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Nerve growth factor modulates the drug sensitivity of neurotransmitter release from PC-12 cells

(calcium channel/pheochromocytoma/dihydropyridine/dibutyryl cAMP/differentiation)

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ABSTRACT **The release of catecholamines from adrenal chromaffin cells is known to be blocked by dihydropyridines, such as nitrendipine, and enhanced by others, such as BAY K8644. On the other hand, release from sympathetic neurons is predominantly insensitive to these agents. Release of [^{3}H]norepinephrine from undifferentiated PC-12 pheochromocytoma cells resembles that from chromaffin cells in that it is extremely sensitive to dihydropyridines. Following differentiation, however, release of catecholamine becomes predominantly insensitive to both nitrendipine and BAY K8644. Under both growth conditions, release remains completely blocked by 3 mM Co^{2+} or by removal of Ca^{2+} from the release media. Dose–response curves to K^+ show that following differentiation, cells become more sensitive, releasing transmitter at lower K^+ concentrations. In contrast, depolarization-induced uptake of $^{45}Ca^{2+}$ remains sensitive to dihydropyridines and shows similar sensitivity to K^+ stimulation in both growth conditions. These results can be explained by invoking a model involving dihydropyridine-sensitive and -insensitive types of voltage-sensitive calcium channels.**

The biochemical identification of ion channels has been greatly aided by the use of specific drugs or toxins that bind with high affinity to the channel protein (1–3). In the case of voltage-sensitive Ca^{2+} channels, there are several classes of drugs that have been shown to interact with voltage-sensitive Ca^{2+} channels in smooth, cardiac, and skeletal muscle (3). Of these agents, the dihydropyridines (DHP) are the most potent and specific. Some DHP (antagonists) block voltage-sensitive Ca^{2+} channels (4, 5) whereas others cause them to remain open for increased periods of time (agonists) (6, 7). Radioactive DHP have been used to label voltage-sensitive Ca^{2+} channels in muscle, endocrine cells, and neurons (3, 8–11). The nervous system contains a high concentration of specific [^{3}H]nitrendipine binding sites (11).

In neurons and most endocrine cells, the influx of Ca^{2+} through voltage-sensitive Ca^{2+} channels acts as the initial trigger for release of hormones or neurotransmitters (stimulus/secretion coupling) (12). Thus, drugs that block voltage-sensitive Ca^{2+} channels should inhibit the evoked release of neurotransmitters. However, in spite of the fact that the nervous system contains many specific DHP "receptors", it has often been observed that such drugs do not alter neurotransmitter release (3). This has given rise to the suggestion that both drug-sensitive and -insensitive voltage-sensitive Ca^{2+} channels exist (13). Indeed, a few reports have indicated that DHP-sensitive $^{45}Ca^{2+}$ uptake (14) and neurotransmitter release (15, 16) can occur in some instances. Moreover, electrophysiological and fluorescence data have revealed the presence of both DHP-sensitive and -insensitive Ca^{2+} currents in neurons and other cells (17–19).

The evoked release of catecholamines from adrenal chromaffin cells is highly sensitive to modulation by DHP (9). However, evoked release of transmitter from sympathetic nerves has been shown to be completely insensitive or very slightly sensitive to these same agents (3, 16, 20–22). The PC-12 pheochromocytoma cell line can be cultured under various conditions (23, 24). Normally its properties are similar to those of adrenal chromaffin cells. However, as with authentic chromaffin tissue, under appropriate culture conditions, PC-12 cells can be differentiated to resemble sympathetic neurons (23, 24). Interestingly, we show here that this change to a neuron-like morphology is accompanied by a change in the DHP sensitivity of catecholamine release.

MATERIALS AND METHODS

Materials. Nitrendipine and BAY K8644 were gifts from Alexander Scriabine (Miles). $^{45}Ca^{2+}$ was purchased from Amersham. [^{3}H]Norepinephrine was from New England Nuclear. All other materials were obtained from commercial sources.

