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DEPENDENCE OF CYTOSOLIC CALCIUM IN DIFFERENTIATING RAT PHEOCHROMOCYTOMA CELLS ON CALCIUM CHANNELS AND INTRACELLULAR STORES

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SUMMARY

1. The rat clonal pheochromocytoma cell line (PC12) was used to study changes in the free intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) that are related to the distribution of L-type (dihydropyridine-sensitive) and N-type (ω-conotoxin-sensitive) calcium channels during nerve growth factor (NGF)-induced outgrowth of neurites. Changes in $[Ca^{2+}]_i$ during K^+ depolarization were recorded by means of Fura-2 single-cell microfluorimetry.

2. The basal $[Ca^{2+}]_i$ of cells at rest was not altered by long-term treatment with NGF, neither in the cell bodies nor in the growth cones. K^+ depolarization of the cells caused a rise in $[Ca^{2+}]_i$.

3. The dihydropyridine (DHP) nifedipine alone, or together with ω-conotoxin (ω-CgTX), were similarly effective in inhibiting the K^+-induced increase in $[Ca^{2+}]_i$ in untreated and NGF-treated cell bodies, arguing for a preferential distribution of L-type Ca^{2+} channels in this cell area. By contrast, after 6–7 days exposure to NGF the K^+-induced initial transient rise of $[Ca^{2+}]_i$ in growth cones was very sensitive to ω-CgTX, whereas nifedipine affected only the sustained rise.

4. PC12 cells also contain caffeine- and inositol trisphosphate (IP_3)-sensitive intracellular Ca^{2+} stores. Addition of 30 mM-caffeine caused a fast transient rise in $[Ca^{2+}]_i$. The extent of filling of the caffeine-sensitive pool affected basal $[Ca^{2+}]_i$. These Ca^{2+} storage sites were empty under normal culture conditions. However, a single K^+ depolarization caused filling of the stores, followed by spontaneous depletion (50% in about 5 min) after wash-out of high $[K^+]_o$. When the caffeine-sensitive stores were empty, the rise in $[Ca^{2+}]_i$ was attenuated during submaximal depolarization. Caffeine-sensitive Ca^{2+} stores were also present in some growth cones, though with much smaller capacities than in cell bodies.

5. Mobilization of Ca^{2+} from the IP_3-sensitive store, by bradykinin exposure, was found to be independent of the caffeine-sensitive pool. There was no apparent 'cross-talk' between both Ca^{2+} pools.

6. We conclude that changes in $[Ca^{2+}]_i$ in cell bodies depend on both membrane Ca^{2+} channels and intracellular Ca^{2+} stores. During NGF-induced differentiation

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there is a predominance of N-type Ca^{2+} channels in growth cones, while Ca^{2+} stores are of minor importance in these structures.

INTRODUCTION

In neurons, as in other excitable cells, free intracellular calcium concentration ($[Ca^{2+}]_i$) is regulated by the concerted action of membrane Ca^{2+} channels, intracellular Ca^{2+} stores, Ca^{2+} binding proteins and Ca^{2+} extrusion systems (Carafoli, 1987; Blaustein, 1988; Miller, 1988). The relationship between Ca^{2+} entry across the plasma membrane and intracellular Ca^{2+} stores of neurons is only poorly understood. Ca^{2+} entry occurs primarily through voltage-gated channels in the plasma membrane (Miller, 1987; Tsien, Lipscombe, Madison, Bley & Fox, 1988), while intracellular Ca^{2+} stores are thought to serve as Ca^{2+} buffers. Recently it was shown that caffeine-sensitive Ca^{2+} stores play an important role in amplifying or attenuating the effects of Ca^{2+} influx in frog sympathetic neurons (Lipscombe, Madison, Poenie, Reuter, Tsien & Tsien, 1988*a*, *b*; Thayer, Perney & Miller, 1988*b*).

The clonal cell line (PC12) derived from rat pheochromocytoma has become an important system for studying many aspects of neuronal differentiation and function. They respond to nerve growth factor (NGF) by acquiring neuronal characteristics (Greene & Tischler, 1976). They have been used in studies of second messenger pathways (Gatti, Madeddu, Pandiella, Pozzan & Meldolesi, 1988; Meldolesi, Volpe & Pozzan, 1988), of responses to bradykinin (Fasolato, Pandiella, Meldolesi & Pozzan, 1988; Appell & Barefoot, 1989) and of neurotransmitter release (Kongsamut & Miller, 1986; Reber, Porzig, Becker & Reuter, 1990). In addition, much effort has been devoted to the characterization of the expression of Ca^{2+} channels in this cell line (Streit & Lux, 1987, 1989; Plummer, Logothetis & Hess, 1989; Garber, Hoshi & Aldrich, 1989; Usowicz, Porzig, Becker & Reuter, 1990).

In this work, we report experiments designed to study Ca^{2+} influx and Ca^{2+} release from intracellular stores during NGF-induced differentiation of PC12 cells by using the Fura-2 microfluorimetry technique in combination with a video-imaging system (Grynkiewicz, Poenie & Tsien, 1985). This approach allowed us to analyse and compare changes in $[Ca^{2+}]_i$ in different localized areas of single cells. We used pharmacological tools to differentiate between various types of Ca^{2+} channels and intracellular Ca^{2+} release sites. Electrophysiological experiments had previously shown that some PC12 cell clones preferentially express N-type over L-type Ca^{2+} channels during NGF-induced differentiation (Plummer *et al.* 1989; Usowicz *et al.* 1990). Our results suggest that these N-type channels are predominantly inserted into growth cones. Furthermore, we studied the interaction between Ca^{2+} influx through these voltage-gated Ca^{2+} channels and intracellular Ca^{2+} stores. We found caffeine- and inositol trisphosphate (IP_3)-sensitive Ca^{2+} stores which do not seem to interact. Ca^{2+} influx may be amplified by Ca^{2+} release from the caffeine-sensitive pool. Ca^{2+} stores are more important in cell bodies than in growth cones.

METHODS

Tissue culture. A PC12 cell clone that acquires neuronal characteristics within 4 days of NGF treatment was kindly provided by Dr U. Otten, University of Basle, Switzerland. Cells were plated

at a density of 5000 cells/cm^2 on poly-L-lysine-coated cover-slips placed in a Petri dish (diameter 8·7 cm). The cells were incubated in Dulbecco's modified Eagle minimal essential medium (high-glucose DMEM) supplemented with 5% fetal calf serum (FCS) and 10% horse serum (HS). The medium also contained (per ml) 250 ng amphothericin B, 100 U penicillin and 100 μg streptomycin. Morphological differentiation of PC12 cells was induced by reducing the serum concentration to 5% HS and by adding NGF (7S fraction, 100 ng/ml). Medium changes and NGF addition were repeated every other day.

$[Ca^{2+}]_i$ measurements. Cytosolic free Ca^{2+} was measured with the Ca^{2+}-sensitive fluorescent dye Fura-2 (Grynkiewicz *et al.* 1985). Cover-slips with cells were glued to a hole in a Petri dish by means of Vaseline. The cells were kept in a solution consisting of 140 mM-NaCl, 5 mM-KCl, 1·5 mM-$MgCl_2$, 2 mM-$CaCl_2$ and 10 mM-HEPES–NaOH (pH 7·4) and were loaded with dye by incubation in 1 μM-Fura-2 acetoxymethylester in dimethyl sulphoxide (DMSO; 0·1%) for 15 min at 37 °C. This length of time was sufficient to obtain a homogeneous dye loading. On the other hand, prolonged incubation at this temperature led to accumulation of the dye in subcellular compartments followed by a complete loss of the dye, probably due to cell damage.

