Habitation in response to repetitive depolarization of PC12 cells can be used as a model for memory processes at the molecular level. In response to depolarization by high external potassium, a triphasic elevation in internal calcium levels occurred. Calcium elevation was maximal immediately after addition of the stimulus (phase 1), followed by a 2-min period in which the calcium level decreased (phase 2), leading to a new steady-state level which was higher than in the unstimulated cell (phase 3). In response to repetitive depolarizations, the calcium level in phase 1 was reduced by as much as 43%, and phase 3 was reduced by as much as 40%. By measuring the relationship between calcium elevation and secretion, it was shown that measured reductions in calcium levels were correlated with neurosecretory habituation. One of the components responsible for the reductions in calcium levels was a tetraethylammonium-sensitive potassium channel, and the habituation of this channel was reversed by addition of 4&phorbol 12-myristate 13-acetate.

Habituation is the process by which an organism adapts by diminishing its response to repeated stimulation. Habituation occurs at many levels, from complex multicellular organisms to single cells (1). In a number of experimental systems, habituation has been shown to be a function of reduced synaptic function (2-4). Reduction of synaptic function has been shown to result from reduced neurotransmitter release of voltage-gated calcium channels (9). Both of these models invoke the presynaptic cell as the primary effector of the behavioral process. If the presynaptic cell is responsible for habituation, it could be accounted for by a simple adaptation process, much like adaptation processes already described in single cell organisms, such as chemotaxis in the bacterium (10).

McFadden and Koshland (11) have demonstrated that neurosecretory habituation can occur in single mammalian cells. To do this they studied PC12 cells, a clonal pheochromocytoma cell line initially developed by Greene and Tischler (12). PC12 cells, when grown in the presence of nerve growth factor, terminally differentiate and take on many of the properties of noradrenergic sympathetic neurons (12, 13). In response to repetitive depolarizations, PC12 cells secrete reduced levels of norepinephrine (11), and this reduced secretion meets many of the criteria for habituation initially defined by Thompson and Spencer (14).

When PC12 cells are presented with high (56 mM) external potassium, the plasma membrane is depolarized, allowing calcium entry through voltage-gated calcium channels (15, 16). Voltage-gated calcium entry in these cells occurs predominantly through the dihydropyridine-sensitive L-type calcium channel, which is responsible for most calcium entry from the exterior in response to depolarization (17). As calcium entry is required for secretion to occur (16), we have investigated the role of calcium in the mechanism of secretory habituation in PC12 cells.

**EXPERIMENTAL PROCEDURES**

*Measurement of Internal [Ca2+]—NGF-differentiated PC12 cells were removed from the Cytodex beads they were grown on by gentle vortexing. The beads were allowed to settle for 2 min, and the cells were removed and spun at 3,200 rpm for 0.5–1 min. The supernatant was removed and the cells were dispersed in low K+ buffer at 37°C. An equivalent number of cells were partitioned into three sterile 15-ml tubes, and the volume increased to 10 ml with low K+ buffer. Fura 2 AM was added to a final concentration of 1–2 μM (0.1% (v/v) dimethyl sulfoxide) to two of the three tubes, and all three tubes were incubated for 50–60 min at 37°C. After fura 2 loading, the cells were spun for 0.5–1 min at 3,200 rpm, washed in 1 ml low K+ buffer and spun for 1 min at 3,200 rpm, after which the two sets of cells that had been loaded with the dye were taken up in 1.5 ml of low K+.

**The abbreviations used are: TEA, tetraethylammonium chloride; PMA, 4&phorbol 12-myristate 13-acetate; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.**
buffer in separate 4-ml polystyrene cuvettes and placed in an SLM 4800S fluorimeter for [Ca$$^{2+}$$] measurements. The cells were stirred gently (1–2 Hz) and kept at 37 °C throughout the experiment. Fluorescence intensity was measured interchangeably at 340 and 380 nm excitation wavelengths with emission always measured at 510 nm. Each data point represented the average of 10 independent measurements at each wavelength taken over a 5-s time interval. Internal [Ca$$^{2+}$$] was measured using the 340:380 ratio technique (18).

