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Factors affecting habituation of PC12 cells to ATP

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Extracellular ATP triggers catecholamine secretion from PC12 cells by activating ionotropic purine receptors. Repeated stimulation by ATP leads to habituation of the secretory response. In this paper, we use amperometric detection to monitor the habituation of PC12 cells to multiple stimulations of ATP or its agonist. Cells habituate to 30 μM ATP slower than they do to 300 or 600 μM ATP. Modifying external Mg^{2+} affects the response of cells to 30 μM ATP, but does not affect habituation, suggesting that habituation does not necessarily correspond to either stimulus intensity or cellular response. Mg^{2+} affects the initial response of PC12 cells to 2MeSATP in a manner similar to ATP. Increasing external [Mg^{2+}] to 3.0 mM, however, eliminates habituation to 2MeSATP. This habituation can be partially restored by costimulation with 100 μM UTP. Background application of UTP increases habituation to both ATP and 2MeSATP. This suggests that ATP-sensitive metabotropic (P_2Y) receptors play a role in the habituation process. Finally, although Ca^{2+} influx through voltage-operated calcium channels does not appear to contribute to secretion during ATP stimulation, blocking these channels with nicardipine increases habituation. This suggests a role for voltage-operated calcium channels in the habituation process.

Keywords: voltage-operated calcium channels; PC12 cells; habituation; inactivation; P_2X receptors.

While ATP is commonly known as an energy storage molecule, it also serves as a neurotransmitter. ATP activates both ionotropic (P_2X) receptors, triggering neurosecretion, and metabotropic (P_2Y) receptors, which induce the production of inositol phosphates, diacylglycerol and cyclic AMP, and inhibit L-type calcium channels [1].

PC12 cells are a convenient model for ATP-induced secretion. When stimulated, these cells release catecholamines, ATP, and a wide variety of other neurotransmitters and neuromodulators [2,3]. Several ligands, including purinergic and cholinergic ligands [3,4], trigger Ca^{2+} influx, which activates exocytotic catecholamine secretion. ATP, for example, activates a ligand-gated cation channel permeable to Na^+ and Ca^{2+}, triggering exocytosis [3,5–7]. Several factors modify the response of PC12 cells to ATP, including stimulus intensity [8], exposure to neuromodulators [9] and previous stimulations that the cell may have experienced [8,10].

One such modification is habituation, which is defined as the progressive decrease in the response of a cell to repetitively applied stimulations. Cheever and Koshland [8,10] correlated habituation of the exocytotic response of PC12 cells to ATP with a decrease in Ca^{2+} influx during ATP stimulation, elegantly demonstrating that habituation to ATP is ultimately due to inactivation of the ionotropic P_2X receptors.

The results of some studies have suggested that the P_2X_2 receptors found in PC12 cells do not readily inactivate [11,12]. The studies cited, however, examined ion channels expressed in HEK cells and oocytes. Cellular components necessary for desensitization in the native environment of the channels might not be present in the transfected cells. Indeed, recent work by Ding and Sachs [13] shows desensitization of P_2X_2 channels in HEK cells under when the cell membrane is punctured in the presence of external Ca^{2+}. We are therefore comfortable supporting the interpretation of Cheever and Koshland.

Work by Chow and Wang [9] has suggested that phosphorylation of receptor-channels is necessary for habituation. They transfected cells that do not normally express P_2X channels with P_2X_2 receptor-channel cDNA from PC12 cells. By measuring ion influx triggered by ATP stimulation, they demonstrated that the response of the cell to brief stimulations with ATP did not desensitize unless the cell was treated with 8-Br-cAMP or the purified catalytic subunit of PKA. Recent work by Chen and Bobbin [14] supports this finding by showing that increasing protein kinase A phosphorylation of the P_2X receptor down-regulates P_2X activity. Other groups [15,16] have examined the structural nature of P_2X channels that allows habituation.

In this paper we show that habituation is not a necessary consequence of stimulation, and suggest that habituation is controlled by metabotropic receptors acted upon concomitantly with ATP activation of ionotropic receptors. We also show that when ATP depolarizes cells, the subsequent opening of L-type Ca^{2+} channels does not enhance secretion but does decrease habituation.

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Abbreviation: VOCC, voltage-operated calcium channel.