Changes in the fluorescence intensity of Fura-2 were recorded with a video-imaging system consisting of an inverted microscope equipped with epifluorescence optics (Diaphot-TMD, Nikon) and an image intensifier attached to a video camera (Videoscope International, Washington, DC). Images were digitized at 8-bit resolution and stored as pictures of 320 × 256 pixels in a video-frame processor (Leutron AG, Glattbrugg, Switzerland). The video-frame processor was controlled for recording and analysis of data via an interface connected to a COMPAQ 386/25 MHz computer. The excitation light was provided by two high-pressure mercury lamps whose light beams were passed through a 340 or 380 nm filter, respectively. The 380 nm light was reflected by 90 deg into the exciting lightpath by means of a mirror prism. A computer-controlled shutter mechanism (Vincent Associates, Inc., Rochester, NY, USA) allowed switching between the two excitation wavelengths. The wavelengths for emission fluorescence were selected by a 520–560 nm interference filter. Calibration of Fura-2 fluorescence in terms of $[Ca^{2+}]_i$ was calculated from the ratio of 340/380 nm excitation fluorescence values, assuming a dissociation constant (K_d) for the Fura-2–Ca^{2+} complex of 224 nM (Grynkiewicz *et al.* 1985). The hardware configuration of the video-frame processor was programmed for storage of a maximum of forty recordings at both excitation wavelengths (two-image acquisition mode). These data were used for calculation and display of video images representing $[Ca^{2+}]_i$ in false colour mode. The logarithm of the calculated calcium concentrations between 0·01 and 1 μM was correlated with pixel intensity values between 0 and 255. Pixel intensities were represented by colours. Alternatively, the intensities of the recorded fluorescence signals at the two excitation wavelengths were read directly from defined areas of the cell depicted on the videoscreen (on-line mode). The $[Ca^{2+}]_i$ values were calculated from the equation (Grynkiewicz *et al.* 1985):

$$[Ca^{2+}]_i = \frac{(R - R_{min})\, F^{380}_{free}}{(R_{max} - R)\, F^{380}_{sat}} K_d,$$

where R_{min} and R_{max} are the respective minimal and maximal fluorescence ratios, and $F^{380}_{free}/F^{380}_{sat}$ is the fluorescence ratio of a Ca^{2+}-free and Ca^{2+}-saturated solution measured at 380 nm wavelength. They were determined, after adequate adjustments of the set-up, from the fluorescence signal of Ca^{2+}-free or Ca^{2+}-saturated salt solution droplets (1 μM-Fura-2, 140 mM-KCl, 10 mM-HEPES–NaOH, pH 7·4, 10 mM-EGTA or 10 mM-$CaCl_2$, respectively) which were dispersed in *n*-octanol (10–20 μm in diameter). Autofluorescence from unloaded cells was not detectable under our conditions.

Cells were kept at room temperature in a Petri dish filled with 2 ml of medium. Depolarization-induced Ca^{2+} influx into the cells was produced by exchanging the normal medium with solutions containing K^+ concentrations between 40 and 210 mM. Sodium chloride was isosmotically replaced by KCl up to 140 mM. In order to avoid cell damage, the high-K^+ solution was added by means of a pipette at the rim of the Petri dish about 1·5 cm apart from the cell under investigation. Optimal mixing was achieved by four times suction and ejection of the solution. Caffeine was added from a 90 mM stock solution prepared in the actual bathing medium. All other drugs were pipetted into the bathing solution at appropriate concentrations. Whenever possible, data are presented as means ± S.E.M.

Patch clamp experiments. Whole-cell Ba^{2+} currents through Ca^{2+} channels were measured in essentially the same way as described by Usowicz *et al.* (1990).

Materials. Tissue culture reagents were purchased from Gibco Corp., Basel, Switzerland or from Boehringer Mannheim Corp., Rotkreuz, Switzerland. Fura-2 pentapotassium salt and Fura-2 acetoxymethyl ester (Fura-2/AM) were obtained from Molecular Probes Inc., Eugene, OR, USA. Nifedipine was a gift from Professor Hoffmeister, Bayer AG, Leverkusen, FRG. ω-Conotoxin GVIA (ω-CgTX) was purchased from Peninsula Laboratories, Belmont, CA, USA. All other chemicals were obtained from Fluka AG, Buchs, Switzerland.

RESULTS

The goal of this work was to investigate functional parameters that are involved in the calcium homeostasis of PC12 cells before and after NGF-induced differentiation. PC12 cells showed a clear response to the addition of NGF in the culture medium. Morphological differentiation of the cells occurred within 4–7 days as judged by the stop of cell division, enlargement of cell bodies, neurite extension and the presence of growth cones (Usowicz *et al.* 1990). During NGF-induced differentiation, free intracellular calcium concentration ($[Ca^{2+}]_i$) was measured with Fura-2 in cell bodies and growth cones. No significant differences in $[Ca^{2+}]_i$ were observed in cell bodies before (72 ± 3 nM, $n = 19$) and after (78 ± 5 nM, $n = 15$) 6–7 days of exposure to NGF, or in the newly formed growth cones (82 ± 6 nM, $n = 6$) after NGF-induced differentiation.

Transient rises of $[Ca^{2+}]_i$

The presence of voltage-dependent L- and N-type Ca^{2+} channels in PC12 cells has recently been described in detail (Plummer *et al.* 1989; Usowicz *et al.* 1990). Therefore, we investigated changes in $[Ca^{2+}]_i$ during membrane depolarization by means of elevating the external K^+ concentration ($[K^+]_o$). Figure 1 shows the rise of $[Ca^{2+}]_i$ during K^+ depolarization in the cell body and in the growth cone. With increasing $[K^+]_o$ in the range of 20–75 mM the time-lag between medium change and the beginning of the rise in $[Ca^{2+}]_i$ became shorter. $[K^+]_o$ above 75 mM reduced the peak response, while leaving the time-lag constant (data not shown). A significant difference in the average peak response to 75 mM $[K^+]_o$ was observed between undifferentiated (442 ± 5 nM, $n = 24$) and NGF-differentiated cells (662 ± 15 nM, $n = 19$). After 6–7 days of exposure to NGF maximum $[Ca^{2+}]_i$ in growth cones during K^+ depolarization reached values (602 ± 21 nM, $n = 11$) similar to those in differentiated cell bodies. Figure 1*B* shows a plot of the standardized $[Ca^{2+}]_i$ against the $[K^+]_o$ in the bathing medium. A sigmoidal curve was obtained with a half-maximal response at 43 ± 5 mM $[K^+]_o$ ($n = 4$) in differentiated cell bodies and growth cones. Assuming an intracellular $[K^+]$ of 110 mM, this would correspond to a membrane potential of −24 mV as calculated by the Nernst equation. Experiments with growth cones were more difficult than those with cell bodies, probably due to their more fragile structures and irreversible damage at high $[Ca^{2+}]_i$.