Cell Culture. PC-12 rat pheochromocytoma cells were cultured as described (10, 23), with slight modifications. The PC-12T subclone was cultured in Dulbecco's modified Eagle's medium (DMEM from GIBCO) supplemented with 10% (vol/vol) heat-inactivated horse serum (KC Biological, Lenexa, KS), 5% (vol/vol) fetal bovine serum (KC Biological), and penicillin/streptomycin (100 units/ml and 100 μg/ml, respectively, from GIBCO). The PC-12P subclone was cultured in RPMI 1640 (GIBCO) rather than DMEM. The PC-12T subclone was from Lawrence Toll (SRI International, Menlo Park, CA) and the PC-12P subclone was provided by Mark Bothwell (Princeton University). The data from both subclones were similar and were pooled in all calculations. For release assays, cells were subcultured onto 60-mm tissue culture plates (Falcon) coated with rat tail collagen (25). Nerve growth factor-treated cells were grown in the presence of 1 mM dibutyryl cAMP and 50 ng of nerve growth factor/ml (7S nerve growth factor from Sigma) for about 2 weeks (range 10–21 days) (24).

[^{3}H]Norepinephrine Release Assays. Release assays were performed essentially as described by Shalaby *et al.* (26). Briefly, cells were loaded with 2 ml of medium containing [2,5,6 ^{3}H]norepinephrine at a concentration of 3 μM (specific activity approximately 1.65 μCi/ml; 1 Ci = 37 GBq) for about 60 min at 37°C in a Hepes-buffered assay medium (26). This loading is inhibited by 10 μM desmethyl imipramine or by incubation on ice. Cells were then washed eight times with 3 ml of buffer each over a half-hour period in a shaking water bath at 37°C. Release was assessed by replacing the medium in the plate every 5 min over 25 min and collecting 1-ml

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Abbreviation: DHP, dihydropyridine(s).

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fractions for liquid scintillation counting. Media containing higher K^+ concentrations (choline chloride concentrations reciprocally reduced) were added at the third fraction. When drugs were used, they were preincubated for 10 min (i.e., added to the first two fractions). At the end of the release period, plates were extracted with 3 ml of 0.2% $NaDodSO_4$ to determine the amount of tissue ^3H remaining. Release is expressed as a percentage of radioactivity present in the cells prior to the release period. Typically, basal release was approximately 1200 cpm per plate per 5 min, stimulated release was about 12,000 cpm per plate per 5 min, and counts remaining on the plate at the end of the experiment were about 90,000 cpm.

$^{45}Ca^{2+}$ Uptake Assays. Calcium uptake assays were performed as described by Freedman *et al.* (27) except the incubation media used were those in Shalaby *et al.* (26). The sodium concentration in these Hepes-buffered media was kept constant to eliminate any contribution by sodium–calcium exchange at these relatively long incubation times (min). Briefly, cells grown in 60-mm dishes were preequilibrated in a shaking water bath at 37°C for 10 min during which time any drugs were added. At time zero, preequilibration media were replaced by media of different K^+ concentrations, containing 1 μCi of $^{45}CaCl_2$/ml, and after 5 min, plates were aspirated and washed four times in ice cold 175 mM choline chloride/2 mM EGTA baths. Plates were then extracted with 5 ml of 0.2% $NaDodSO_4$. One milliliter was taken for liquid scintillation counting, and the remainder was used for a protein assay.

Protein Assays. Protein assays were performed according to the fluorescence method of Avruch and Wallach (28) by using bovine serum albumin as standard.

RESULTS

Fig. 1 shows the morphology of PC-12 cells grown under normal and under differentiating conditions. PC-12 cells normally appear round and quite devoid of processes similar to neurites (23). Following differentiation, however, cells put out many neurites and take on a highly neuronal appearance (23, 24). They possess many properties of sympathetic ganglion cells (29).

Fig. 2*A* shows some data from a typical release experiment. Cells preloaded with [^3H]norepinephrine rapidly release catecholamine upon depolarization with 70 mM K^+, release returning quickly to prestimulation values when the stimulus is removed. As demonstrated by ourselves (26) and others (30, 31), evoked release of catecholamine from undifferentiated cells is greatly enhanced by the voltage-sensitive Ca^{2+} channel agonist BAY K8644 and blocked by the voltage-sensitive Ca^{2+} channel antagonist nitrendipine. Dose–response curves for these two effects are shown in Fig. 2 *B* and *C*. It can be seen that in addition to being very effective, the DHP are also very potent, producing significant effects at low concentrations. Interestingly, careful inspection of Fig. 2 *A* and *C* shows that even at high concentrations (1 μM) nitrendipine only blocks about 95% of evoked release. Addition of 3 mM Co^{2+} or stimulation in a Ca^{2+} free medium, however, completely abolished evoked release.