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Materials and methods

PC12 cell culture

PC12 cells were grown on cell culture dishes in Dulbecco's modified Eagle's medium with 10% (v/v) horse serum and 5% (v/v) fetal bovine serum, supplemented with 50 IU·mL^{-1} penicillin and 50 μg·mL^{-1} streptomycin. No nerve growth factor was added to solution. Cells were nevertheless observed to differentiate in culture, suggesting the presence an endogenous growth factor. The culture medium was replaced once every 3 days, and the cells were passed to avoid confluence.

One day prior to an experiment, cells from culture dishes were transferred to Petri dishes containing cytodex 3 beads. Cell-coated beads were then loaded into an HPLC fitting (total volume 62 μL) which served as a cell chamber. This was then connected to the flow-through apparatus (described below) and placed in a water bath maintained at 30 °C.

Flow-through apparatus

Exocytosis of the PC12 cells was measured with an amperometric detector mounted in a flow-through apparatus. Pressurized air was used to move the contents of the buffer solution bottles through polyethylene lines to a six-port injection valve. Stimulants were added to the background solution without affecting the pressure or flow rate of the system. From the valve, solution traveled to the cell chamber, flowed over the beads, and passed over an amperometric detector set at 0.45 V. Catecholamines that passed over the electrode were oxidized, generating a current proportional to their concentrations, which was recorded on a chart recorder. Intensity of response was measured as the maximum amplitude of current generated during the secretory response to a given stimulation. Peak amplitudes generally ranged from 1 to 50 nA. Current across the electrode was monitored for the full duration of the experiment.

Cell stimulation in flow-through apparatus

Stimulation of the cells was accomplished using a six-port injection valve. Solution containing either ATP or its analogs was injected into the 100 μL loading loop of the injector valve. When it was time to stimulate the cells, the valve was switched so that the solution flowed through the loading loop to the cell chamber. At a flow rate of 1 mL·min^{-1}, the cells were stimulated for ≈ 6 s. Norepinephrine standards were used to determine the response of the detector and the dispersion of ATP and its analogs during stimulation. These tests indicated that stimulants loaded in the loading loop were diluted approximately threefold by the time they reached the test chamber. All stimulants were therefore injected into the loading loop at three times the desired concentration.

In all experiments, the cells were given a single reference stimulation in Locke's solution (in mM: 154 NaCl, 5.6 KCl, 2.2 $CaCl_2$, 1.2 $MgCl_2$, 10 glucose, 5 HEPES, pH 7.3) prior to switching to test conditions (Fig. 1A). This was carried out to ascertain if the test conditions affected the response of the cell to the stimulant being used. During habituation the cells were stimulated once every 5 min. If the background solution of the cells was switched from the standard Locke's solution to a modified solution, e.g. a Locke's solution with 100 μM UTP, the cells were allowed 10 min to adjust to the change in conditions before the habituation stimulations were begun.

This reference stimulation was also carried out to normalize the results of each study. The distribution and configuration of the cells on the beads was not generally uniform. This not only makes it impossible to count the cells, but also interferes with determining active cell numbers using other methods, such as total protein assay, which do not reflect the degree to which cells have access to medium. Data were therefore recorded as ratios (described in data analysis). By doing this, we consider only the secretory sites of the cells that are exposed to the medium.

In contrast to experiments in which plates of cells are stimulated for minutes to measure habituation, our experiments are for much shorter times and the amount of catecholamine release is under 1% of cell content. Direct evidence that the habituation we observe is not depletion of secretion-ready granules is shown by the data of Fig. 2 (bars 6 and 10), 4, and 5. In 3.0 mM Mg^{2+}, ATP and 2MeSATP cause equivalent secretion but very different degrees of habituation.

Data analysis

To determine the effect of a test condition on the response of PC12 cells to a stimulant, the first response of cells under test conditions was divided by the response of the cells to an identical stimulation under control conditions given 10 minutes earlier (Fig. 1, B/A). To allow comparisons of the relative amplitude of cellular responses, each response was scaled to a standard, in this case 300 μM ATP under control conditions. This was accomplished by multiplying the effect of each condition to a stimulus (B/A) by the ratio of the cellular response of that stimulus to 300 μM ATP (F/G). The term 'scaled response' will refer to the response of PC12 cells to a stimulus under a particular condition that has been normalized to the response of PC12 cells to 300 μM ATP under control conditions. The scaled response of PC12 cells to ATP and 2MeSATP in the various conditions studied are shown in Fig. 2.

