, the host of functions that rely on $[Ca^{2+}]_i$ for their regulation are believed to be affected to different extents or at different times depending on their localization within the stimulated cells.

The present report concerns Ca^{2+} waves, i.e., intracellular events defined by their nondecremental propagation and distinct orientation, previously described in a number of cell types, although with variable properties. Waves appear, in fact, mostly planar in muscle (Neylon et al., 1990; Ishide et al., 1990; Takamatsu and Wier, 1990; Blatter and Weir, 1992; Iino et al., 1993), endothelial (Jacob, 1990*a*) and epithelial cells (Roone et al., 1990, 1991; Kasai and Augustine, 1990; Kasai et al., 1993); spiral, with complex organization in eggs (Lechleiter et al., 1991). Based on these phenomena, the cytosol can be considered, at least on a theoretical ground, as an excitable medium which operates together with the plasma membrane in the control of cell electrochemical computation. The overall consequences of this interplay are expected to be particularly relevant in the case of cells, such as neurons, which exhibit complex geometry and perform spatiotemporal integrations of signals. The results herewith reported, obtained in the widely used neuronal model, the PC12 cells (Greene and Tischler, 1976) differentiated with NGF, demonstrate that waves do develop and run longitudinally along the neurites, directed either to-

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wards or away from the cell soma, when receptors coupled to the hydrolysis of polyphosphoinositides (PPI)[1] are activated. The requirements we hypothesize for the development of these waves are the specific distribution at the cell surface of the triggering receptors, and the widespread distribution along the neurites of inositol 1,4,5-trisphosphate ($InsP_3$)-sensitive Ca^{2+} stores, two properties recognized to exist also in at least some types of neurons (Starke et al., 1989; Nomura et al., 1994; Satoh et al., 1990). The hypothesis of Ca^{2+} waves in the latter type of cells, with profound consequences on their activity, deserves therefore to be investigated.

Materials and Methods

Cell Preparation

PC12 cells from the parent population and from the clone No. 64 were prepared and cultured according to the procedures described previously (Grohovaz et al., 1991) in DME containing 2 mM glutamine, 10% horse serum, and 5% fetal calf serum. For experimental use, cells were plated on glass coverslips, serum deprived for 24 h, and then treated with 50 ng/ml mouse 2.5S NGF for at least 1 wk in complete DME.

Fura-2 Loading

At the beginning of each experiment the cells were incubated with fura-2-AM (2–5 μM) for 45 min at 37°C, washed, further incubated in the medium to allow de-esterification of the dye and finally incubated in a Krebs-Ringer solution buffered with Hepes (KRH) containing (mmoles/l): 125 NaCl, 5 KCl, 1.2 $MgSO_4$, 1.2 KH_2PO_4, 2 $CaCl_2$, 6 glucose, 25 Hepes-NaOH, pH 7.4.

Fluorimeter Measurement of $[Ca^{2+}]_i$

Suspensions of fura-2-loaded cells (1×10^6 cell/ml), supplemented with 250 μM sulfinpyrazone (to prevent dye leakage), were transferred into a thermostatted cuvette (37°C), maintained under continuous stirring and analyzed in a Perkin Elmer LS-5B fluorimeter (see details in Zacchetti et al., 1991).

Videomicroscopy Measurement of $[Ca^{2+}]_i$

At the beginning of each experiment the coverslips were transferred in a temperature-controlled microincubator chamber designed to fit the stage of the light microscope. Stimulants and blockers were administered to the cells bathed either in KRH, in Ca^{2+}-devoid medium (KRH without Ca^{2+} added) or in Ca^{2+}-free medium (Ca^{2+}-devoid medium with 1 mM EGTA). The delivery and mixing time was <1 s. The digital fluorescence-imaging microscopy system was built around a Zeiss inverted IM 35 light microscope as already described (Grohovaz et al., 1991). Briefly, excitation light at 340 or 380 nm was provided by two 150 W xenon lamps; fluorescence images were collected by a low-light level CCD camera (Hamamatsu Photonics, Herrsching, Germany) and fed into a digital image processor (Argus 100; Hamamatsu Photonics) coupled to a host computer where video frames were digitized and integrated in real time. Ratioing and/or $[Ca^{2+}]$ calculation were carried out pixel by pixel on pairs of corresponding 340 and 380 images according to Grynkiewicz et al. (1985). Fast rate temporal analyses (8–12 ratios/s) were low pass filtered by a Fourier-based procedure to remove high frequency components.

Measurement of Inositol Phosphates

Production of $InsP_3$ and total inositol phosphates (InsPs) was measured in cell preparations pre-incubated for 24 h with 1 μCi/ml [^{3}H]myoinositol in BME supplemented with 0.2% horse serum and 0.1% fetal calf serum. Before application of the stimuli the cells were detached, washed and resuspended in KRH either with or without Ca^{2+}. After application of the stimuli, reactions were stopped with an equal volume of ice-cooled trichloroacetic acid (15% solution), and radioactive InsPs were separated by ion-exchange liquid chromatography as described by Jackson et al. (1987). For total InsPs experiments the cells were stimulated for ten minutes in the presence of 10 mM Li^+. Values are expressed as percentage of the basal obtained in unstimulated PC12 cells.

