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Habituation in the single cell: Diminished secretion of norepinephrine with repetitive depolarization of PC12 cells

(memory/protein phosphorylation/protein kinase C/phorbol esters/calcium)

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ABSTRACT **Neuronally differentiated PC12 cells secrete decreasing amounts of [^{3}H]norepinephrine when repetitively stimulated by depolarizing concentrations of potassium ion. The decreasing response shows attributes that have been classically ascribed to response habituation, a behavior commonly observed in nervous systems but found here in a homogeneous cell type. Alteration of the habituation pattern was caused by activators of the protein kinase C pathway and of voltage-gated calcium channels.**

Memory is defined as "the modification of behavior by experience" (1) and is generally now considered to involve changes induced at the cellular or synaptic level of neurons. Several systems in which stimuli alter subsequent behavior—long-term potentiation (2) and habituation (3)—have been used as rudimentary models of memory, and more complex systems (4) have been shown to have characteristics that overlap with these simpler systems. It would be of theoretical interest and practical value if a single cultured cell system were shown to have these properties because it would establish that feedback from different types of cells is not necessary. Such a system would allow the biochemistry of the process to be studied more effectively because many identical cells would be available.

PC12 cells, derived from a rat pheochromocytoma, have been widely used as a model neuronal system (5). When cultured with nerve growth factor, PC12 cells develop long neuritic outgrowths (6), become electrically excitable (7), and take on many of the biochemical traits of sympathetic noradrenergic neurons (5). Membrane depolarization, mediated by raising the K^+ concentration in the medium, evokes neurosecretion of catecholamines from these cells (8). Ca^{2+} influx through voltage-gated channels is the proposed second messenger for neurosecretion (9). Phorbol ester treatment increases secretion by PC12 cells (10), presumably through the activation of protein kinase C (11).

To test whether neuronally differentiated PC12 cells would be an appropriate model system for habituation and memory, cells were presented with repetitive membrane depolarization, and their neurotransmitter secretion was measured. The resulting cellular behavior satisfies several of the criteria that have been used to classify habituation in organisms (12). The effects of a Ca^{2+}-channel agonist and protein kinase C activation by phorbol esters were also tested. The results reveal that habituation occurs within single cells and is affected by important second messenger systems.

EXPERIMENTAL PROCEDURES

Cells and Media. The rat pheochromocytoma cell line PC12 was obtained from the American Type Culture Collection and was cultured on dextran beads in the presence of nerve growth factor for 7–10 days. Culture medium was formulated as RPMI 1640 growth medium/10% heat-inactivated horse serum (56°, 30 min)/5% fetal bovine serum/penicillin (20 units·ml^{-1})/streptomycin (20 units·ml^{-1})/20 mM sodium Hepes, pH 7.4 (6). Test media for secretion measurements were formulated as reported (8). Low K^+ medium was 125 mM NaCl/4.8 mM KCl/1.3 mM $CaCl_2$/1.2 mM Mg SO_4/1.2 mM potassium phosphate/5.6 mM glucose/1 mM ascorbate, and 25 mM Hepes, pH 7.4. High K^+ medium was 69 mM NaCl/56 mM KCl and otherwise identical to low K^+ test medium. Phorbol 12-myristate 13-acetate (PMA) (Sigma) and Bay K 8644 (Calbiochem) were added to media using dimethyl sulfoxide vehicle at a final concentration of 0.1% (vol/vol), a concentration of vehicle that had no measurable effect on cellular secretion.

[^{3}H]Norepinephrine Loading of Cells. Cells (5×10^6) on dextran beads were suspended in 5 ml of low K^+ medium to which was added [^{3}H]norepinephrine (L-[7,8-^{3}H]norepinephrine; 20 μCi (1 Ci = 37 GBq) in 20 μl of vehicle containing 20 mM acetic acid/ethanol (9:1); 30 Ci mmol^{-1} (Amersham). The suspensions were then incubated (37°, laboratory atmosphere) for 1 hr with the radiolabel, with gentle swirling every 15 min. Approximately half of the radiolabel in the medium was taken up by the cells in this time and was evidently stored in secretory granules (13, 14) because we found that >80% of the intracellular radiolabel would sediment after lysis of the cells with digitonin. The cell-covered beads (10^6 cells; 5×10^4 beads; 4×10^6 dpm) were then transferred to a perfused cell-chamber constructed from a plastic-syringe barrel (0.15-ml internal volume) and with a glass-fiber filter at its outlet.