Effects of Ca^{2+} *channel blockers*

The transient elevation of $[Ca^{2+}]_i$ during K^+ depolarization can be separated into four phases: (1) an initial basal level, (2) a rise to a transient maximum, (3) a decrease to an elevated steady-state level, and (4) a new resting level after

repolarization. We expected that influx of Ca^{2+} through voltage-gated channels contributes to phases 2 and 3, since absence of extracellular Ca^{2+} prevented the rise of $[Ca^{2+}]_i$ during K^+ depolarization. In order to investigate this possibility further, we analysed the effects of the L- and N-type Ca^{2+} channel blockers nifedipine and

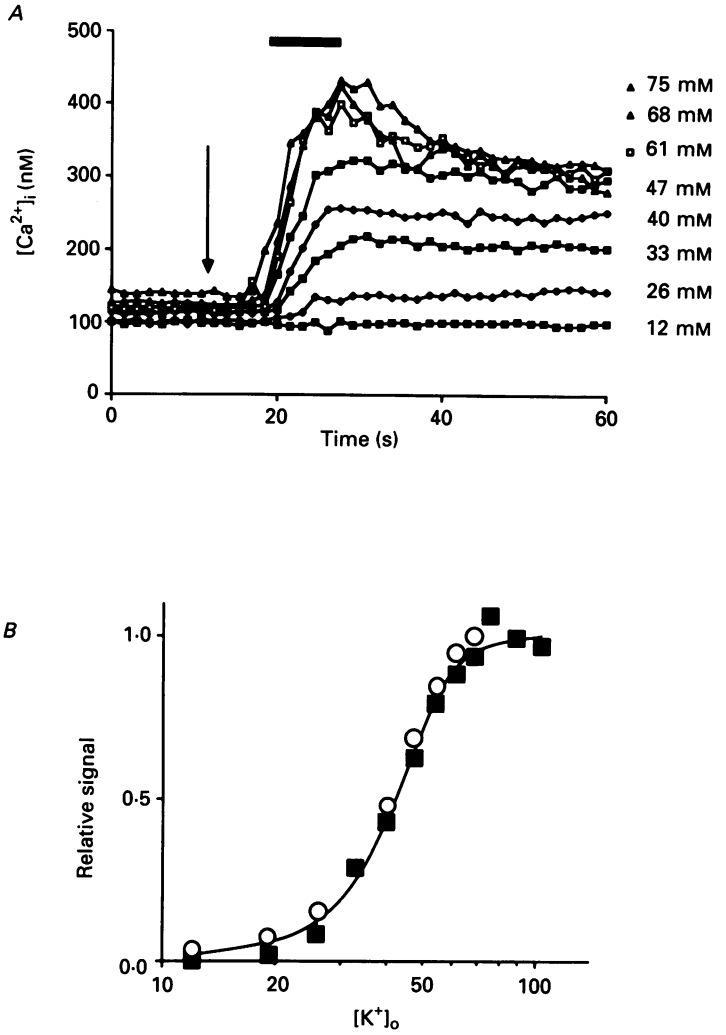

Fig. 1. Relationship between $[Ca^{2+}]_i$ and $[K^{z+}]_o$ in differentiated PC12 cells. *A*, changes in $[Ca^{2+}]_i$ were measured as described in Methods. The same cell was consecutively exposed to increasing $[K^+]_o$ between 12 and 75 mM. The cell was washed with low-K^+ medium (5 mM) and kept at rest for at least 2 min between two depolarizations. *B*, normalized response of $[Ca^{2+}]_i$ in the cell body and the growth cone of two separate cells. Data from Fig. 1*A* were analysed by calculating the area under the curves as marked by the horizontal bar. Shown are values of the cell body (■) normalized to the response at 75 mM $[K^+]_o$. Data obtained from a growth cone (○) in an independent experiment are superimposed. Two other experiments gave similar results.

ω-CgTX on the K^+-induced rise of $[Ca^{2+}]_i$ in the cell bodies before and after NGF treatment. Averaged traces of recordings from several undifferentiated (*A*) and differentiated (*B*) cells in the presence and absence of the blockers are shown in Fig. 2. Nifedipine (10 μM) caused similar inhibition of the initial rise of $[Ca^{2+}]_i$ in the cell

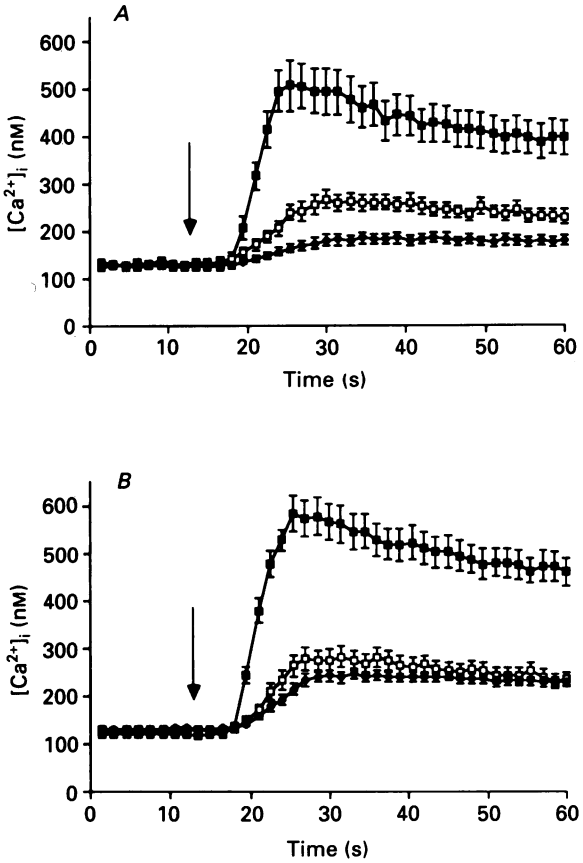

Fig. 2. Effects of nifedipine and ω-CgTX on $[Ca^{2+}]_i$ transients evoked by K^+ depolarization in cell bodies. $[Ca^{2+}]_i$ was measured in the two-image acquisition mode. Cells were depolarized by elevating $[K^+]_o$ to 70 mM (arrow). Results were combined from six undifferentiated cells (*A*, 0 day NGF) and fourteen differentiated cell bodies (*B*, 6 day NGF). Shown are the calculated mean traces $\pm$S.E.M. of changes in $[Ca^{2+}]_i$: controls (■); 10 μM-nifedipine (□): 10 μM-nifedipine plus 500 nM-ω-CgTX (◆). Pre-incubation with the Ca^{2+} channel blockers lasted for 5 min. Cells were kept at 5 mM $[K^+]_o$ for 2–5 min between two consecutive depolarizations.

body before and after NGF treatment (64 and 65%, respectively). The additional presence of 500 nM-ω-CgTX reduced the response further by 19 and 11%, respectively. However, despite the presence of both drugs at saturating concentrations (Usowicz *et al.* 1990), elevation of $[Ca^{2+}]_i$ was not blocked completely.