Measurement of Ca^{2+} Release from Microsomes

PC12 cells were homogenized in an intracellular buffer (containing, in mmoles/l: 5 NaCl, 100 KCl, 2 $MgCl_2$, 2 KH_2PO_4, 25 Hepes-KOH, 0.1 phenylmethylsulfonil fluoride, pH 7.4) by passing them through a ball-bearing homogenizer with a clearance of 0.012 mm (EMBL Lab Workshop, Heidelberg, FRG; see Balch et al., 1984). The suspension was then centrifuged at 10,000 *g* for 30 s in a microfuge. The supernatant was collected and stored frozen at −80°C until use.

For the Ca^{2+} release assay aliquots of post-mitochondrial supernatant were thawed and diluted (5–10 mg protein/ml) with the intracellular buffer in the thermostatted cuvette of a fluorimeter (Perkin Elmer LS-50). Fura-2 free acid (1 μM) and an ATP regenerating system (phosphocreatine 5 mM and creatine phosphokinase 20 U/ml) were then added and uptake of Ca^{2+} was started by adding 3 mM ATP at 37°C. At the end of the uptake process, monitored by the decrease of fluorescence (excitation at 340 nm and emission at 495 nm), microsomal suspensions were rapidly equilibrated at 18° or 37°C. $InsP_3$ was then administered and the fluorescence values recorded with a time resolution of 0.1 s. Fluorescence values from different experiments were normalized according to the maximal values obtained after administration of 5 μM ionomycin.

Results

Spatiotemporal Aspects of $[Ca^{2+}]_i$ Responses

Our initial digital imaging videomicroscopy experiments were carried out on fura-2–loaded, NGF-differentiated PC12 cells stimulated while bathed in Ca^{2+}-containing KRH medium. Fig. 1 compares, in a single cell, the different patterns of response observed at 37°C after either depolarization or activation of a receptor coupled to PPI hydrolysis. When depolarized by elevating the extracellular K^+ (50 mM) the cells revealed, after a short latency, a sharp $[Ca^{2+}]_i$ increase initially confined to the distal portion of the neurites (Fig. 1 *A*), i.e., where voltage-operated Ca^{2+} channels have been reported to be concentrated (Streit and Lux, 1989; Reber and Reuter, 1991). Increase in the soma appeared after several seconds and remained of lower level with respect to the neurite signal. More striking was the response when PPI hydrolysis was induced by 100 nM bradykinin (BK). The peptide promoted first a $[Ca^{2+}]_i$ transient in the neurites, followed shortly thereafter by the appearance of the signal in the soma (Fig. 1 *B*), a response largely maintained when BK was administered in a Ca^{2+}-free medium. Restoration of physiological $[Ca^{2+}]$ in the medium after the exhaustion of the initial transient resulted in a second, small $[Ca^{2+}]_i$ elevation beginning in the neurites that, usually, was not followed by the spreading of the signal to the cell body (not shown).

The results with BK reported so far demonstrate that the $[Ca^{2+}]_i$ signal is largely sustained not by influx from the extracellular space but by release from intracellular stores, and suggest that this signal is initiated in the neurites and then extended to the cell body. In order to investigate the latter problem, cells exposed to the peptide while bathed at 37°C in the Ca^{2+}-devoid medium were imaged at fast rates (8–12 ratios/s) and signals were recorded from three windows placed ~25-μm apart from each other, the first more distal,

1. *Abbreviations used in this paper*: BK, bradykinin; $[Ca^{2+}]_i$, cytosolic Ca^{2+} concentration; CCh, carbachol; $InsP_3$, inositol 1,4,5-trisphosphate; $InsP_x$, inositol phosphates; KRH, Krebs-Ringer solution buffered with Hepes; PPI, polyphosphoinositides.