The effects of DHP on evoked release of [^3H]norepinephrine from differentiated PC-12 cells, however, were strikingly different. As observed (32), differentiated PC-12 cells release less norepinephrine than undifferentiated cells upon depolarization by 70 mM K^+ (12.2 ± 0.22% undifferentiated, 8.66 ± 0.20% differentiated, $n = 6$). Release of [^3H]norepinephrine from differentiated PC-12 cells was only slightly enhanced by BAY K8644 (Fig. 2*B*) and was very poorly inhibited by nitrendipine even at high concentrations (Fig. 2*C*). The maximal inhibition achieved by 1 μM nitrendipine varied between 74.6 and 0% depending on the culture ($n = 6$); the mean inhibition was 32.6%. Thus release of norepinephrine under these conditions is predominantly resistant to modulation by DHP. In spite of this, in differentiated as in undifferentiated cells, 3 mM Co^{2+} or Ca^{2+}-free medium blocked evoked transmitter release virtually completely, indicating that the calcium source to trigger release remains extracellular.

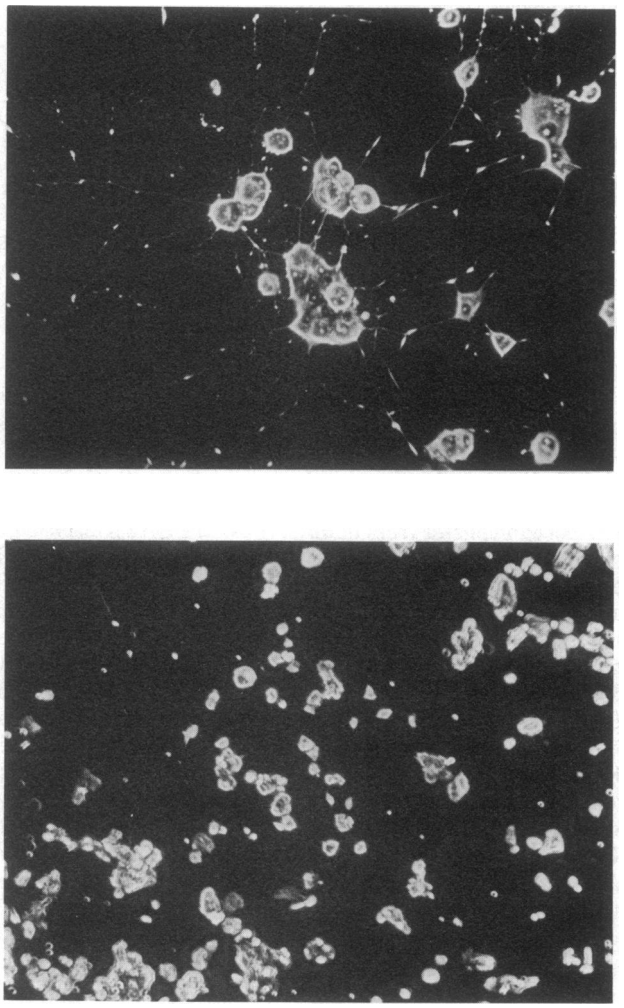

FIG. 1. PC-12 rat pheochromocytoma cells cultured with 1 mM dibutyryl cAMP and 50 ng of nerve growth factor/ml for 2 weeks (*Upper*) and in the absence of such treatment (*Lower*).

Depolarization-induced uptake of $^{45}Ca^{2+}$ into PC-12 cells was also examined. As with [^3H]norepinephrine release, absolute uptake of $^{45}Ca^{2+}$ in response to 70 mM K^+ was lower in the differentiated state. However, Fig. 3 *A* and *B* illustrate that the drug sensitivity of the evoked $^{45}Ca^{2+}$ uptake was similar under both growth conditions. Thus, the percentage enhancement of $^{45}Ca^{2+}$ uptake produced by BAY K8644 was at least as great in the differentiated state (Fig. 3*A*). Moreover in both growth states, nitrendipine (1 μM) reduced evoked $^{45}Ca^{2+}$ uptake completely to values not significantly different from basal or those found in the presence of 3 mM Co^{2+} (Fig. 3*B*).