One of the two sets of fura 2 AM-loaded cells was used for the experiment and the other to control for the rate of leakage of the fura 2 from the cells. When PC12 cells were incubated at 37 °C, they extruded a small portion of the dye into the extracellular medium. This extrusion was linear with time for at least 40 min after the experiment was begun. None of the experiments presented lasted any longer than 40 min. To control for leakage, the amount of dye extruded from both sets of loaded cells (those involved in the experiment and the leakage control) were quantified at the end of the experiment by filtering the buffers that contained the cells through a 0.2 μm filter (Acrodisc) and measuring the fluorescence intensity at 510 nm (340 nm excitation). None of the agents added to the cells showed any change in the amount of dye present in the extracellular space. None of the agents added to the cells showed any change in the amount of dye present in the extracellular space.

**Secretion Assays**—Cells were washed with low K+ buffer and incubated in 1 ml of low K+ containing 1 μCi/ml [3H]norepinephrine (42 mCi/mmol) for 1 h at 37 °C, after which they were washed four times with 1 ml of low K+. The low K+ buffer was removed for the experiment and the appropriate stimulus added. The cells were stimulated for 30 s, after which the supernatant was collected, spun for 3 min at 5,000 rpm, and a portion was counted by liquid scintillation counting. The cells were lysed in ice cold 0.4 M perchloric acid, spun for 13,000 rpm for 3 min, and a portion was counted by liquid scintillation counting.

**Flow-through Secretion Measurements**—PC12 cells grown on Cytodex beads were washed in 5 ml of low K+ at 37 °C and then incubated in low K+ at 37 °C in the presence of 20 μCi of [3H]norepinephrine (42 mCi/mmol) for 1–1.5 h, after which they were transferred to a flow-through apparatus and stimulated and described previously (11). Briefly, approximately 106 cells were placed in a perfusion chamber at 37 °C. This chamber was connected to a peristaltic pump which maintained a constant flow rate of 0.28 ml/min. The perfusion medium was changed by using a computer-controlled solenoid valve (MKS Instruments). The cells were allowed to clear the scintillation fluid (2.2 ml/min) and monitored on-line with a flow-through scintillation counter (Flo-One, Radiomatic Instruments). At the end of each experiment, the cells were lysed in 0.2% Triton X-100 to determine the total amount of radioactive neurotransmitter present in the cells, and the amount of radioactive norepinephrine secreted was corrected for the ongoing loss of radiolabel.

**RESULTS**

**Habituation Correlates with Reduced Calcium Entry**—To determine whether changes in calcium levels were responsible for habituation of norepinephrine secretion, cells were maintained in low potassium (low K+) and stimulated with varying numbers of 5-min high potassium (high K+) stimulations (Fig. 1). Repetitive depolarizations led to reduced calcium levels in these cells. Calcium elevation was maximal immediately after the stimulus was presented (phase 1). This was followed by a 2-min period in which a decrease in calcium levels was seen (phase 2), after which the calcium level remained at a steady-state level above that seen in the unstimulated cell (phase 3). Calcium elevations in phase 1 and phase 3 were reduced by prior high K+ stimulation. Following one 5-min high K+ prestimulation, phase 1 for the second high K+ stimulus was reduced by 25%. In response to 2 prestimulations, phase 1 was reduced further to 40% of the response in previously unstimulated cells. A third prestimulation led to a reduction of 43%. One prestimulation led to a 16% reduction in phase 3, two to a 36% reduction, and three to a 40% reduction.