[^{3}H]Norepinephrine Secretion Measurements. Cellular secretion of [^{3}H]norepinephrine was measured by a method adapted from previous studies (15). Medium was delivered to the cell chamber by a peristaltic pump (0.28 ml·min^{-1}), the stream of medium exiting the cell chamber was mixed with a stream of scintillation fluid (2.2 ml·min^{-1}), and the mixed streams then entered a flow-through scintillation detector (Flo-One Beta; Radiomatic Instruments and Chemical, Tampa, FL). The number of cpm of radioactivity passing through the scintillation counter was updated and recorded every 0.1 min. Secretion was evoked by switching the medium coming into the cell chamber to high K^+. The lag time between switching to high K^+ and the appearance of the response has been subtracted in all figures. [^{3}H]Norepinephrine secretion measurements have been corrected for the ongoing loss of radiolabel and are displayed as the percentage of remaining cellular [^{3}H]norepinephrine released per min. The total amount of radiolabel present in the cells was determined after lysis of the cells by detergent at the end of each experiment with 1% (vol/vol) Triton X-100. During 1- to 3-hr experiments, between 10 and 30% of the initial cellular radiolabel was released, depending on the number of stimu-

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Abbreviation: PMA, 4β-phorbol 12-myristate 13-acetate.

lations applied. Response sizes were measured by calculating the areas under the [^{3}H]norepinephrine peaks after correction had been made for the ongoing loss of radiolabel.

RESULTS

Cellular Habituation with Repetitive Membrane Depolarization. The effect of repetitive stimulation with high K^+ depolarization on norepinephrine secretion was studied in PC12 cells. A single presentation of high K^+ is known to stimulate norepinephrine secretion from PC12 cells (8). The response of the cells, defined as the amount of [^{3}H]norepinephrine secreted with each high K^+ stimulus, was found to diminish steadily with certain patterns of repetitive stimulation, such as the presentation of high K^+ for 1 min every 5 min (Fig. 1).

Other temporal patterns of stimulation were tested, and the decrease in the response was found to depend on the frequency of high K^+ stimulation (Fig. 2). Habituation was strongest with frequent stimulation, such as with 1-min stimulations every 5 or every 10 min, in which the response decreased to ≈25% and 80% of its initial size, respectively. The response size remained nearly constant when 1-min stimulations were presented every 20 min.

Habituation also depended on the duration of the high K^+ stimulations. Five-minute stimulations at a frequency of every 10 min led to a decrease in the response that was twice as fast as did 1-min stimulations applied at this same frequency. The response was constant with 5-min stimulations every 40 min.

To correct for the possibility that the decrease in [^{3}H]norepinephrine release arose artifactually from the depletion of cellular radiolabel, the secretion data shown here have been corrected for ongoing loss of cellular radiolabel, determined by lysing the cells at the end of each experiment and measuring their content of [^{3}H]norepinephrine. Also, the intracellular content of radiolabel was depleted in constant proportion with the total intracellular store of catecholamine, which was assayed by standard biochemical procedures. Finally, control measurements showed that the released radiolabel cochromatographed with genuine norepinephrine. Thus, the diminishing size of the response peaks accurately reflects a decrease in norepinephrine secretion.

To test the duration of habituation, prolonged rest periods were allowed during repetitive K^+ stimulation (Fig. 3). In a typical experiment, high K^+ stimulations were presented every 5 min until over half of the cellular response had been lost through habituation. The cells were then allowed to rest for 20 min, after which a significant restoration in the response was found. Subsequent repetitive stimulation led to further habituation. A partial increase of the response was again observed after another 20-min rest interval was allowed.