Thus, catecholamine release in the differentiated state is considerably less drug sensitive than in the undifferentiated state although the sensitivity of evoked $^{45}Ca^{2+}$ uptake is similar in both cases. How may this be explained? One clue is suggested by the data in Fig. 4. It can be seen that in the differentiated state, evoked release of catecholamine is achieved at lower external K^+ concentrations, half-maximal release occurring at approximately 40 mM K^+ in undifferentiated cells and 20 mM K^+ in differentiated cells. On the other

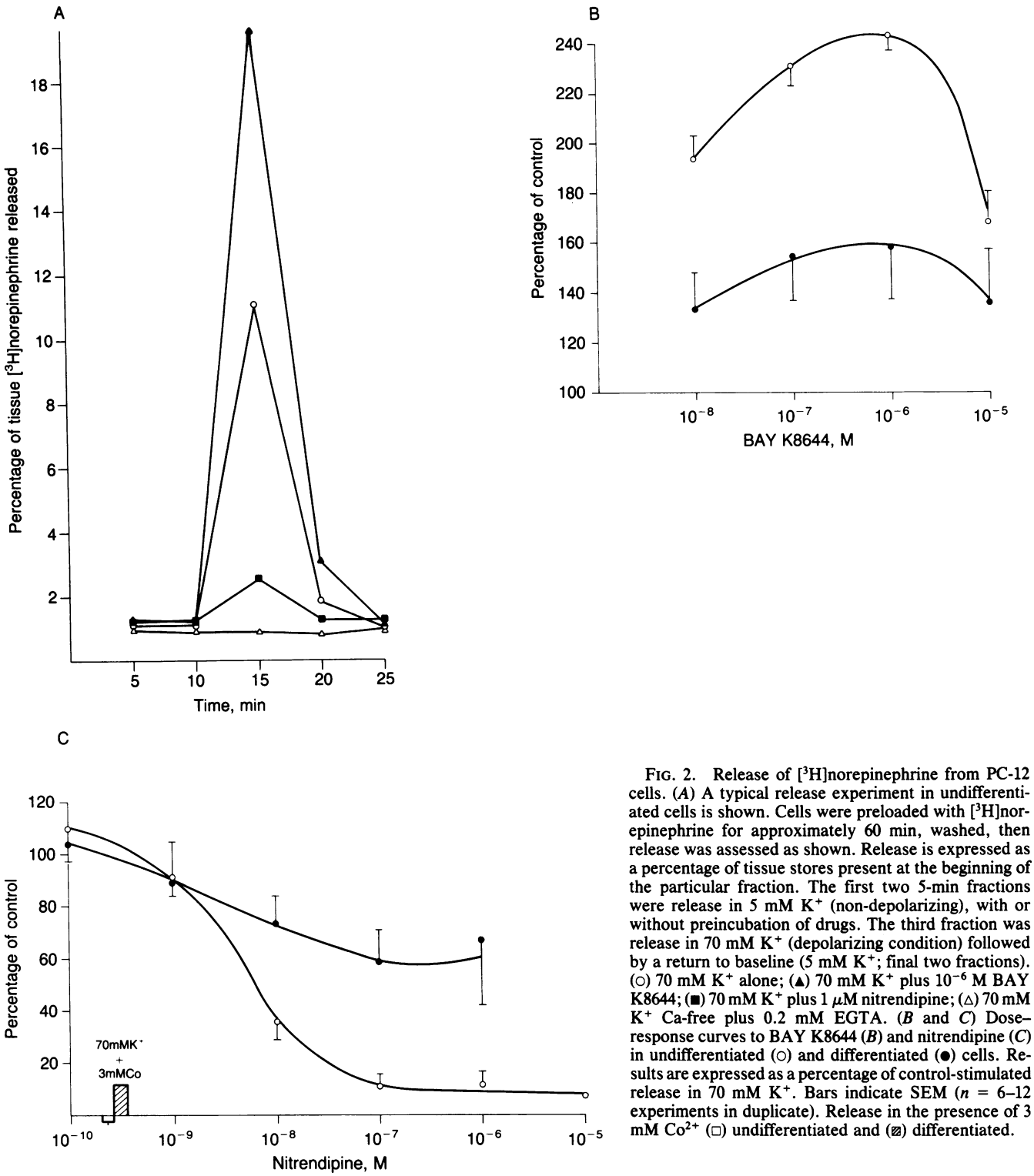

FIG. 2. Release of [^{3}H]norepinephrine from PC-12 cells. (*A*) A typical release experiment in undifferentiated cells is shown. Cells were preloaded with [^{3}H]norepinephrine for approximately 60 min, washed, then release was assessed as shown. Release is expressed as a percentage of tissue stores present at the beginning of the particular fraction. The first two 5-min fractions were release in 5 mM K^+ (non-depolarizing), with or without preincubation of drugs. The third fraction was release in 70 mM K^+ (depolarizing condition) followed by a return to baseline (5 mM K^+; final two fractions). (○) 70 mM K^+ alone; (▲) 70 mM K^+ plus 10^{-6} M BAY K8644; (■) 70 mM K^+ plus 1 μM nitrendipine; (△) 70 mM K^+ Ca-free plus 0.2 mM EGTA. (*B* and *C*) Dose–response curves to BAY K8644 (*B*) and nitrendipine (*C*) in undifferentiated (○) and differentiated (●) cells. Results are expressed as a percentage of control-stimulated release in 70 mM K^+. Bars indicate SEM (n = 6–12 experiments in duplicate). Release in the presence of 3 mM Co^{2+} (□) undifferentiated and (▨) differentiated.