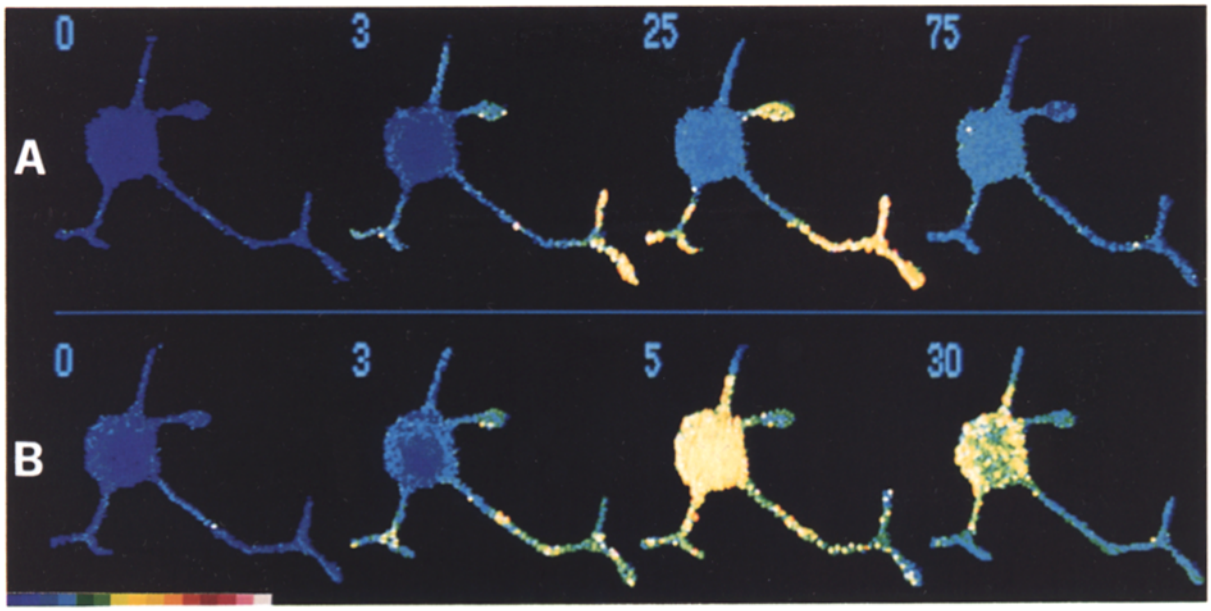

Figure 1. $[Ca^{2+}]_i$ responses induced by KCl and BK in a NGF-differentiated PC12 cell challenged at 37°C. In this and in the other figures, the numbers indicate seconds after administration of the stimulus (time *0*) and the intensities of fluorescence ratio values are represented according to the calibration bar (bottom in *B*), where lowest values are coded blue. Administration of 50 mM KCl into complete KRH medium caused a large and transient response mainly confined to the neurites (*A*). The same cell, washed and then stimulated with 100 nM BK while bathed in Ca^{2+}-free medium, exhibited an initial elevation of $[Ca^{2+}]_i$ in the neurites and a delayed, but quantitatively larger, response in the cell body (*B*).

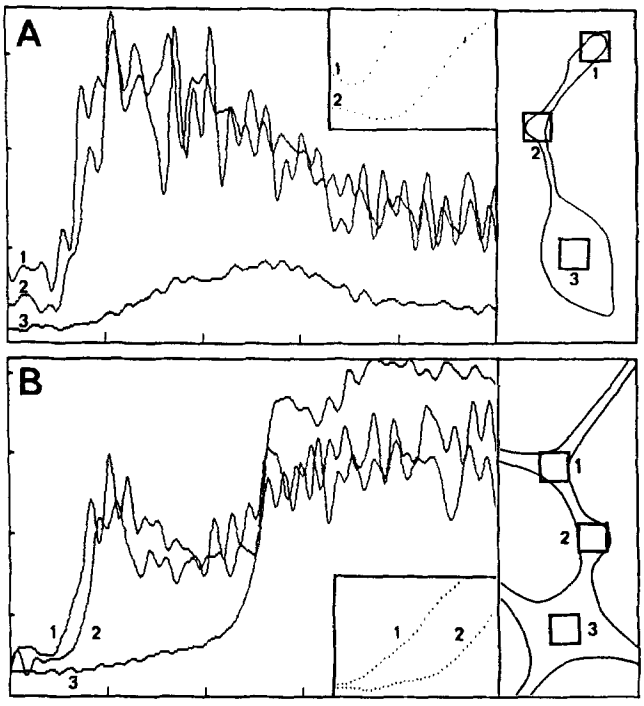

Figure 2. Time course of $[Ca^{2+}]_i$ responses upon stimulation with BK (100 nM) of two NGF-differentiated PC12 cells challenged at 37°C while bathed in Ca^{2+}-devoid medium. *A* and *B* illustrate the $[Ca^{2+}]_i$ temporal changes recorded at fast rates (*A*, 8 ratios/s; *B*, 12 ratios/s) in three regions of the cell. The recording windows, positioned as illustrated in the drawings to the right, were separated from each other of ~25 μm. The $[Ca^{2+}]_i$ rises appeared first in the more distal region of the neurite (*1*), after a short delay in the proximal one (*2*), and eventually (several seconds) in the soma (*3*). The initial few seconds of the plots in *A* and *B* are shown on an expanded scale in the insets to highlight the delay between the onset of the signal in regions *1* and *2* (dots indicate single ratio values). Once established, the signals from the neurite windows were noisy. In the soma the $[Ca^{2+}]_i$ increases were most variable, sometimes remaining low (*A*), more often rising according to a foot-upstroke kinetics to reach high values (*B*).

the second more proximal in the neurites, and the third over the cell body (see Fig. 2, *A* and *B*).

The results obtained not only confirmed the delay of the cell body, but also revealed an earlier appearance of the signal (0.8–1.5 s) in the distal compared to the proximal region of the neurite (Fig. 2, *A* and *B*) suggesting the occurrence of waves running along the latter at the apparent rate of 17–30 μm/s. In addition, once established, the neurite signal appeared not constant, but exhibited small oscillations of high frequency that could not be appropriately distinguished from the background.