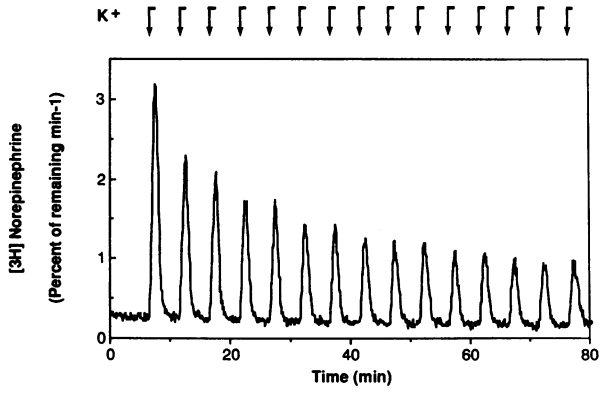

FIG. 1. [^{3}H]Norepinephrine secreted from PC12 cells with repetitive depolarizing stimulation. [^{3}H]Norepinephrine-loaded cells were placed in a perfused chamber (see *Experimental Procedures*) and stimulated with high K^+, as shown by arrows and horizontal bars. High K^+ stimulations were presented for 1 min every 5 min.

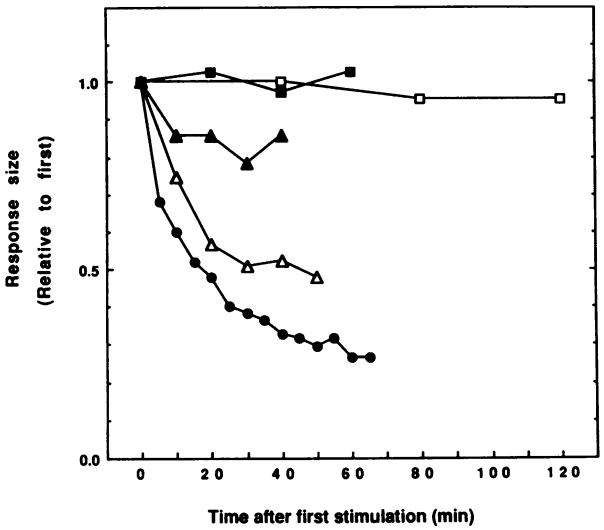

FIG. 2. Size of [^{3}H]norepinephrine response peaks depending on the frequency and duration of depolarizing stimulation. Five experiments are summarized on a scale normalized to the size of the first response peak in an experiment. Each point represents the area beneath a detected peak of norepinephrine elicited by high K^+. ●, ▲, and ■, stimulations of 1-min duration; △ and □, stimulations of 5-min duration. Frequencies of stimulation in the five experiments were as follows: ●, stimulation every 5 min (data replotted from Fig. 1); ▲, △, stimulation every 10 min; □, stimulation every 20 min; and ■, stimulation every 40 min.

Ca^{2+} and Phorbol Ester Disruption of Habituation. The dihydropyridine drug Bay K 8644 (16), an activator of voltage-gated Ca^{2+} channels, was tested for its ability to disrupt habituation. Application of Bay K 8644 to habituated cells increases secretion (Fig. 4). Here, Bay K 8644 was applied continuously, beginning at the time of the fifth stimulation by high K^+, and the increase in secretion was rapid in onset. This and the next two responses to high K^+ were restored in size to within the range of the initial peak size. It appears, then, that increased Ca^{2+} entry into the cell, due to this channel activator, can overcome habituation. The secretory response size then decreased with further high K^+ stimulation, despite continued exposure to Bay K 8644 (Fig. 4), the decrease likely being due to a detrimental effect of Bay K 8644 because exposure to this agent beyond the time shown

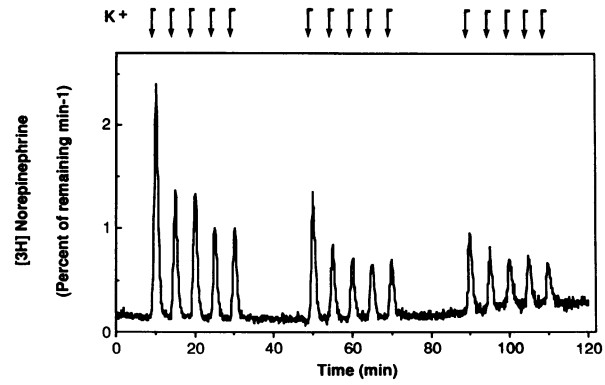

FIG. 3. [^{3}H]Norepinephrine secreted from PC12 cells with repetitive depolarizing stimulation showing the effect of rest. High K^+ stimulation was for 1 min every 5 min, except at two intervals when the period between stimulations was 20 min.