Habituation of the cells to a stimulant under different conditions (as shown in Figs 3–6) is reported as relative response, which is defined as the ratios of the amplitude of each response (B,C,D,E) in the run to the amplitude of the initial response of that run (B). Habituation will be recorded in text as a percentage of the fourth stimulation relative to the first stimulation of the habituation test. That is $(E/B) \times 100\% \pm$ SEM.

Habituation data was analyzed with two-way ANOVAS with repeated measures followed by Bonferroni's *post-hoc* tests. One-way ANOVAS were used to determine significant differences in secretory responses. Analysis was carried out using SPSS 9.0 for Windows (SPSS Inc.). Significant differences were assumed at $P < 0.05$. Constraints in growing conditions, apparatus requirements, and resources often made it impractical to run a full complement of control runs per experiment. Only one or two control runs therefore typically accompanied each set of experimental runs. The

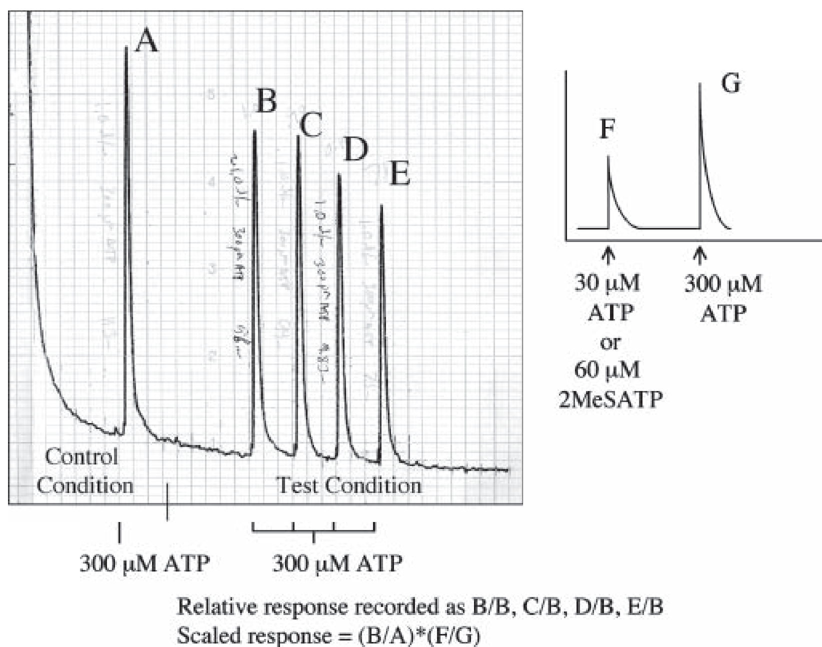

Fig. 1. Method for data analysis. Cells were stimulated once under control conditions (A), switched to test conditions, allowed 10 min to adjust to changes in conditions, and given four stimulations (B–E) spaced 5 min apart. Comparisons between stimulants (30 μM ATP and 300 μM ATP, for example) were made by stimulating individual groups of PC12 cells with both stimulants (F,G) under control conditions. The effect of test conditions on cellular response to a stimulus was determined by dividing the peak current generated by the first stimulation under test conditions (B) by the peak current generated under control conditions (A). The ratio of F/G was then used to scale the cellular responses to the various stimuli and conditions to a single standard, 300 μM ATP under control conditions (Fig. 2). Habituation was recorded as the peak current of each stimulation in test conditions (B,C,D,E) divided by the peak current of the first stimulation in test conditions (B). The line in the recording has been enhanced to allow easier visualization.