The results obtained at 37°C were hard to analyze in detail because of the high frequency of the events and the poor signal to noise ratio of the images. To reveal the spatial and temporal patterns of the release-sustained events induced by BK, the role of temperature was next investigated. Fig. 3 *A* illustrates the results obtained in 12 out of 14 responsive cells to which BK was administered at 18°C in Ca^{2+}-free medium. In these cells no response appeared during the first several seconds. Thereafter, $[Ca^{2+}]_i$ rises were seen to begin asynchronously at the tip of various neurites, and then propagate along the shafts as waves much slower, and thus better appreciated, than the events revealed at 37°C. The right panel of Fig. 3 *A* summarizes the results obtained in the illustrated cell. The waves, occurring in three out of four neurites, are shown to run at apparently constant rate (2–4 μm/s), with neither loss of signal at the leading edge nor decline behind

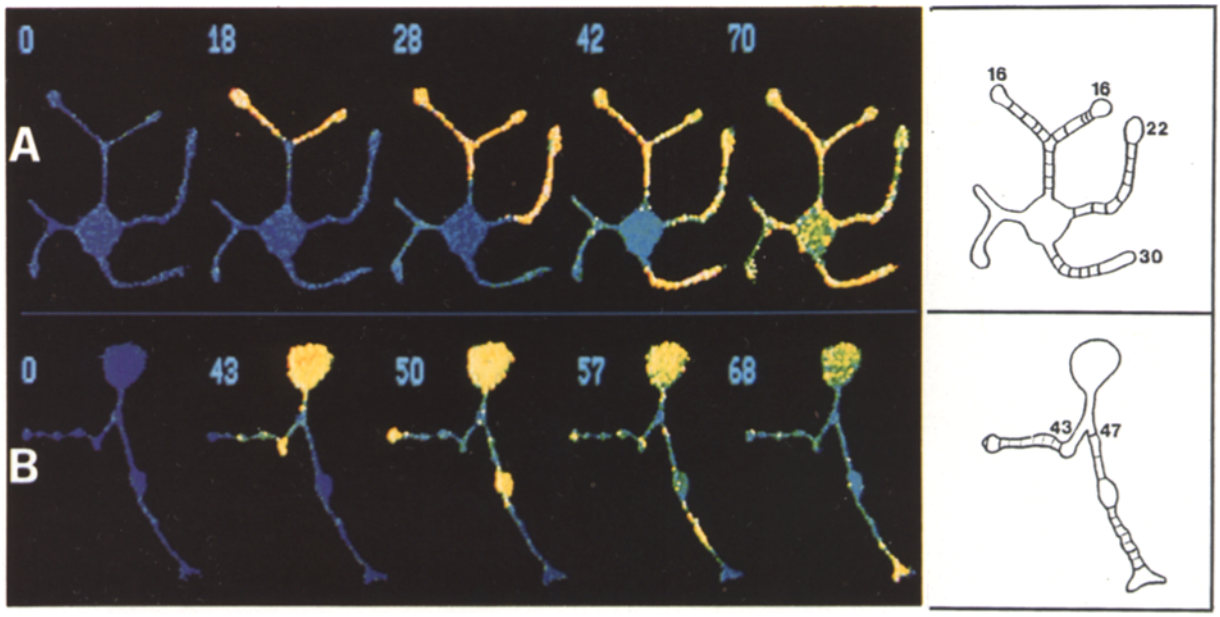

Figure 3. Ca^{2+} waves with opposite orientation induced in two differentiated PC12 cells by BK and ATP administered at 18°C in the Ca^{2+}-free medium. Application of the agonists in the Ca^{2+}-free medium activated Ca^{2+} release from intracellular stores. With 100 nM BK, after various and marked delays, $[Ca^{2+}]_i$ rose at the tip of three neurites and then developed into slow, autoregenerative waves (tides) directed towards the cell body (*A*). In the cell stimulated with 100 μM ATP the signal appeared in the cell body and propagation of the Ca^{2+} waves was oriented centrifugally, appearing however more as bands (*B*). The drawings to the right summarize the $[Ca^{2+}]_i$ events elicited in the neurites of the two cells. Numbers indicate the time when waves first appeared in each neurite. Transversal lines (one second apart) mark the progression of the waves as revealed by the whole set of images. Sharp boundaries (always occurring with the BK tides) are marked by continuous lines; confused boundaries (often appearing with the ATP-induced bands) by dotted lines.

(tide propagation; see Dupont and Goldbeter, 1992) (Fig. 3 *A*). At the interface with the soma the waves became no longer appreciable, replaced by a slow, en block $[Ca^{2+}]_i$ rise.

The next question raised was whether and to what extent the Ca^{2+} wave pattern observed was specific for BK or could be reproduced by other PPI-hydrolysis–coupled receptor agonists. Treatment at 18°C in Ca^{2+}-free medium with one such stimulant, ATP (100 μM), did induce neurite Ca^{2+} waves which however could be differently oriented. In fact, in 10 out of 16 cells the waves originated in the body of the cell and travelled centrifugally (Fig. 3 *B*). Only in two they were centripetal, while in the remaining four responsive cells no spatio-temporal differences were appreciated between soma and neurites (not shown). An additional difference of the ATP- versus BK-induced waves concerned propagation. With the nucleotide, in fact, only some waves appeared as tides, while others propagated more as moving bands, without sharp boundaries at either edges (Fig. 3 *B*). In these cells the appreciation of the wave progression was not as distinct as in those treated with BK (compare the right panels of Fig. 3, *A* and *B*). In the soma the ATP-induced $[Ca^{2+}]_i$ rises (Fig. 4 *B*) were steeper than those induced by BK (Fig. 4 *A*), with peak response values variable and often higher than those observed in the neurites. In all experimental conditions, however the $[Ca^{2+}]_i$ elevations in the soma consisted of biphasic rises, with an initial slower phase, the foot, followed by a rapid phase, the upstroke (see Figs. 2 *B* and 4 *C*), which occurred without any clear sign of oriented propagation.