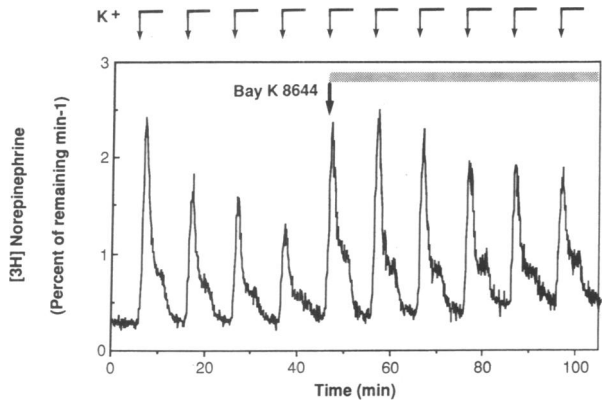

FIG. 4. Effect of the Ca^{2+} channel-specific drug, Bay K 8644, on [^{3}H]norepinephrine secretion during repetitive depolarizing stimulation. [^{3}H]Norepinephrine-loaded cells were stimulated with high K^+ for 5 min every 10 min, as shown by arrows and horizontal bars. Bay K 8644 (1 μM) was applied to the cells at the time shown by the bold arrow and was present for the remainder of the experiment, as shown by the shaded horizontal bar.

resulted in a precipitous decrease in secretion that was not restored by removal of the drug.

Phorbol esters were found to increase the cellular response in a variable manner, depending on whether the cells are habituated or not. When cells were first habituated by repetitive depolarizing stimulation, a transient application of PMA resulted, within 5 min, in an increase in the size of the response of ≈50% over that expected had habituation been unaffected (Fig. 5*A*). The response size then decreased again with further high K^+ stimulation, decreasing to the previously habituated size within ≈60 min of the PMA application.

In the same experiment, a second dose of PMA was applied 90 min after the first, and the response was only slightly increased (Fig. 5*A*). The failure of a second dose of PMA to increase the response as effectively as the first shows that simple washout of the first dose is not the reason for the eventual decrease in the response.

PMA interferes with later development of habituation (Fig. 5*B*). When PMA was applied to cells before frequent depolarizing stimulations, no habituation was observed for over 30 min, and the response size then diminished only slightly over the next 60 min. Prior treatment with PMA can thus inhibit the habituation, causing cells to secrete nearly constant amounts of norepinephrine, with a pattern of repetitive high K^+ stimulation that would otherwise produce very obvious habituation.

To test the possibility of a reciprocal effect, an experiment was performed in which PMA was first applied to cells and high K^+ stimulations were then presented sufficiently infrequently that little habituation would be expected. An unusual pattern of secretion was observed (Fig. 5*C*), in which the size of the secretory response increased from the first to the second high K^+ stimulation by ≈80% and then remained at this high level for the next hour. This result shows that the effects of PMA continue to build for at least 20 min in the absence of habituation. PMA is thus most effective in increasing secretion in the absence of habituation.