Prolonged K^+ depolarization caused, after the initial peak, a persistent elevation of $[Ca^{2+}]_i$ (phase 3). Therefore, the question arose, whether Ca^{2+} influx through the

plasma membrane continued under these conditions. Figure 3*A* shows that, after 3 min K^+ depolarization, a reduction of $[K^+]_o$ to 5 mM lowered $[Ca^{2+}]_i$ within 10 s. Similarly, nifedipine in the presence of high $[K^+]_o$ caused a rapid fall of $[Ca^{2+}]_i$ (Fig. 3*B*). However, such an effect could not be obtained with ω-CgTX (Fig. 3*C*). This

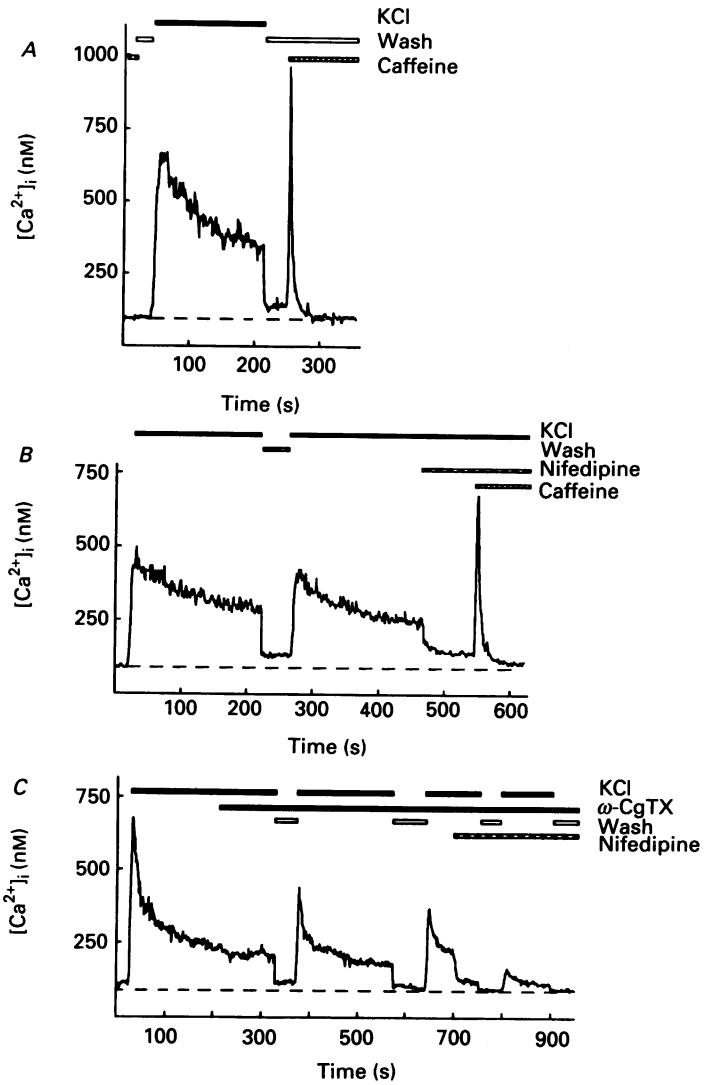

Fig. 3. Effects of caffeine and Ca^{2+} channel blockers on $[Ca^{2+}]_i$ in undifferentiated cell bodies at two different $[K^+]_o$ (5 and 75 mM). $[Ca^{2+}]_i$ was measured with the on-line mode at 1·4 Hz. The time axis represents actual recording time. The horizontal bars above each trace indicate the time period during which external conditions were changed. *A*, effect of 30 mM-caffeine on $[Ca^{2+}]_i$ before and after a first challenge with high $[K^+]_o$; *B*, effect of 10 μM-nifedipine and 30 mM-caffeine during the late phase of K^+ depolarization; *C*, effect of 500 nM-ω-CgTX and 10 μM-nifedipine during K^+ depolarization.

argues for a maintained Ca^{2+} influx through L-type Ca^{2+} channels during phase 3. ω-CgTX reduced the initial peak (phase 2) of $[Ca^{2+}]_i$ during K^+ depolarization (Fig. 3*C*). This indicates that N-type Ca^{2+} channels are more involved in the initial rise in $[Ca^{2+}]_i$ than in its steady-state level during maintained depolarization. The level of

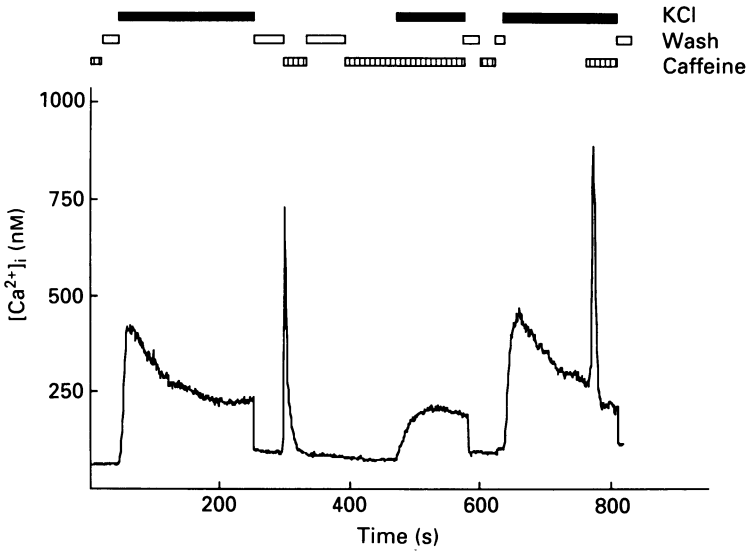

Fig. 4. Effect of caffeine on Ca^{2+} mobilization, Ca^{2+} influx and $[Ca^{2+}]_i$ during K^+ depolarization. Changes in $[Ca^{2+}]_i$ were measured with the on-line method at 1·4 Hz. The time axis represents actual recording time. Recording was stopped during part of the wash-out periods. Changes in the external conditions are marked by the horizontal bars. Cells were exposed to either 30 mM-caffeine and/or 70 mM-KCl. In order to mobilize Ca^{2+} from internal stores by caffeine, the freshly cultured undifferentiated cells had to be depolarized at least once in the presence of external calcium (2 mM). The first and second challenges with caffeine were performed at low $[K^+]_o$ (5 mM), the third one at high $[K^+]_o$ (70 mM).

$[Ca^{2+}]_i$ after repolarization (phase 4) or after nifedipine exposure was slightly above the original (phase 1) resting $[Ca^{2+}]_i$ (Fig. 3*A* and *B*). Subsequent addition of caffeine (30 mM), which has been shown to mobilize Ca^{2+} from intracellular stores in peripheral neurons (Neering & McBurney, 1984; Lipscombe *et al.* 1988*b*), produced a fast transient rise in $[Ca^{2+}]_i$ after which the original basal level *before* K^+ depolarization was reached (Fig. 3*B* and *C*). This suggests that leakage of Ca^{2+} from filled caffeine-sensitive stores contributes to the basal level of $[Ca^{2+}]_i$. When the stores are filled basal $[Ca^{2+}]_i$ is higher than when they are depleted by caffeine.

Characterization of intracellular Ca^{2+} stores

Caffeine did not lead to mobilization of Ca^{2+} from internal stores of freshly cultured PC12 cells kept in normal bathing solution ($n = 10$). However, a single K^+ depolarization (> 10 s) was sufficient to condition, in both undifferentiated and NGF-differentiated cells, for a caffeine-induced Ca^{2+} release (Fig. 4). The presence of caffeine during K^+ depolarization prevented the filling of the stores. After return to low $[K^+]_o$, a second exposure to caffeine did not produce the usual $[Ca^{2+}]_i$ transient.