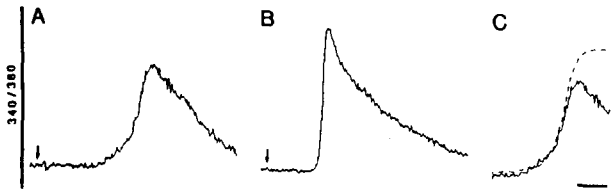

Figure 4. $[Ca^{2+}]_i$ responses in the cell soma after administration of either BK or ATP (0.1 and 100 μM, respectively) at 18°C in Ca^{2+}-free medium. *A* and *B* show the time course of the $[Ca^{2+}]_i$ rises in the soma of the two cells illustrated in Fig. 3, *A* and *B*, respectively. Arrows indicate the time when the agonists were added (bar = 60 s). In *C* the traces of *A* and *B* are superimposed, one (*BK*) kept as in *A*, the other (ATP, *dashed line*) drawn on an expanded time scale (bar = 9 s). Notice that the kinetics of the $[Ca^{2+}]_i$ rise in the soma is in both cases of the foot-upstroke type, although the rise time of the response is almost sevenfold faster after ATP than after BK.

Mechanisms of Ca^{2+} Release

From our previous work (Zacchetti et al., 1991) it is known that PC12 cells express a single, rapidly exchanging store which can release its Ca^{2+} via two independent intracellular channels, i.e., the receptors for caffeine/ryanodine and for $InsP_3$. An isolated clone, No. 64, however, differs from the parent line because of its unresponsiveness to caffeine/ryanodine (Zacchetti et al., 1991) and can therefore be used to establish whether the ryanodine receptor plays or not a key role in the wave process. Since the clone responds only weakly to BK, and more strongly to CCh and ATP, experiments

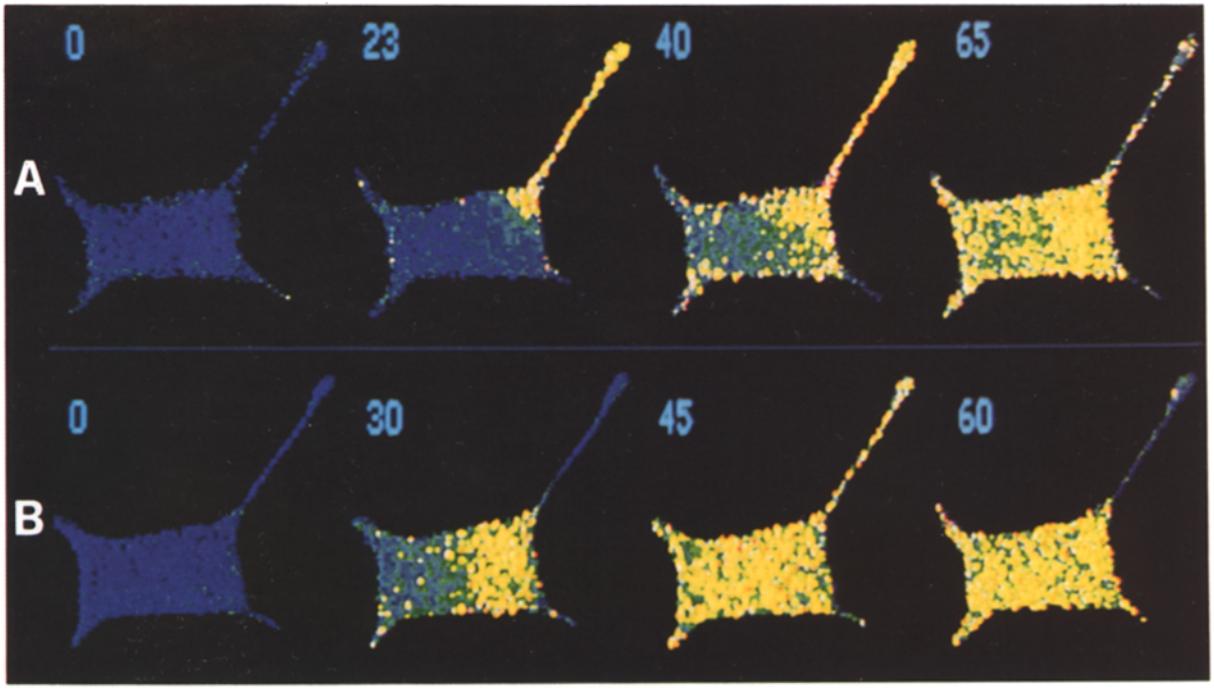

Figure 5. Ca^{2+} waves with opposite orientation induced by CCh and ATP in a single cell from the caffeine-insensitive PC12 clone No. 64 incubated at 18°C in the Ca^{2+}-free medium. Administration of 500 μM CCh induced in the neurite a Ca^{2+} wave oriented centripetally (*A*). Subsequent administration (after 15-min washes) of 100 μM ATP elicited a wave of similar intensity but opposite direction (*B*).