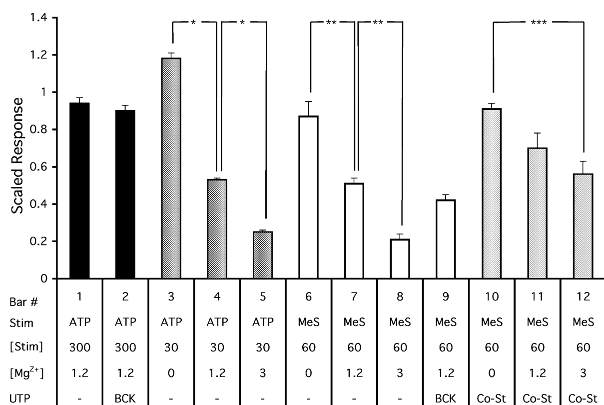

Fig. 2. Initial responses of PC12 cells to stimulation by ATP and 2MeSATP. Responses were normalized as described in the Materials and methods and Fig. 1. 'BCK' indicates the presence of 100 μM UTP in the background solution. 'Co-St' indicates the use of 100 μM UTP as a costimulant. An asterisk indicates a significant difference from 30 μM ATP under test conditions to 30 μM ATP under control conditions ($P < 0.05$). Double asterisks indicate a significant difference between the response of PC12 cells to 60 mM 2MeSATP under test conditions and 60 μM 2MeSATP under control conditions ($P < 0.05$). The triple asterisks indicates a significant difference between the response of PC12 cells to stimulation with 60 μM 2MeSATP/100 μM UTP in 0 mM Mg^{2+} and the response to an identical stimulation in 3.0 mM Mg^{2+} ($P < 0.05$).

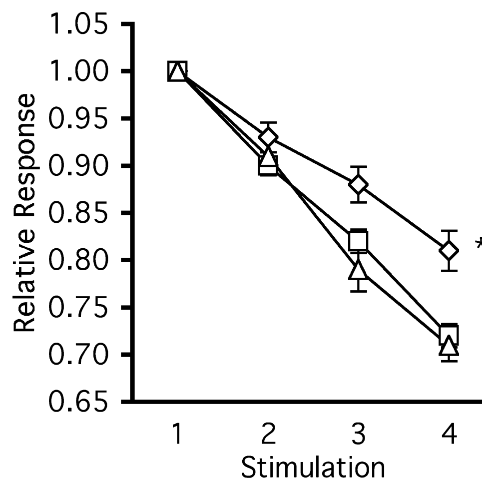

Fig. 3. Effect of [ATP] on habituation of PC12 cells to ATP. Cells were stimulated with 30 μM ATP (◇, $n = 14$), 300 μM ATP (□, $n = 16$), or 600 μM ATP (△, $n = 3$). Asterisk indicates a significant difference from the habituation of cells to 300 μM ATP ($P < 0.05$). Error bars denote one SEM.

control group was run to make sure that the cells and conditions of that day were performing in the same manner that they had on previous occasions. The experimental groups were then compared with the accumulated total of