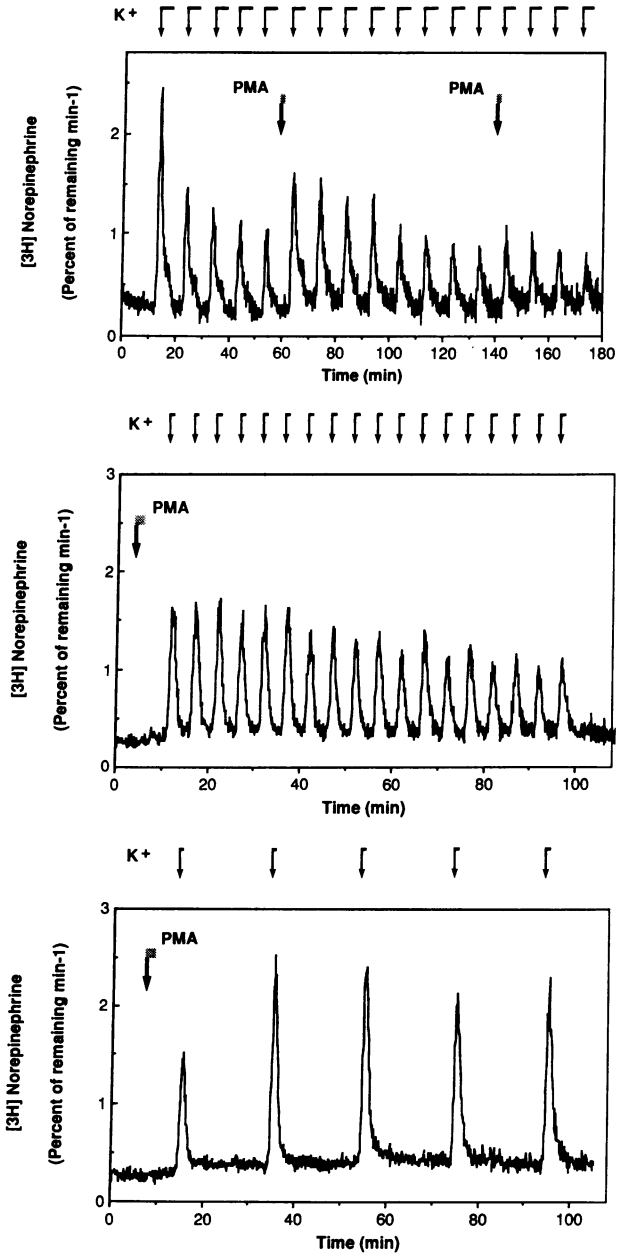

FIG. 5. Effect of PMA on [^{3}H]norepinephrine secretion with high K^+ stimulation at the times shown by arrows and for the durations shown by horizontal bars. PMA (0.1 μM) was applied to the cells for 2 min at the times shown by bold arrows. (*A*) PMA applied during frequent stimulation; high K^+ stimulation was for 5 min every 10 min. (*B*) PMA applied before frequent stimulation; high K^+ stimulation was for 1 min every 5 min. (*C*) PMA applied before infrequent stimulation; high K^+ stimulation was for 1 min every 20 min.

DISCUSSION

Autonomous Cellular Habituation. These studies indicate that neuronally differentiated PC12 cells are capable of habituation, often termed the simplest form of learning. Habituation has previously been studied in cellular detail in many model systems, including vertebrates (17, 18); invertebrates (19–21); polysynaptic pathways such as the mammalian spinal reflex (22); monosynaptic pathways in molluscs (3, 23, 24); crustaceans (25); and monosynaptic reflex pathways in amphibians (26). In *Aplysia*, homosynaptic depression through decreased Ca^{2+} entry into sensory neurons has been proposed as a mechanism of habituation (27). In each of the above systems, the presence of cell–cell interactions has left open the question as to whether such a memory can occur in the single cell, in contrast to communication between cells.

Synaptic input between cells, as the mechanism of habituation in PC12 cells, seems unlikely, because few cell-to-cell synapses are seen in microscopic examination (5), and it would therefore have to be argued that any such synapses would have enormous quantitative effects. Any diffusible regulatory molecule secreted by the cells into their culture

medium would have only autoregulatory effects because of the uniformity of the cells. The habituation of PC12 cells described here thus reflects autonomous biochemical regulation of the single cell.

While several terms may be used to describe a decrease in a response (desensitization, adaptation, depression, inhibition), the term habituation seems most appropriate for the observed cellular behavior because it explicitly serves to describe changes in behavior with repeated stimulation. Nine criteria for habituation in complex organisms have been enumerated by Thompson and Spencer (12, 19), and we have evaluated the behavior of PC12 cells with respect to several of these. The primary criterion of decreased responsiveness with repetitive stimulation is clearly observed when cells are repetitively stimulated by high K^+ medium. A second criterion, increased habituation with increased frequency of stimulation, is also met by repetitively stimulated cells. This frequency dependence shows that the decreasing response is not simply due to deterioration of the cells in the course of the experiment.