(n = 5) were carried out by using the latter two agonists. Fig. 5 illustrates the results in a cell of this clone exposed first to CCh (*A*) and then, after exhaustive washes, to ATP (*B*). As can be seen, the $[Ca^{2+}]_i$ responses to the two agonists initiated at the tip of the neurites and in the cell body, respectively, and then invaded the rest of the cell, i.e., the Ca^{2+} waves developed in the No. 64 clone cells according to the patterns typically observed in cells of the parent PC12 population when exposed to BK and ATP. These results indicate that caffeine/ryanodine-sensitive channels are not required in either the generation or the spreading of Ca^{2+} waves, and indirectly indicate that $InsP_3$ receptors are involved.

The possibility that the observed Ca^{2+} wave responses were induced solely by the activation of $InsP_3$ receptors was further studied by biochemical experiments. To investigate whether the rates of $InsP_3$ production were consistent with the different $[Ca^{2+}]_i$ responses, parallel batches of PC12 cells were exposed to BK at the two temperatures investigated, and PPI hydrolysis was estimated by $InsP_3$ formation. The rate of $InsP_3$ rise at 18°C was found to be slower than at 37°C, so that the peak was reached only after 30 s and the increase was half of that obtained at 37°C (Fig. 6). At later incubation times, the $InsP_3$ values were similar at both temperatures.

The kinetics of the Ca^{2+} release responses induced directly by $InsP_3$ at 37° and 18°C were investigated at the subcellular level, using total microsomes of PC12 cells incubated in an intracellular-type medium containing fura-2-free acid. As shown in Fig. 7 *A*, when the microsomes were tested at the lower temperature not only the kinetics of the release induced by a sub-maximal $InsP_3$ concentration were slowed down, but also a delay appeared. At higher $InsP_3$ concentrations the differences between the two temperatures, although less pronounced, were still evident (Fig. 7 *B*). Taken together the data of Figs. 6 and 7 indicate that low temperature has profound effects on the kinetics of $InsP_3$ production and Ca^{2+} release from intracellular stores.

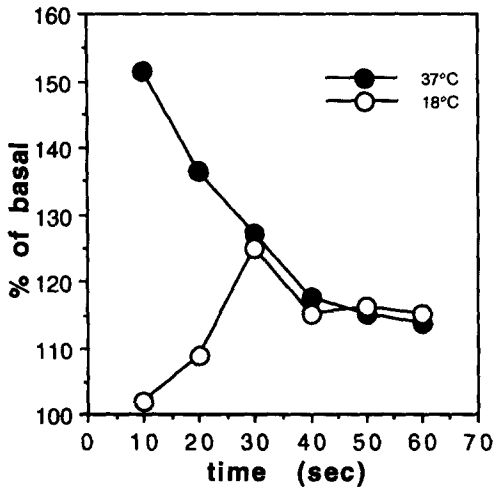

Figure 6. Time course of $InsP_3$ production induced by BK at 37° and 18°C in PC12 cells. Following administration of 100 nM BK, $InsP_3$ accumulation (*% of basal*) reached the maximal values within the first 10 s at 37°C (●) and after ~30 sec at 18°C (○). At later times $InsP_3$ levels were comparable at both temperatures. Procedures and conditions were as described under Materials and Methods.

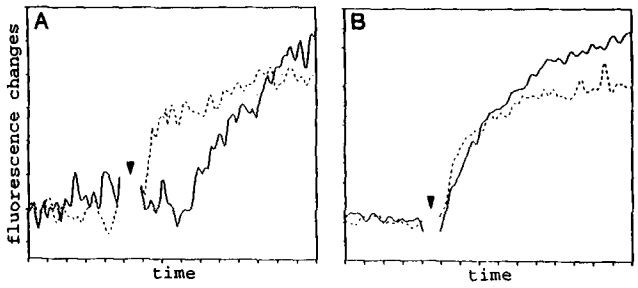

Figure 7. Kinetics of $InsP_3$-induced Ca^{2+} release responses from PC12 microsomes. The traces show the time-course of fura-2 (free acid) signal in microsome suspensions treated with 2 (*A*) or 10 (*B*) μM InsP3 (*arrowheads*) at either 37°C (*dashed lines*) or 18°C (*solid lines*). Fluorescence changes (in ordinate) are given as arbitrary units, and timing (in abscissa) by ticks drawn 1-s apart. Traces shown are typical of results obtained with four independent preparations. See Materials and Methods for details of Ca^{2+} uptake conditions and fluorescence value normalization.