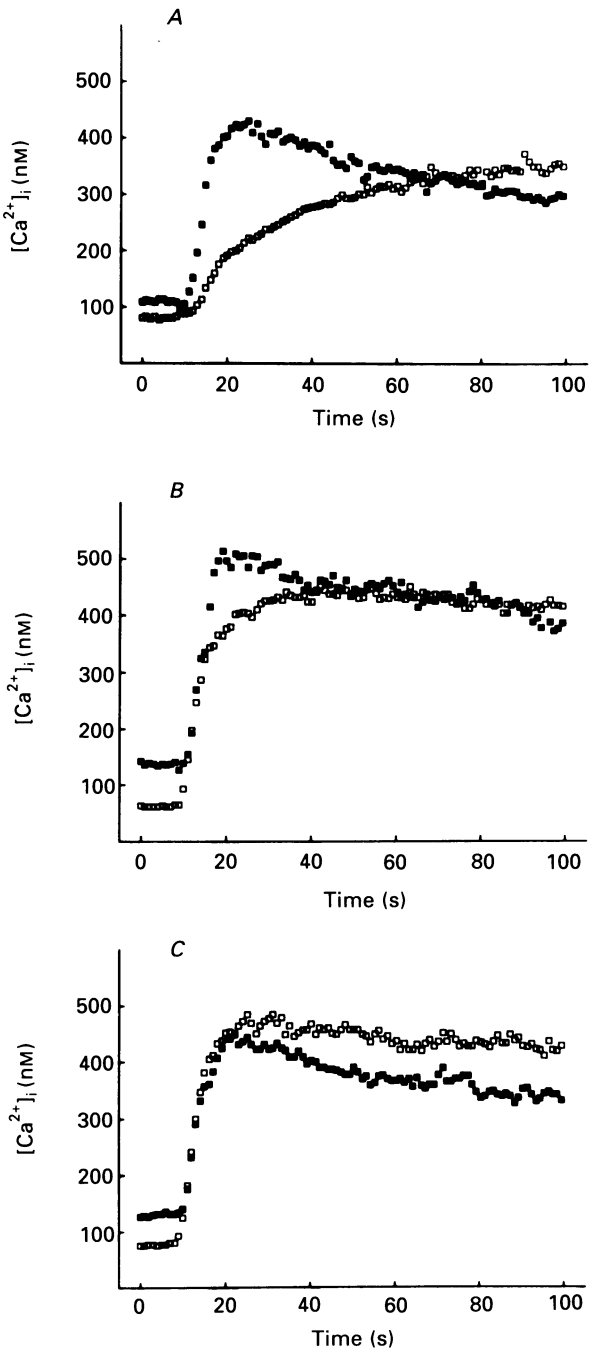

Fig. 5. The response of $[Ca^{2+}]_i$ towards elevated $[K^+]_o$ depends on the state of filling of the caffeine-sensitive Ca^{2+} stores. NGF-differentiated cells with emptied (□) or filled (■) stores were depolarized by elevating $[K^+]_o$ to 35 mM (*A*), 45 mM (*B*) and 70 mM (*C*). Changes in $[Ca^{2+}]_i$ were measured at 1·4 Hz. The caffeine-sensitive stores were filled by a single K^+ depolarization and emptied by challenge with 30 mM-caffeine followed by an

However, a single subsequent depolarization in the absence of caffeine was enough to fill the stores (Fig. 4). These recordings also show that the caffeine-induced Ca^{2+} release was membrane potential independent. The first release was initiated at low $[K^+]_o$, whereas the second one was elicited at high $[K^+]_o$. The amount of Ca^{2+} that could be released from caffeine-sensitive stores was related to the level of $[Ca^{2+}]_i$ achieved during the preceding K^+ depolarization. Small changes in $[Ca^{2+}]_i$ during moderate depolarizations (30 mM-KCl; see Fig. 1) caused less filling of the stores than large changes in $[Ca^{2+}]_i$ during strong depolarization.

Caffeine inhibited the K^+-induced rise of $[Ca^{2+}]_i$ when the caffeine-sensitive stores were depleted (Fig. 4). The reduction in peak $[Ca^{2+}]_i$ by caffeine was more pronounced in native cells ($66 \pm 15\%$, $n = 3$), than in cells after 6-day NGF treatment ($24 \pm 4\%$, $n = 3$). A possible explanation for this reduction is an inhibition of Ca^{2+} influx through voltage-gated Ca^{2+} channels by caffeine. However, electrophysiological measurements of whole-cell Ca^{2+} currents ($n = 14$) showed no noticeable effect of caffeine (30 mM), while Ba^{2+} currents through Ca^{2+} channels in undifferentiated cells revealed only a small ($13 \pm 4\%$; $n = 4$) inhibition. At present we have no satisfactory explanation for the inhibitory effect of caffeine.

Caffeine-sensitive stores contribute to the rise in $[Ca^{2+}]_i$ during K^+ depolarization. For example, an influx of Ca^{2+} during depolarization could be amplified by an additional release of Ca^{2+} from intracellular stores. If the stores were depleted by caffeine, K^+ depolarization produced a smaller increase in $[Ca^{2+}]_i$. We addressed the question of whether the caffeine-sensitive stores contribute to the overall rise in $[Ca^{2+}]_i$ during K^+ depolarization by a Ca^{2+}-induced Ca^{2+} release mechanism (Lipscombe *et al.* 1988*b*). We observed that the state of filling of the caffeine-sensitive stores influenced the rate of rise in $[Ca^{2+}]_i$ (Fig. 5). The rise was faster if the stores were filled prior to depolarization. This effect was membrane potential dependent and was less pronounced at higher $[K^+]_o$, presumably because stronger depolarization causes more voltage-gated Ca^{2+} channels to open. Such an effect would be consistent with Ca^{2+}-induced Ca^{2+} release, since during modest depolarization Ca^{2+} influx through Ca^{2+} channels contributes less to the overall rise in $[Ca^{2+}]_i$ than at stronger depolarization, while the contribution of intracellular stores is larger. Another argument in favour of Ca^{2+}-induced Ca^{2+} release is the fact that K^+ depolarization in the absence of $[Ca^{2+}]_o$ cannot release Ca^{2+} from filled caffeine-sensitive stores. Like in frog sympathetic ganglion cells (Lipscombe *et al.* 1988*b*) an influx of Ca^{2+} during K^+ depolarization is required for such a release. Another possible explanation for the effect shown in Fig. 5 is a hyperpolarization of the membrane due to opening of Ca^{2+}-activated K^+ channels (Fasolato *et al.* 1988) by caffeine-induced Ca^{2+} release. This could lead to a larger availability of Ca^{2+} channels to open and, therefore, to a larger Ca^{2+} influx during the subsequent K^+ depolarization. This explanation, however, is highly unlikely since bradykinin has the same effect on Ca^{2+}-activated K^+ channels, but does not potentiate changes in $[Ca^{2+}]_i$ during K^+ depolarization.

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intensive wash-out of the drug. Depending on the state of filling of the stores, basal $[Ca^{2+}]_i$ was below or above 100 nM. *A* and *B* were recorded from the same cell; *C* is taken from an independent experiment. Similar results have been seen in undifferentiated cells (data not shown).