We next investigated whether the calcium reductions seen in PC12 cells correlated with the reductions in norepinephrine secretion. To do this, we assayed changes in calcium levels during phase 1 and norepinephrine secretion at various external potassium concentrations (Fig. 2). The resulting data allowed us to generate a curve describing the relationship between calcium changes in response to high K+ and norepinephrine secretion. Calcium elevation showed a sigmoidal relationship with respect to secretion. Using the fourth order polynomial equation, $Y = (7.2857 \times 10^{-5} + (2.3203 \times 10^{-7}) (X)) - (3.6640 \times 10^{-5}(X^2)) + (2.3470 \times 10^{-7}(X^3)) - (3.6640 \times 10^{-9}(X^4)))$, we fit this curve with a correlation coefficient of 0.987. Using this equation, the 43% reduction in the phase 1 calcium level with repetitive high K+ stimulations (Fig. 1) corresponds to a 66% reduction in norepinephrine secretion. The maximal neurosecretory habituation for stimulus lengths of 1 min in this (Fig. 3) study was 68%, and in previous studies...
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FIG. 3. Norepinephrine secretion to repetitive high potassium stimulations with and without TEA. At 30 min, the following stimulus paradigm was initiated: 1 min high K+ (HK; 25 mM sodium and 100 mM sucrose), 4 min low K+, 1 min high K+ with 50 mM tetraethylammonium chloride (HK + TEA; 25 mM sodium and 50 mM TEA), 4 min low K+. The stimulus paradigm was repeated 15 times. During the second 4 min low K+ incubation of the ninth repetition, 100 nM PMA (0.1% (v/v) dimethyl sulfoxide) was added.

(11) it was 72%. The reduction in calcium levels we have measured (Fig. 1) can account for 97 and 92% of these values, respectively. Integrating the expected secretion over a 5-min period, the total calcium level reduction in the fourth high K+ stimulation corresponded to a 45% reduction in norepinephrine secretion relative to the first stimulation. The decrease measured previously in response to secretion during analogous 5-min stimulations was 49%. Therefore, the reduction in calcium levels can account for 92% of the secretory reduction in response to longer stimulations as well. This suggests that the calcium decreases observed in response to repetitive stimulations could account for the level of neurosecretory habituation seen.

Habituation Involves a Voltage-gated Potassium Channel—Since elevating external potassium was the means of depolarizing the cells, we investigated the effects of three potassium channel blockers, TEA, cesium chloride, and apamin, in order to determine their effects on secretion. TEA is a general blocker of all potassium channels; cesium also blocks most potassium channels, but sometimes shows a different specificity than TEA; and apamin is a peptide which specifically blocks the calcium-activated potassium channel (19, 20).

Cells were stimulated with high K+ alternately in the presence and absence of 50 mM TEA (Fig. 3). The presence of TEA inhibited norepinephrine secretion from the cells. Moreover, TEA-blocked stimuli habituated at a slower rate than the unblocked stimuli. This suggested that one or more of the potassium channels closed more frequently as the extent of habituation increased, and therefore TEA, which binds to potassium channels in the open state (21, 22), no longer had as much of an effect.

We next repeated the experiment with 50 mM cesium replacing TEA in the alternate stimulations (Fig. 4). Cesium also inhibited norepinephrine secretion, although to a lesser extent than TEA. However, cesium-blocked stimuli habituated at the same rate as unblocked stimuli. This could be a result of the fact that cesium blocked less secretion than TEA, or it could result from a difference in the specificity of blocked channels.

We next tested the effect of apamin (Fig. 5). Apamin reduced norepinephrine secretion very little after the first stimulation, and unlike TEA, this reduction slightly increased during subsequent stimulations. The small effect seen with apamin suggests that the calcium-activated potassium channel was not responsible for the effect seen with TEA.

FIG. 4. Norepinephrine secretion to repetitive high potassium stimulations with and without cesium. At 31 min, the following stimulus paradigm was initiated: 1 min high K+ (HK; 25 mM sodium and 100 mM sucrose), 4 min low K+, 1 min high K+ with 50 mM cesium chloride (HK + Cs; 25 mM sodium and 50 mM cesium chloride), 4 min low K+. The stimulus paradigm was repeated 12 times. During the second 4 min low K+ incubation of the sixth stimulation, 100 nM PMA (0.1% (v/v) dimethyl sulfoxide) was added.

FIG. 5. Norepinephrine secretion to repetitive high potassium stimulations with and without apamin. At 26 min, the following stimulus paradigm was initiated: 1 min high K+ (HK), 4 min low K+, 1 min high K+ with 4 μM apamin (HK + AP), 4 min low K+. The stimulus paradigm was repeated 10 times.

FIG. 6. Internal calcium changes in response to TEA or cesium addition during stimulation by high K+. Internal calcium levels were measured in response to high K+ (HK; 25 mM sodium and 100 mM sucrose, open circles), high K+ in the presence of TEA (HK + TEA; 25 mM sodium and 50 mM TEA chloride, open squares), and high K+ in the presence of cesium (HK + Cs; 25 mM sodium, 50 mM cesium chloride, darkened circles) as described under “Experimental Procedures.”