It has been suggested that $InsP_3$ production can be locally stimulated by $[Ca^{2+}]_i$ elevation, independently from receptor activation, with possible implications for both generation and propagation of Ca^{2+} waves (Meyer, 1991). To verify whether this mechanism was operative in PC12 cells, the accumulation of InsPs was studied at 37° and 18°C and compared to the $[Ca^{2+}]_i$ variations obtained at 37°C (those at 18°C exhibited slower kinetics but reached similar maximal values) in different experimental conditions. When the three receptor agonists—BK, CCh, and ATP—were administered together in the Ca^{2+}-free medium, InsPs accumulation doubled and the $[Ca^{2+}]_i$ response reached high values, yet it remained transient (Fig. 8, lane *A*). These results were compared with those obtained using ionomycin, a Ca^{2+} ionophore. Fig. 8 (lane *B*) shows that when ionomycin (5 μM) was administered in a Ca^{2+}-free medium, thus directly depleting internal stores, it induced a $[Ca^{2+}]_i$ response analogous to that elicited by receptor activation, but no significant generation of InsPs. When the experiments were carried out in Ca^{2+}-containing medium, the $[Ca^{2+}]_i$ response to 1 μM ionomycin was considerable, yet InsPs accumulation was not raised over basal values (Fig. 8 *C*). Only when ionomycin concentration was increased to 5 μM (in complete KRH) the $[Ca^{2+}]_i$ response became maximal and sustained and was accompanied by a significant elevation of InsPs (Fig. 8 *D*). Each experiment was performed at both 18° and 37°C and the results were essentially the same, except for the 5 μM ionomycin experimental condition, at which InsPs accumulation was significantly reduced at the lower temperature (Fig. 8 *D*).

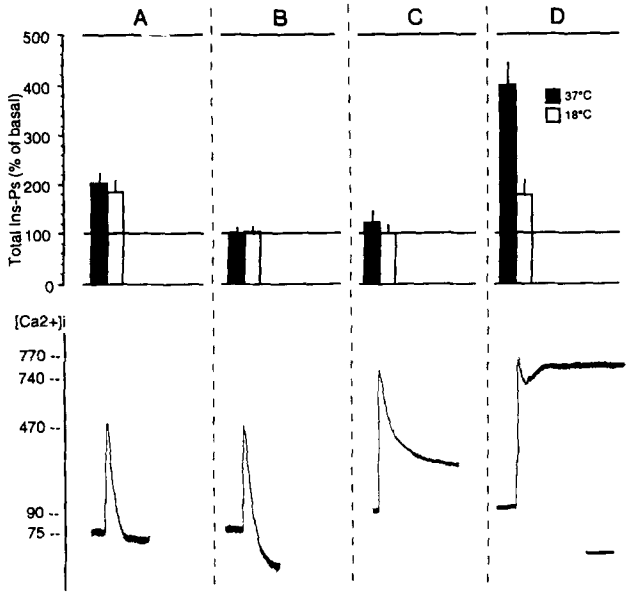

Figure 8. Ca^{2+}-activated phospholipase C assayed in different experimental conditions. Total InsPs production(% of basal ± SD) in PC12 cells (stimulated at either 37° or 18°C, ■ and □, respectively) is shown in the upper panels; the corresponding $[Ca^{2+}]_i$ responses, representative of at least three experiments carried out at 37°C and expressed in nM are illustrated in the bottom panels (time bar = 1 min). Cells were stimulated as follows: (*A*) combination of 500 μM CCh, 100 nM BK, and 100 μM ATP in Ca^{2+}-free medium; (*B*) 5 μM ionomycin in Ca^{2+}-free medium; (*C*) 1 μM ionomycin in complete KRH; and (*D*) 5 μM ionomycin in complete KRH. Procedures and conditions were as described under Materials and Methods.

Discussion

The fundamental observation of this work is the appearance of nondecremental Ca^{2+} waves of constant speed running along the neurites of PC12 cells differentiated by pretreatment with NGF and incubated in Ca^{2+}-free medium. Processes of this kind were not reported in spite of previous extensive videomicroscopy studies carried out by us and others in both PC12 (Grohovaz et al., 1991; Reber and Reuter, 1991) and neurons (Lipscombe et al., 1988; Silver et al., 1990). Only during the preparation of this article, preliminary results have appeared suggesting in neurons (of earth worm and Xenopus) the occurrence of waves which however, in contrast to those herewith described, largely depend on the influx of extracellular Ca^{2+} (Gu et al., 1994; Ogawa et al., 1994). Our present results provide evidence that at 37°C (the temperature employed in previous studies), the events induced by BK were so fast that they could be appreciated only when the imaging apparatus was operated at rates much higher than those used previously. When the temperature was decreased (a procedure widely used experimentally, especially in electrophysiology) the dynamic events induced by BK (and also by other receptor agonists) were revealed as bona fide waves, sustained by the autoregenerative release of Ca^{2+} from intracellular stores and requiring, for their generation, the activation of PPI-hydrolysis-coupled receptors, no matter whether the B_2 (Fasolato et al., 1988) or the P_{2u} (Lustig et al., 1993; otherwise referred to as P_{2y2}, Abbracchio and Burnstock, 1994) or the M_3 (Michel et al., 1989) receptors. In most cases the waves we observed appeared as tides. Only in experiments carried out with ATP they sometimes appeared like moving bands (as defined by Dupont and Goldbeter, 1992), with no sharp boundaries at their edges. This could reflect the concomitant activation in PC12 cells of two types of ATP-sensitive receptors, not only the P_{2u}, but also the P_{2w} (Fasolato et al., 1990) which is expressed only in a fraction of the cells (Zacchetti, D., unpublished observation). Since the P_{2w} receptor is a Ca^{2+}-permeable channel, it could favor the rapid

disposal of the cytosolic Ca^{2+} into the extracellular, EGTA-containing medium, and thus modify the kinetics of the waves triggered by P_{2u} receptor activation.