Since filling of the caffeine-sensitive Ca^{2+} stores occurs during a single K^+ depolarization, the question arises, how fast the stores are depleted at rest. This question was explored in experiments of the kind illustrated in Fig. 6*A*. It shows caffeine-induced Ca^{2+} release in a cell after increasing time periods at rest (0·5–10 min)

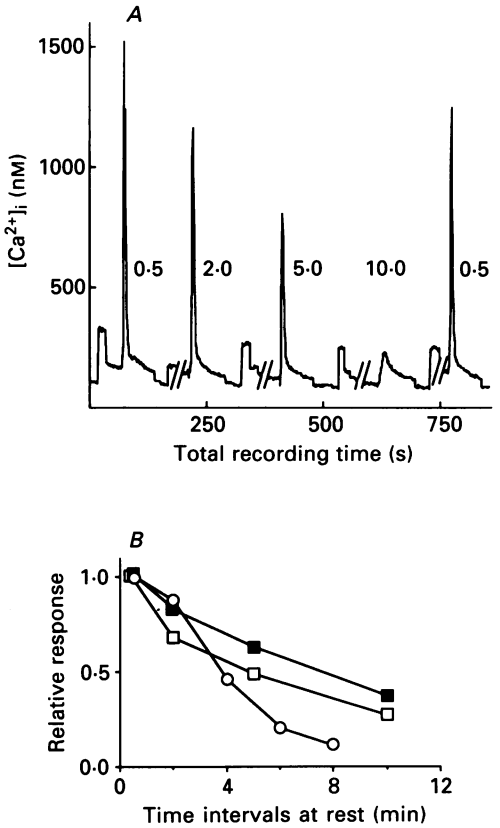

Fig. 6. Spontaneous depletion of the caffeine-sensitive Ca^{2+} stores of differentiated cells at rest. *A*, the decrease in the caffeine-induced $[Ca^{2+}]_i$ transients with increasing time-lags between wash-out of high $[K^+]_o$ and caffeine application. High $[K^+]_o$ (70 mM) was required to fill the stores before each caffeine exposure. Recording was stopped (interruption of traces) part of the time when high $[K^+]_o$ was added and subsequently washed out. The time-lags (0·5, 2·0, 5·0, 10·0 min) are indicated beside or above the response peaks. Basal $[Ca^{2+}]_i$ (160, 136, 116, 99 nM) decreased with prolonged time-lags. *B*, kinetics of spontaneous depletion of the Ca^{2+} stores. The net increases in $[Ca^{2+}]_i$ during 30 mM-caffeine addition were integrated in a time interval of 10 s. Values were normalized to the response after a time-lag of 0·5 min. Shown are the results from three different cells.

following 3 min K^+ depolarizations. In three such experiments the half-time of depletion of the stores was approximately 4–6 min (Fig. 6*B*).

Bradykinin is another substance that causes Ca^{2+} release from intracellular stores in PC12 cells (Fasolato *et al.* 1988; Appell & Barefoot, 1989). In the presence of this peptide inositol 1,4,5-trisphosphate (IP_3) accumulates in the cells and causes a release of Ca^{2+} (Fasolato *et al.* 1988; Appell & Barefoot, 1989). In addition,

bradykinin may also stimulate Ca^{2+} influx (Fasolato *et al.* 1988; Appell & Barefoot, 1989). We were interested in a possible interaction between caffeine- and IP_3-releasable Ca^{2+} pools. Bradykinin transiently increased $[Ca^{2+}]_i$ to 404 ± 60 nM in six out of twelve NGF-differentiated cells. Figure 7 shows a change in $[Ca^{2+}]_i$ during K^+

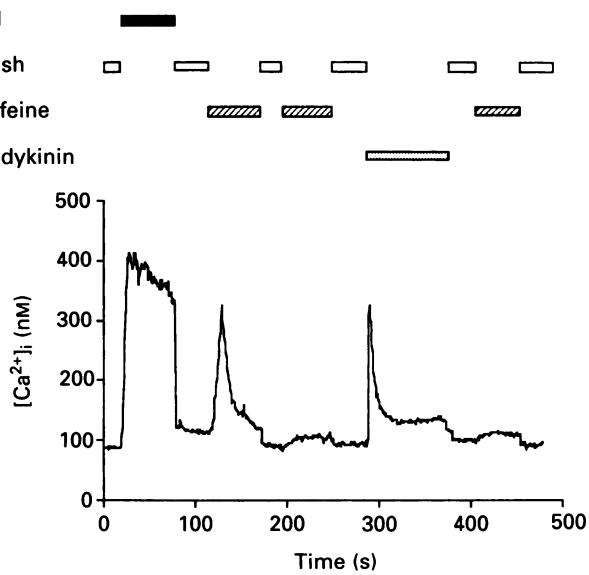

Fig. 7. Separation of bradykinin- and caffeine-sensitive Ca^{2+} stores. A 6 day NGF-treated cell was exposed to 75 mM $[K^+]_o$, 30 mM-caffeine or 500 nM-bradykinin as indicated. $[Ca^{2+}]_o$ was 2 mM during the whole experiment. Although caffeine evokes only a small rise in $[Ca^{2+}]_i$ during the second and third addition, a large response to bradykinin is still seen.

depolarization, followed by two caffeine exposures, the second of which was very small because the stores were largely depleted. Subsequent addition of bradykinin, however, caused a large transient rise followed by a sustained elevation of $[Ca^{2+}]_i$. Moreover, Ca^{2+} released after bradykinin application was not taken up into the caffeine-sensitive pool, since another exposure to caffeine had little effect (Fig. 7). This experiment indicates that the caffeine- and IP_3-releasable pools are separate and do not communicate. Caffeine had little effect on $[Ca^{2+}]_i$ if it was applied during the maintained phase of the bradykinin response. Absence of extracellular Ca^{2+} slightly reduced the size of the initial peak and eliminated the sustained phase of the change in $[Ca^{2+}]_i$ induced by bradykinin ($n = 3$). The latter effect is consistent with a small Ca^{2+} influx caused by bradykinin through undefined pathways (Fasolato *et al.* 1988). Electrophysiological measurements of whole-cell Ba^{2+} currents through voltage-dependent Ca^{2+} channels did not show any change in these current components by bradykinin ($n = 4$).

Ca^{2+} entry in growth cones

So far we have primarily presented results obtained from cell bodies. However, after 6–8 days exposure of the PC12 cells to NGF they had extended neurites that were several hundred micrometres long. Growth cones at the end of the processes

were viewed with 400–1000 times magnification. In addition to broad cones, finger-like processes could be seen (Aletta & Greene, 1988). Fura-2 fluorescence images were obtained from the main flat portion of the growth cone. Figure 8 illustrates simultaneous recordings of the Fura-2 signal in a body and a growth cone from two