hand, Fig. 4*B* shows that the sensitivity of $^{45}Ca^{2+}$ uptake to increasing K^+ concentrations was unchanged in the two growth modes.

DISCUSSION

In most cases the voltage-sensitive Ca^{2+} channels that provide an influx of Ca^{2+} that triggers the release of neurotransmitters have been shown to be insensitive to organic voltage-sensitive Ca^{2+} channel blocking drugs such as DHP (3). This appears to be true for sympathetic neurons (3, 20–22). Studies in our laboratory (43) have shown that the evoked release of [^{3}H]norepinephrine from cultured superior cervical ganglion neurons of newborn rats is completely DHP resistant. On the other hand, release of catecholamines from adrenal chromaffin cells is extremely sensitive to DHP (9). Adrenal chromaffin cells and sympathetic neurons are both derived from neural crest and are thus closely related. PC-12 pheochromocytoma cells normally resemble adrenal chromaffin cells, but they can be converted into cells resembling sympathetic neurons by nerve growth factor (23, 29, 32). We have shown here that the drug sensitivity of catecholamine release from PC-12 cells in the two growth modes closely reflects that of chromaffin cells and sympathetic neurons. Thus, the PC-12 system should be a useful experimental system for investigating this phenomenon.

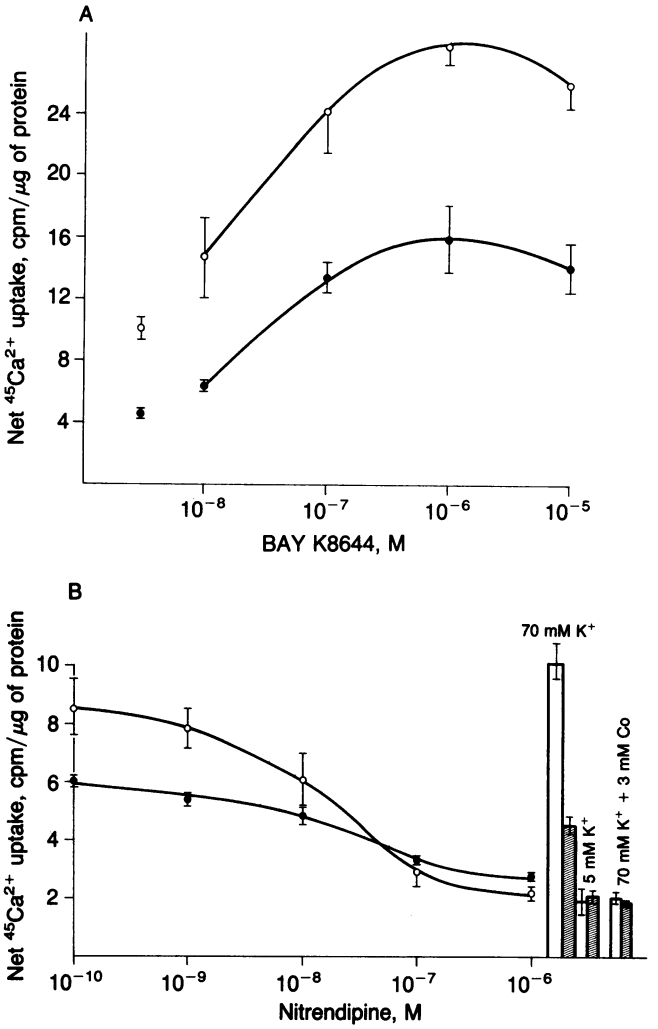

FIG. 3. $^{45}Ca^{2+}$ uptake into differentiated (●; ▨) and undifferentiated (○; □) PC-12 cells over 5 min stimulated by 70 mM K^+. Dose–response curves to BAY K8644 (*A*) and nitrendipine (*B*) are shown. Points at left in *A* are uptake in the absence of drug. Bars indicate SEM (n = 2–6).