In the soma $[Ca^{2+}]_i$ increased most often according to a foot-upstroke pattern, no matter whether this developed as the primary response after application of ATP or as the secondary response following the invasion by the waves triggered by BK in the thin neurites. At present, the possibility cannot be excluded that waves did indeed develop also in the PC12 soma but were not appreciated by conventional fluorescence microscopy (see Lechleiter et al., 1991; and Lipp and Niggly, 1993). However, the peculiar kinetics of the $[Ca^{2+}]_i$ responses (see also Jacob et al., 1988; Blatter and Weir, 1992; Iino et al., 1993) suggests in the soma the occurrence of a threshold-triggered process.

When the cells bathed in the Ca^{2+}-containing KRH medium were depolarized with high K^+, $[Ca^{2+}]_i$ rose often considerably in the neurites (as expected because of the preferential localization of voltage-gated Ca^{2+} channels), however waves were not generated. Exposure of the same cells to BK induced similarly localized initial $[Ca^{2+}]_i$ rises which however evolved as waves. Thus, a localized $[Ca^{2+}]_i$ rise is insufficient to trigger the autoregenerative process unless accompanied by receptor activation. This conclusion is further confirmed by our results with ionomycin, that exclude wave propagation to be sustained by a Ca^{2+}-dependent, positive feedback on phospholipase C activity (Eberhard and Holz, 1988; Meyer and Stryer, 1991). On the other hand, the positive results obtained with the clone No. 64, which is devoid of the caffeine/ryanodine receptor (Zacchetti et al., 1991), exclude the latter to be necessarily involved. From these data we conclude that the waves in PC12 neurites can be triggered only when: (*a*) an appropriate PPI hydrolysis-coupled receptor is activated; and (*b*) the ensuing release of $InsP_3$ leads to the activation of the intracellular cognate receptors.

Consistent with the above conclusions is also the most unexpected result we obtained, i.e., the bidirectional orientation of the waves, either towards or away from the soma depending on the agonist used, observed even in the very same cell. In our opinion a result of this type can only be due to a differential distribution of the corresponding surface receptors, and therefore of the initial $InsP_3$-mediated Ca^{2+} release responses. Additional evidence in favor of the involvement of $InsP_3$ can be deduced from the comparison of the imaging results with those obtained by biochemical analysis of both intact cells and isolated microsomes, the subcellular fraction where most of the $InsP_3$-sensitive Ca^{2+} stores are recovered. In particular, the delay of the wave insurgency at 18°C appears to correlate well with the delay of the $InsP_3$ accumulation observed in the cells exposed to receptor agonists. Moreover, the delay and slowing down of Ca^{2+} release observed when isolated microsomes were directly treated with the second messenger at 18°C (see also Shuttleworth and Thompson, 1991) could contribute significantly to the slowing down of the wave propagation if we assume that Ca^{2+} waves are due to the sequential release from $InsP_3$-sensitive stores (DeLisle and Welsh, 1992; Miyazaki et al., 1992; Iino and Endo, 1992; Iino et al., 1993; Lechleiter and Clapham, 1992) located along the neurites.

Taken together, our experimental evidence indicates that, in order to sustain propagation of the waves along the neurites, $InsP_3$ and Ca^{2+} need to operate coordinately. The $InsP_3$ may first act by priming its receptors which however become activated only when the diffusing calcium ions induce $[Ca^{2+}]_i$ rises to levels (up to 250 nM) known to positively modulate its function (Iino, 1990; Finch et al., 1991; Bezprozvanny et al., 1991; Parys et al., 1992). The mechanistic phenomena that sustain waves so far discussed appear to be largely the same as those that sustain $[Ca^{2+}]_i$ oscillations. Oscillations however differ from waves inasmuch as they rhythmically initiate at a specialized region of the cytoplasm, the pacemaker, which is believed to be particularly sensitive to $InsP_3$ and $[Ca^{2+}]_i$ variations (D'Andrea et al., 1993; Kasai et al., 1993).

In conclusion, our results demonstrate that in PC12 cells differentiated by treatment with NGF, Ca^{2+} waves can run bidirectionally, depending on the stimulus along thin neurites. The conditions for the generation and propagation of these waves that can be hypothesized based on the indirect evidence we have obtained are: (*a*) the differential distribution on the cell surface of the receptors that initiate the signal transduction cascade; and (*b*) the widespread distribution along the neurites of the $InsP_3$-sensitive Ca^{2+} stores.

PC12, a line originated from a rat pheochromocytoma, represents one of the best known neuronal model. The possibility that at least some neurons express waves of the type identified here in PC12 should therefore be kept into consideration. Because of the key role of Ca^{2+} in cell regulation, waves could be involved not only in the control of functions common to other cell types, but also in nerve cell–specific functions, both at short term (e.g., the differential release of clear and dense-core vesicles; Verhage et al., 1991) and long term (e.g., neurite growth; Silver et al., 1989; Mattson et al., 1990; Davenport and Kater, 1992). Even more important, via the functional interaction with the plasma membrane, the waves could contribute to the overall excitability and differential processing of inputs, thus playing a dynamic role in the electrochemical computation that nerve cells carry over.

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