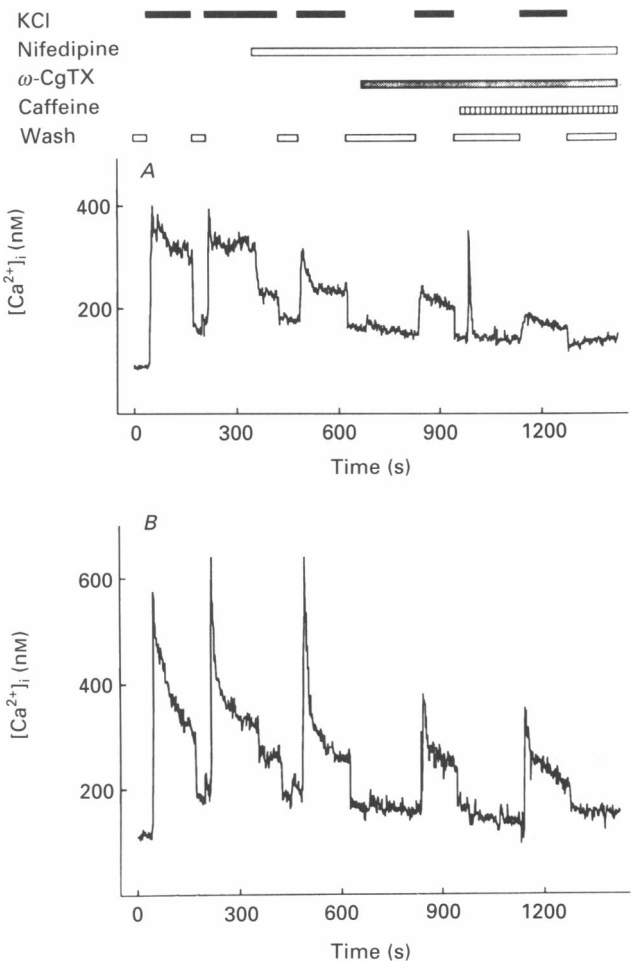

Fig. 8. Comparison of the actions of Ca^{2+} channel blockers on the cell body and growth cone of NGF-differentiated cells. Data were simultaneously recorded from a cell body (*A*) and a nearby growth cone (*B*) after 6 days of NGF treatment. Because of the length of the neurites at this stage of differentiation and the limited optical field of the video camera, the areas of interest had to be depicted from two separate cells, which were in close proximity on the video monitor. 70 mM $[K^+]_o$, 10 μM-nifedipine, 500 nM-ω-CgTX and 30 mM-caffeine were added to the external bathing solution as indicated by the bars. Recording was stopped during the wash cycles (0·5 min) and addition of ω-CgTX (5 min), but not during the application of nifedipine.

separate but neighbouring cells in the same dish. Unfortunately it was not possible to measure Ca^{2+} signals in the soma and growth cone of the same cell, because after 7 days of differentiation these structures were too far apart to visualize them

simultaneously. Therefore, well-developed growth cones which were closely approaching another cell body had to be used. However, in all experiments ($n = 5$) where simultaneous recordings from cell bodies and growth cones were done, the differences were always similar to those illustrated in Fig. 8. Resting levels of $[Ca^{2+}]_i$

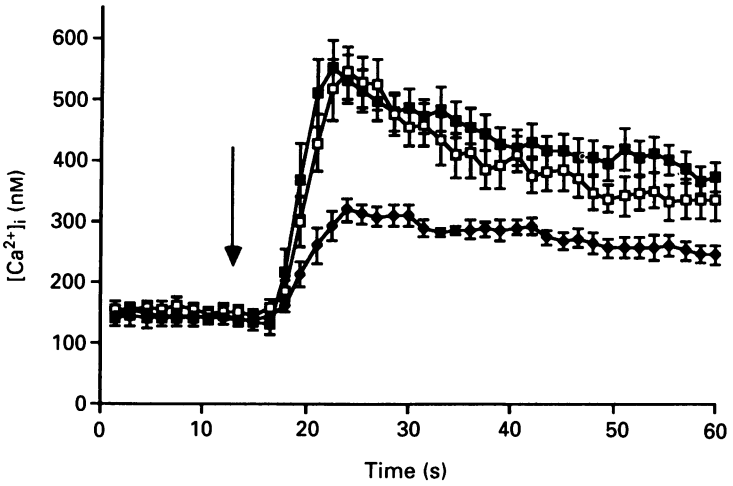

Fig. 9. Effects of nifedipine and ω-CgTX on $[Ca^{2+}]_i$ transients evoked by K^+ depolarization in growth cones. Cells were depolarized by elevating $[K^+]_o$ to 70 mM (arrow). Results were combined from seven growth cones after 6–7 days of treatment. Shown are the calculated mean traces ±S.E.M. of changes in $[Ca^{2+}]_i$; control (■), 10 μM-nifedipine (□); 10 μM-nifedipine plus 500 nM-ω-CgTX (◆). Incubation with the Ca^{2+} channel blockers lasted for 5 min. Cells were kept at 5 mM $[K^+]_o$ for 2 min between two consecutive depolarizations.

were similar in both structures. K^+ depolarization caused rapid changes in $[Ca^{2+}]_i$ with more pronounced initial peaks in the growth cone. At later times during K^+ depolarization, nifedipine (10 μM) reduced the steady elevation of $[Ca^{2+}]_i$ in both structures, while the initial peak response during the following depolarization was inhibited only in the cell body. By contrast, ω-CgTX had a stronger effect on the initial change in $[Ca^{2+}]_i$ during K^+ depolarization in the growth cone. Subsequent caffeine exposure led to a transient rise in $[Ca^{2+}]_i$ only in the cell body. In nine out of fourteen growth cones an increase in $[Ca^{2+}]_i$ after caffeine exposure could be observed which, however, was much smaller (280±40 nM; $n = 9$) than in cell bodies (720±75 nM; $n = 10$). These results indicate that caffeine-sensitive Ca^{2+} stores are much less developed in growth cones than in cell bodies. The same is true for bradykinin responses (data not shown).

Recent electrophysiological experiments have shown that N-type channels are preferentially expressed over L-type channels during NGF-induced differentiation of PC12 cells (Plummer *et al.* 1989; Usowicz *et al.* 1990). The data shown in Fig. 8 indicate that many of the N-type channels accumulate in the growth cones. This conclusion is substantiated further by the results shown in Fig. 9 (averaged traces from seven growth cones). Nifedipine (10 μM) had only a minimal (2%) effect on the peak change in $[Ca^{2+}]_i$ and a more pronounced effect on the later phase during K^+

depolarization. When ω-CgTX (500 nM) was added, the initial rise in $[Ca^{2+}]_i$ was inhibited by 42%. This is different from cell bodies where nifedipine caused a much larger inhibition than ω-CgTX (Fig. 2). At present we have no explanation for the residual component of K^+ depolarization-induced change in $[Ca^{2+}]_i$, after blockade of N- and L-type Ca^{2+} channels. However, addition of 1 μM-Cd^{2+} together with the other two Ca^{2+} channel blockers completely eliminated this component in two growth cones (data not shown).

DISCUSSION

In this study we have demonstrated that an elevation of $[Ca^{2+}]_i$ in PC12 cells can occur through several pathways. Two types of voltage gated Ca^{2+} channels in the plasma membrane, the L- and N-type channels, are responsible for Ca^{2+} influx into the cell during depolarization (Plummer *et al.* 1989; Usowicz *et al.* 1990). Both channel types can be separated by pharmacological tools, such as nifedipine and ω-CgTX. In addition, at least two types of intracellular Ca^{2+} stores are present in these cells. One store can be depleted by caffeine and the other by IP_3. This is similar to other neuronal cells (Thayer *et al.* 1988*b*; Burgoyne, Cheek, Morgan, O'Sullivan, Moreton, Berridge, Mata, Colyer, Lee & East, 1989).