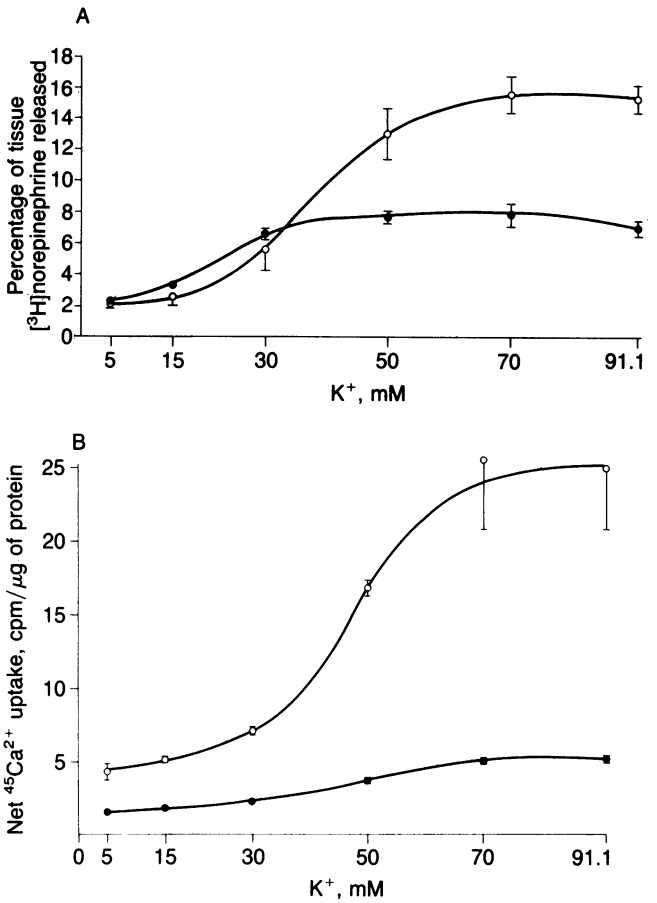

FIG. 4. Dose–response curves to K^+. Differentiated (●) and undifferentiated (○) PC-12 cells. (*A*) [^{3}H]Norepinephrine release, expressed as a percentage of tissue stores released, is plotted against K^+. Bars indicate SEM (n = 4–6 plates). (*B*) $^{45}Ca^{2+}$ uptake, expressed as cpm/μg of protein, is plotted against K^+. Bars indicate SEM (n = 2–4 plates).

What is the basis for this change in sensitivity? Our studies imply that the voltage-sensitive Ca^{2+} channels providing Ca^{2+} for transmitter release are predominantly DHP sensitive in undifferentiated (chromaffin-like) PC-12 cells and predominantly DHP insensitive in differentiated (neuron-like) PC-12 cells. Indeed, several kinds of voltage-sensitive Ca^{2+} channels have now been reported in neurons (17, 33). One type of channel (L-type) is activated by strong depolarizations and is DHP sensitive. It inactivates slowly. Another type (T-type) has a low threshold and is DHP insensitive. This type of voltage-sensitive Ca^{2+} channel inactivates rapidly. Both L- and T-type channels have been found in numerous cell types including neurons (17, 18, 33, 34). A third type of voltage-sensitive Ca^{2+} channel (N) so far only reported in dorsal root ganglion cells has biophysical characteristics between L and T (17, 33). However, it also inactivates rapidly and is DHP insensitive. Electrophysiological data from PC-12 cells is rather scanty (35–39). There are some indications that at least two types of voltage-sensitive Ca^{2+} channels may be found. It has been reported that in undifferentiated cells, although the evoked release of transmitter is blocked by DHP, the Ca^{2+} spike recorded in the same cells is quite unaffected (35). Significantly, these investigators found that cells had to be preconditioned at hyperpolarized membrane potentials to produce good Ca^{2+} spikes. Under voltage clamp, hyperpolarizing steps are required to remove inactivation from both T and N (drug resistant) voltage-sensitive Ca^{2+} channels (17). Secondly, it has also been reported that under voltage clamp the Ca^{2+} current recorded in undifferentiated PC-12 cells was only partially blocked by DHP when evoked from hyperpolarized holding potentials but was completely blocked when evoked from depolarized holding potentials (39). This again suggests the presence of two channel types, although other explanations are also possible.