We next tested whether the reduction of norepinephrine secretion seen with cesium and TEA was the result of their ability to lower calcium levels (Fig. 6). Both cesium and TEA increased the resting calcium level in the unstimulated cell by 20 nM. Taking into account the shifted base line, the phase 1 calcium elevation in response to high K+ was reduced by 21%
for cesium and by 26% for TEA. Using the relationship between calcium changes and norepinephrine secretion in Fig. 2, this corresponded to a 25% reduction in secretion for cesium, and a 36% reduction for TEA. These calculated reductions were within 4% of measured values (Figs. 3 and 4). Thus, if one were to assume that calcium was solely responsible for secretion, the ability of these ions to reduce secretion could be the result of their ability to lower calcium levels.

Fig. 7 summarizes the effects of TEA, cesium, and apamin on high K+-evoked secretion with repetitive stimulation. The amount secreted for high K+ in the presence of 50 mM TEA, relative to the high K+ stimulus just preceding it which lacked TEA, went from 60% to 85% from the first pair of stimulations to the 11th pair. Secretion for high K+ in the presence of 50 mM cesium was 71% relative to the high K+ stimulus without cesium just prior to it, and this percentage was constant through the 12th pair of stimulations. The ability of apamin to reduce secretion relative to the high K+ stimulus just prior to it was 70% for the first pair of stimulations, but then went to 90% for the second pair and increased to 85% by the 10th pair of stimulations. The sum of these data suggests that the closure of a voltage-gated potassium channel or channels during habituation led to a reduction in the sensitivity of the cell to potassium. While blockage of norepinephrine secretion can be substantial, 40% with TEA, these values are not sufficient to explain the full amplitude of the decreases seen in habituation.

Phorbol Esters Can Reverse Habituation—PMA alters both internal calcium and norepinephrine secretion in response to depolarization by high potassium (23, 24), through its ability to activate protein kinase C (for review, see Ref. 25). Fig. 8 demonstrates that PMA not only increased norepinephrine secretion from the cells, but also reversed habituation of the TEA-sensitive potassium channel. All secretory peaks shown in Figs. 3 and 4 were integrated and are summarized in Fig. 8 as percentages relative to the first peak in which the stimulus was present. When added at a time when the cells had already been habituated to the stimulus, PMA increased the ability of TEA to block secretion to 40% (Fig. 3), the level in nonhabituated cells. After PMA treatment, TEA-blocked stimuli became habituated at the same rate as unblocked stimuli (Fig. 8B). PMA had no effect on the rate of habituation for cesium-blocked stimuli (Fig. 8A), even though the level of secretion was increased, as with the TEA experiment. These data suggest that PMA addition was able to reactivate the habituated channel, leading to increased potassium sensitivity during depolarization. The ability of PMA to increase

![Diagram](image-url)

**Fig. 7.** Effect of potassium channel blockers on secretion as a function of repetitive stimulations. The percentage of norepinephrine secreted for high K+ stimulation in the presence of TEA (darkened circles), cesium (open squares), and apamin (open circles) relative to the high K+ stimulus immediately preceding it is graphed as a function of the number of pairs of stimulations presented.

![Diagram](image-url)

**Fig. 8.** Effect of PMA on the ability of cesium and TEA to block norepinephrine with repetitive stimulations. The percentage of decreased secretory responsiveness to high K+ (HK, darkened circles) relative to the first high K+ stimulus, and the percentage of decreased secretory responsiveness of cesium (A) and TEA (B, both open circles) to the first high K+ stimulus containing those agents, is graphed as a function of the number of pairs of stimulations presented. At the indicated time, 100 nM PMA (0.1% v/v dimethyl sulfoxide) was added. Error bars indicate standard deviations (n = 2).