We have shown that the caffeine-sensitive Ca^{2+} stores in PC12 cells are depleted with a half-time of about 5 min when the cells are at rest. They are rapidly replenished by Ca^{2+} influx during a single K^+ depolarization. The degree of filling and, hence, leakage of Ca^{2+} from these stores contributes to the resting level of the cytoplasmic $[Ca^{2+}]$. The caffeine-sensitive stores seem to be involved in amplifying the rise in $[Ca^{2+}]_i$ due to Ca^{2+} influx. This could result from a Ca^{2+}-induced Ca^{2+} release mechanism, since membrane depolarization led to Ca^{2+} release from this store only in the presence of external Ca^{2+}, i.e. when Ca^{2+} influx occurred. This result is similar to that obtained by Lipscombe *et al.* (1988*b*) in frog sympathetic ganglion cells and is different from that by Thayer, Hirning & Miller (1988*a*) who found little amplification of $[Ca^{2+}]_i$ transients by this mechanism in rat sympathetic neurons.

The second Ca^{2+} pool is indirectly coupled to bradykinin receptors in PC12 cells. Fasolato *et al.* (1988) have shown that bradykinin causes accumulation of IP_3 and other inositol phosphates in PC12 cells. The transient phase of the rise in $[Ca^{2+}]_i$ after bradykinin addition that Fasolato *et al.* (1988) and ourselves have observed seems to be due to the release of Ca^{2+} from IP_3-sensitive Ca^{2+} stores. The maintained phase of the changes in $[Ca^{2+}]_i$ by bradykinin may result from Ca^{2+} influx (Fasolato *et al.* 1988). Although the mechanism responsible for this Ca^{2+} influx has not yet been identified, it is clear from our experiments that voltage-dependent Ca^{2+} channels are not involved. Moreover, in contrast to the caffeine effects, not all cells responded to bradykinin in our PC12 clone. The reason for this variability is not clear. Possibly the density of bradykinin receptors is sparse.

The two intracellular Ca^{2+} stores do not seem to interact. The caffeine-sensitive pool could be released without an effect on the filling of the stores that could be released by bradykinin exposure and *vice versa*. This finding is important in relation to models concerning cytoplasmic Ca^{2+} oscillations which do suggest such interactions (Goldbeter, Dupont & Berridge, 1990).

A major goal of our study was to compare signals that lead to a rise in $[Ca^{2+}]_i$ in undifferentiated and NGF-differentiated PC12 cells. Fasolato *et al.* (1988) have only studied undifferentiated PC12 cells. However, several groups (Streit & Lux, 1987, 1989; Plummer *et al.* 1989; Usowicz *et al.* 1990) have shown an increase in Ca^{2+} channel density during NGF-induced differentiation of PC12 cells. This increase in Ca^{2+} channel density results from a preferential expression of ω-CgTX-sensitive N-type over DHP-sensitive L-type Ca^{2+} channels (Plummer *et al.* 1989; Usowicz *et al.* 1990). Streit & Lux (1989) found that a particularly high Ca^{2+} channel density appears in the growth cones of NGF-differentiated PC12 cells. It was, therefore, of interest to study possible changes in the mechanisms that are involved in the control of $[Ca^{2+}]_i$ during differentiation of the cells.

There were relatively minor alterations in the pattern of elevations of $[Ca^{2+}]_i$ in cell bodies without and with NGF treatment. The increase in $[Ca^{2+}]_i$ evoked by K^+ depolarization was 50% larger in NGF-differentiated cell bodies which may be accounted for by the higher density of Ca^{2+} channels. The percentage inhibition by Ca^{2+} channel blockers of the depolarization-induced rise in $[Ca^{2+}]_i$ was very similar in undifferentiated and NGF-differentiated cells. About 65% of K^+-evoked $[Ca^{2+}]_i$ changes could be inhibited by nifedipine and an additional 10–20% by ω-CgTX in both untreated and NGF-treated cell bodies. This argues for a preferential presence of L-type Ca^{2+} channels in these structures. This seems to be at variance with the results by Plummer *et al.* (1989) and by Usowicz *et al.* (1990) who found a larger increase in N-type than in L-type Ca^{2+} channels during NGF treatment. However, these authors used holding potentials (−90 mV) for full activation of N-type Ca^{2+} channels that were substantially more negative than the normal resting potential (about −60 mV) of PC12 cells in this study, where a large fraction of N-type Ca^{2+} channels was inactivated (Lipscombe *et al.* 1988; Plummer *et al.* 1989; Usowicz *et al.* 1990). In addition, Usowicz *et al.* (1990) used NGF-differentiated cells in suspension for their electrophysiological studies. These cells do not extend processes, like those kept under our culture conditions, and, therefore, all newly expressed Ca^{2+} channels were sitting in the cell body.

We found a major difference with regard to the $[Ca^{2+}]_i$ response during K^+ depolarization between cell bodies and growth cones. In growth cones of NGF-differentiated PC12 cells there exist only sparse, if any, caffeine-sensitive Ca^{2+} stores. Therefore, in contrast to cell bodies, these stores do not participate to any appreciable extent in the control of $[Ca^{2+}]_i$ in growth cones. This is in agreement with results by Lipscombe *et al.* (1988*a*) in frog sympathetic ganglion cells and with those of Thayer *et al.* (1988*a*) in rat dorsal root ganglion (DRG) neurons. Growth cones from our cells show a much higher sensitivity to ω-CgTX-induced inhibition of the rapid K^+-evoked rise in $[Ca^{2+}]_i$ than cell bodies. This indicates that a large fraction of newly expressed N-type Ca^{2+} channels (Plummer *et al.* 1989; Usowicz *et al.* 1990) is inserted into the plasma membrane of growth cones. The late phase of the rise in $[Ca^{2+}]_i$ in growth cones is sensitive to nifedipine. This is similar, but less pronounced than in cell bodies. It indicates the presence of L-type channels in both structures, though at different densities. Although these channels are a major source for the increase in $[Ca^{2+}]_i$ during K^+ depolarization, a considerable fraction of this rise is not accounted for by opening of N- and L-type Ca^{2+} channels. On the other hand, it is

clear from our results that this fraction also depends on Ca^{2+} influx. We have tested whether a Na_i^+–Ca_o^{2+} exchange could account for this discrepancy, but we found no effect when Na_o^+ was replaced by Li_o^+ which should have increased $[Ca^{2+}]_i$ (unpublished result). We can also exclude the T-type Ca^{2+} channel, since in contrast to Garber *et al.* (1989), we have not found this channel type in our PC12 clone (Usowicz *et al.* 1990). However, other, so far unidentified channels, such as those responsible for maintained Ca^{2+} influx during bradykinin exposure, cannot be excluded.

A final aspect that is important in relation to the preferential accumulation of N-type Ca^{2+} channels in growth cones is their importance for neurotransmitter release. Takahashi, Tsukui & Hatanaka (1985) and Kongsamut & Miller (1986) observed that catecholamine release in undifferentiated PC12 cells is highly sensitive to dihydropyridines, while NGF-differentiated cells become largely insensitive to these drugs. In addition, we found that [^{3}H]dopamine release becomes more sensitive to ω-CgTX after NGF-induced differentiation of PC12 cells (Reber *et al.* 1990). This agrees with the larger inhibition by ω-CgTX of the rise in $[Ca^{2+}]_i$ during depolarization.

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