It should be pointed out that $^{45}Ca^{2+}$ flux studies carried out under the conditions reported here and elsewhere almost exclusively monitor Ca^{2+} influx via DHP-sensitive L-type channels. Thus, incubations are carried out over relatively long time periods and mask the small uptake occurring through rapidly inactivating channels. It is clear from our studies that drug-sensitive (L-type) channels occur in both undifferentiated and differentiated PC-12 cells, and yet the sensitivity of the release process to DHP differs dramatically. To explain this, it may be supposed that drug-sensitive (L-type) and -insensitive (probably N-type) voltage-sensitive Ca^{2+} channels occur in PC-12 cells in both growth modes (35, 39). We suggest that upon differentiation, there is a rearrangement of voltage-sensitive Ca^{2+} channels. Release from neurons occurs mainly from terminal varicosities rather than from the soma, although some release is still possible from this latter region (40). It is thought that voltage-sensitive Ca^{2+} channels cluster around sites on the plasma membrane ("active zones") with which transmitter-containing vesicles fuse during exocytosis (41, 42). A clustering of voltage-sensitive Ca^{2+} channels around active zones has been sug-

gested on morphological and biophysical grounds (41, 42). Interestingly, clusters of drug insensitive N-type voltage-sensitive Ca^{2+} channels have also been detected electrophysiologically (33). Upon differentiation, it is possible that drug-insensitive voltage-sensitive Ca^{2+} channels already present in the cells are grouped into clusters around transmitter release sites in terminal varicosities, or alternatively new channels could be synthesized and inserted. Thus, drug-insensitive channels would have a far greater influence on release in the differentiated state by virtue of their position even though they make a relatively minor contribution to the total depolarization-induced uptake of Ca^{2+} into the cell. This suggested scenario is given some credence by our observation that release in differentiated cells occurs at lower K^+ concentrations. Thus, N-type DHP-insensitive channels activate at somewhat lower thresholds than L-type channels (17). There is apparently no change in PC-12 resting potential in the two growth states (37). It is also possible that L-type channels are preferentially localized in the cell soma. Indeed, it has been recently shown that there is a different voltage dependence for Ca^{2+} uptake into the soma and neuropil of single molluscan neurones (16).

More work is required to establish the feasibility of our hypothesis. Furthermore, DHP-sensitive release is clearly observed in some neurons. For example, in dorsal root ganglion neurons from newborn rats, the evoked release of substance P is very DHP sensitive (43). Why different neurons use predominantly different types of voltage-sensitive Ca^{2+} channels to provide Ca^{2+} for transmitter release is an intriguing question.

Note Added in Proof. Since this paper was submitted, we have found that release under both differentiated and undifferentiated conditions is very sensitive to Cd at micromolar concentrations. In addition, a paper has appeared that has results supporting our findings (44).