**DISCUSSION**

We have demonstrated that calcium levels become habituated in response to repetitive depolarizations by high potassium. The calcium level immediately after high K+ stimulation (phase 1) was reduced by as much as 43% and the elevated steady-state calcium level in cells stimulated for several minutes (phase 3) was reduced by as much as 40% in response to repetitive stimulations with high K+. Based on empirical data (Fig. 2), we derived a mathematical function to describe the relationship between calcium levels and norepinephrine secretion. Using this relationship, measured reductions in calcium levels can account for 97% of the maximal reduction in norepinephrine secretion in this study (Fig. 3) and can account for 92% of the reduction in norepinephrine secretion generated for both short (1 min) and long (5 min) stimulations in previous studies (11). The finding that these reductions in calcium levels were correlated with the decreases measured in norepinephrine secretion demonstrates that neurosecretory habituation to repetitive depolarizations is the result of decreased calcium levels in PC12 cells. If other regulatory factors exist which act on habituation at a point other than calcium homeostasis, they appear to remain constant during this process.

One component responsible for the decreased calcium levels during habituation was a potassium channel. Blockage of high K+-stimulated norepinephrine secretion by TEA was reduced from 40 to 15% as a result of repetitive high K+ stimulations, suggesting that a potassium channel (or channels) was less active in the habituated cell. Two potassium channel blockers, TEA and cesium, were able to reduce phase 1 calcium levels.
by 26 and 21%, respectively. Using the above-mentioned function describing the relationship between calcium and norepinephrine secretion, these reductions can account for the ability of these agents to block secretion within 4%. This demonstrates that blockage of secretion by a potassium channel was the result of its ability to lower calcium levels and is consistent with the finding that calcium alone is responsible for habituation. While potassium channel blockers such as TEA could block a substantial amount of norepinephrine secretion in response to high K+, they could not account for more than half of the maximal level of secretory reduction seen in these (Fig. 3) and other experiments (11), suggesting that there is another component contributing to the calcium reductions responsible for habituation.

Habituation of the potassium channel was reversed by PMA. In habituated cells, the potassium channel blocker TEA was only able to block 15% of the high K+-stimulated norepinephrine secretion (Fig. 3). After PMA addition, the ability of TEA to block secretion returned to 40%, the value measured in nonhabituated cells. Therefore, PMA was able to reactivate potassium channels which had become habituated in response to repetitive depolarizations. This reactivation lasted for at least 1 h after PMA addition, suggesting that PMA could reset these channels for an extended period of time. It has been previously shown that PMA can alter habituation in response to repetitive depolarizations by increasing the amplitude of norepinephrine secretion, as well as decreasing the rate of habituation (11). Our studies confirm these observations and show that PMA decreases neurosecretory habituation by reactivating a habituated potassium channel.

Our results with a clonal cell line are consistent with results seen in other systems. The demonstration of reduced calcium levels during repetitive stimulation is analogous to the calcium current reductions reported for the gill-withdrawal reflex in Aplysia (9). Furthermore, stimulus-dependent closure of a TEA-sensitive voltage-gated potassium current has been demonstrated in molluscs (26). While the lengths of the depolarizing stimulus we have used were necessarily longer than those used in electrophysiological experiments, experiments in molluscan neuron somata demonstrate that recovery of outward potassium currents from inactivation is a slow process, taking at least an order of magnitude longer than the length of the stimulus (26), and this is also the case in PC12 cells (11).

It is important to note that evoking depolarization by increasing extracellular potassium did not evoke an action potential, but a change in equilibrium potential. Thus, if the potassium channel we have shown to contribute to habituation is an outwardly conducting channel, such as a delayed rectifier channel, its closure may have a potentating effect on stimulants other than high K+. Closure of an outward channel may lengthen the duration of an action potential, and this could allow calcium channels to remain open for a longer period of time, as has been shown in the molluscan neuron somata (27), leading to increased secretion. Therefore, habituation of a potassium channel in PC12 cells may potentiate norepinephrine secretion by other agonists which stimulate calcium elevation through the generating of an action potential.

Future work is needed to clarify other aspects of the biochemistry of habituation, in particular the identification of other components responsible for calcium reduction and the factors governing feedback in the cell. However, it is intriguing that many of the complex responses of neurons can be replicated in clonal cells devoid of any synaptic connections. This should allow the detailed elucidation of the biochemistry underlying fundamental neurological phenomena.

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