A third criterion, recovery of responsiveness when stimulation is withheld, is also a property of these cells. At least part of the cellular responsiveness is recovered with a rest period, showing that an endogenous mechanism operates between stimulations in opposition to the down-regulation of secretion with habituation. Responsiveness that is not restored with a rest period does reflect a long-term down-regulation of secretion. Alternatively, PC12 cells may demonstrate a fourth criterion of habituation that is observed in animal systems—i.e., prolonged recovery times caused by depressing a system below its habituated level (12).

A fifth criterion for habituation that was met by the cells was dishabituation of the response by a heterologous signal. A common manipulation in animal studies is the presentation of a second form of stimulation that interrupts habituation, often by increasing the sensitivity of the system (28). PMA and Bay K 8644, activators of protein kinase C and of voltage-gated Ca^{2+} channels, respectively, were shown to dishabituate cells.

The period of information storage, an important feature of any memory mechanism, was deduced on the basis of the longest interval between repetitive stimulations that could produce habituation. The decrease in cellular responsiveness in single PC12 cells can persist for up to ≈20 min. That length of time is characteristic of short-term memory for some organisms and long-term memory for others.

The similarity of habituation in PC12 cells and in the model systems studied by others does not indicate that all biochemical systems will be similar but does indicate that principles of information processing may be similar in these different types of systems. The method we have used to depolarize cells, delivery of external high K^+, does not allow as rapid and precise variation of membrane voltage as do electrophysiological voltage clamp methods. On the other hand, the method has the clear advantage of being free of feedback effects from postsynaptic cells, simplifying the interpretation of the effects of externally applied agents.

Interplay of Second Messenger Systems and Cellular Habituation. Cells are dishabituated by increased Ca^{2+} entry and by PMA treatment, indicating that known second messenger systems can oppose the cellular mechanism of habituation. Bay K 8644 is a dihydropyridine derivative known to activate L-type Ca^{2+} channels in numerous preparations (16, 29, 30). Other laboratories have detected this channel in PC12 cells, though it is reportedly present in reduced numbers after nerve growth factor treatment for several weeks (31–33). In our work, which involved culturing cells for 1 week with nerve growth factor, this channel appeared to contribute to most of the depolarization-mediated secretion by these cells, as shown by the complete blocking of secretion by verapamil (data not shown), an antagonist of the same class of channels.

Ca^{2+} influx is a requirement for depolarization-mediated neurosecretion in PC12 cells. A simple interpretation of dishabituation of PC12 cells by Bay K 8644, then, is that increased Ca^{2+} entry into the cell due to this agent can compensate for a down-regulation of Ca^{2+} signaling during habituation. Down-regulation of Ca^{2+} current in nerve endings has been proposed as a mechanism of habituation in *Aplysia* (27, 34). A major question now is how the Ca^{2+} signal may be down-regulated and how this information is stored between depolarizing stimulations. PMA activates secretion in a variety of Ca^{2+}-dependent systems, presumably through its activation of cellular protein kinase C. It has been proposed that the action of PMA involves regulation of the intracellular Ca^{2+}-sensing mechanism involved in secretion because PMA raises the Ca^{2+}-sensitivity for secretion in cells made permeable to Ca^{2+} (10, 35–37). However, precise interpretation of how PMA affects the habituated cell is complicated by the fact that many proteins are substrates for protein kinase C and many processes may, therefore, be affected by activation of this kinase (38, 39), including a blockade of inward Ca^{2+} currents (40) that could be important in modulating secretion from habituated cells. A further complication is the possibility that PMA may not be absolutely specific as a protein kinase C-activating agent: decreased Ca^{2+} currents due to a protein kinase C-independent action of phorbol esters have been reported, though these measurements were made at a 100-fold higher PMA concentration than used here to dishabituate PC12 cells (41). Finally, PMA treatment not only activates cellular protein kinase C but may lead to the disappearance of the Ca^{2+}-regulated form of the enzyme through proteolytic modification (42–44), possibly explaining our observation of a refractoriness of repetitively stimulated PC12 cells to a second dose of PMA.

Our experiments have shown that neuronally differentiated tumor cells have a memory capacity such that distinct temporal patterns of stimulation, presented over many minutes, can alter the secretory response of the cells. Ongoing work with other agents that can stimulate secretion has indicated that PC12 cells can habituate to other stimuli (45). Understanding habituation in the single cell can help to form a basis for how signals are processed by circuits of neurons.

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