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1. Catterall, W. A. (1980) *Annu. Rev. Pharmacol. Toxicol.* **20,** 15–43.
2. Dolly, J. O. & Barnard, E. A. (1984) *Biochem. Pharmacol.* **33,** 841–858.
3. Miller, R. J. & Freedman, S. B. (1984) *Life Sci.* **34,** 1205–1221.
4. Fleckenstein, A. (1977) *Annu. Rev. Pharmacol. Toxicol.* **17,** 149–166.
5. Tsien, R. W. (1983) *Annu. Rev. Physiol.* **45,** 341–359.
6. Hess, P., Lansman, J. B. & Tsien, R. W. (1984) *Nature (London)* **311,** 538–544.
7. Nowycky, M. C., Fox, A. P. & Tsien, R. W. (1985) *Proc. Natl. Acad. Sci. USA* **82,** 2178–2182.
8. Janis, R. A. & Triggle, D. J. (1983) *J. Med. Chem.* **26,** 775–785.
9. Garcia, A. G., Sala, F., Reig, J. A., Viniegra, S., Frias, J., Fonteriz, R. & Gandia, L. (1984) *Nature (London)* **309,** 69–71.
10. Toll, L. (1982) *J. Biol. Chem.* **257,** 13189–13192.
11. Glossmann, H., Ferry, D. R., Lubbecke, F., Mewes, R. & Hofmann, F. (1982) *Trends Pharmacol. Sci.* **3,** 431–437.
12. Rubin, R. P. (1970) *Physiol. Rev.* **22,** 389–428.
13. Miller, R. J. (1984) *Trends NeuroSci.* **8,** 45–47.
14. Turner, T. J. & Goldin, S. M. (1985) *J. Neurosci.* **5,** 841–849.
15. Middlemiss, D. N. & Spedding, M. (1985) *Nature (London)* **314,** 94–96.
16. Graubard, K. & Ross, W. N. (1985) *Proc. Natl. Acad. Sci. USA* **82,** 5565–5569.
17. Nowycky, M. C., Fox, A. P. & Tsien, R. W. (1985) *Nature (London)* **316,** 440–443.
18. Bean, B. P. (1985) *J. Gen. Physiol.* **86,** 1–30.
19. Connor, J. A. (1985) *Soc. Neurosci. Abstr.* **11,** 176 (abstr.).
20. Cena, V., Garcia, A. G., Khoyi, M. A., Salaices, M. & Sanchez-Garcia, P. (1985) *Brit. J. Pharmacol.* **85,** 691–696.
21. Docherty, J. R., Hyland, L. & Warnock, P. (1984) *Br. J. Pharmacol.* **82,** 213P (abstr.).
22. Beattie, D. T., Cunane, T. C. & Muir, T. C. (1985) *Br. J. Pharmacol.* **84,** 29P (abstr.).
23. Greene, L. A. & Tischler, A. S. (1976) *Proc. Natl. Acad. Sci. USA* **73,** 2424–2428.
24. Heidemann, S. R., Joshi, H. C., Schechter, A., Fletcher, J. R. & Bothwell, M. (1985) *J. Cell Biol.* **100,** 916–927.
25. Bornstein, M. D. (1958) *Lab. Invest.* **4,** 134–140.
26. Shalaby, I. A., Kongsamut, S., Freedman, S. B. & Miller, R. J. (1984) *Life Sci.* **35,** 1289–1295.
27. Freedman, S. B., Dawson, G., Villereal, M. L. & Miller, R. J. (1984) *J. Neurosci.* **4,** 1453–1467.
28. Avruch, J. & Wallach, D. F. H. (1971) *Biochem. Biophys. Acta* **233,** 334–345.
29. Greene, L. A. (1984) *Trends NeuroSci.* **8,** 91–94.
30. Albus, U., Habermann, E., Ferry, D. R. & Glossmann, H. (1984) *J. Neurochem.* **42,** 1186–1189.
31. Greenberg, D. A., Carpenter, C. L. & Cooper, E. C. (1985) *J. Neurochem.* **45,** 990–993.
32. Greene, L. A. & Rein, G. (1977) *Brain Res.* **138,** 521–528.
33. Fox, A. P., Nowycky, M. C. & Tsien, R. W. (1985) *Soc. Neurosci. Abstr.* **11,** 792 (abstr.).
34. Nilius, B., Hess, P., Lansman, J. B. & Tsien, R. W. (1985) *Nature (London)* **316,** 443–446.
35. Ogura, A. & Takahashi, M. (1984) *Brain Res.* **301,** 323–330.
36. Schofield, G. G. & Weight, F. F. (1984) *Soc. Neurosci. Abstr.* **10,** 527 (abstr.).
37. O'Lague, P. H. & Huttner, S. L. (1980) *Proc. Natl. Acad. Sci. USA* **77,** 1701–1705.
38. O'Lague, P. H., Huttner, S. L., Vanden Berg, C. A., Morrison-Graham, K. & Horn, R. (1985) *J. Neurosci. Res.* **13,** 301–321.
39. Kunze, D. L., Hawkes, M. J., Hamilton, S. L. & Brown, A. M. (1985) *Biophys. J.* **47,** 264 (abstr.).
40. Suetake, K., Kojima, H., Inanaga, K. & Koketsu, K. (1981) *Brain Res.* **205,** 436–440.
41. Simon, S. M. & Llinas, R. R. (1985) *Biophys. J.* **48,** 485–498.
42. Pumplin, D. W., Reese, T. S. & Llinas, R. (1981) *Proc. Natl. Acad. Sci. USA* **78,** 7210–7213.
43. Perney, T. M., Hirning, L. & Miller, R. J. (1986) *Biophys. J.* **49,** 200a (abstr.).
44. Takahashi, M., Tsukui, H. & Hatanaka, H. (1985) *Brain Res.* **341,** 381–384.