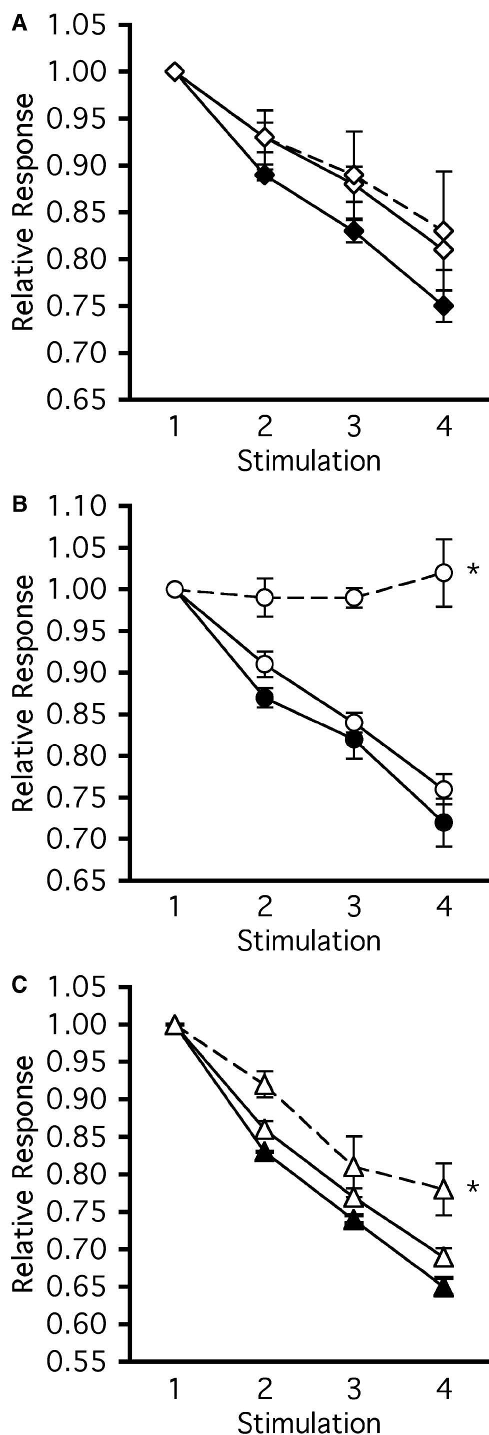

Fig. 4. Effect of Mg^{2+} on the habituation of PC12 cells to 30 μM ATP (A), 60 μM 2MeSATP (B) and 60 μM 2MeSATP with 100 μM UTP (C). All cells were stimulated once in Locke's solution containing 1.2 mM Mg^{2+} before switching to solutions in which the $[Mg^{2+}]$ was adjusted to 0.0 mM Mg^{2+} (solid symbols, solid lines, $n = 3$ for ATP, 3 for 2MeSATP, 3 for 2MeSATP with UTP), 1.2 mM Mg^{2+} (open symbols, solid line, $n = 14$ for ATP, 11 for 2MeSATP, 3 for 2MeSATP with UTP) or 3.0 mM Mg^{2+} (open symbols, dotted lines, $n = 3$ for ATP, 3 for 2MeSATP, 3 for 2MeSATP with UTP). Asterisk indicates a significant difference from the habituation of cells in 1.2 mM Mg^{2+} ($P < 0.05$). Error bars denote one SEM.

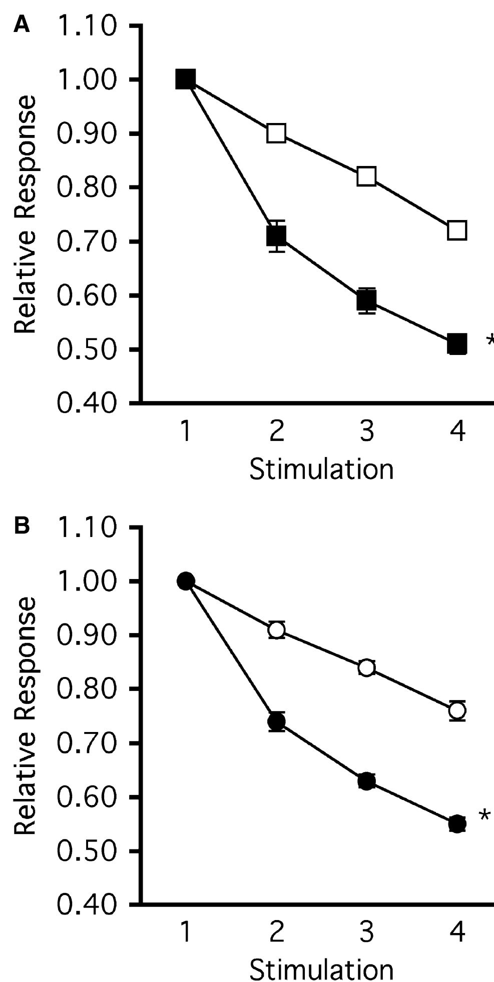

Fig. 5. Effect of prolonged UTP exposure on the habituation of PC12 cells to ATP (A, squares) or 2MeSATP (B, circles). Cells were stimulated with 300 μM ATP or 60 μM 2MeSATP in either a regular Locke's solution (open symbols, $n = 16$ for ATP, 11 for 2MeSATP) or in a background solution containing 100 μM UTP (solid symbols, $n = 3$ for ATP, 3 for 2MeSATP). Asterisks indicate a significant difference from the habituation of cells in the Locke's solution. Error bars denote one SEM.

the control group runs. Analysis of variance within the control runs did not reveal significant variation when the runs were grouped according to day or month, indicating that the degree of habituation observed in response to stimuli is reproducible.

Materials

ATP, $BaCl_2$, $CaCl_2$, Cytodex 3 beads, fetal bovine serum, gramicidin, HEPES, KCl, 2MeSATP, $MgCl_2$, nicardipine, and UTP were obtained from Sigma (St Louis, MO, USA). Glucose and K_2HPO_4 were purchased from Fisher Scientific (Pittsburgh, PA, USA). Horse serum was purchased from Intergen (Purchase, New York, NY, USA). Dulbecco's medium, penicillin, and streptomycin were purchased from Life Technologies, Inc. (Grand Island, NY, USA). PC12 cells were a gift from G. Guroff (NICDH, NIH, Bethesda, MD, USA).

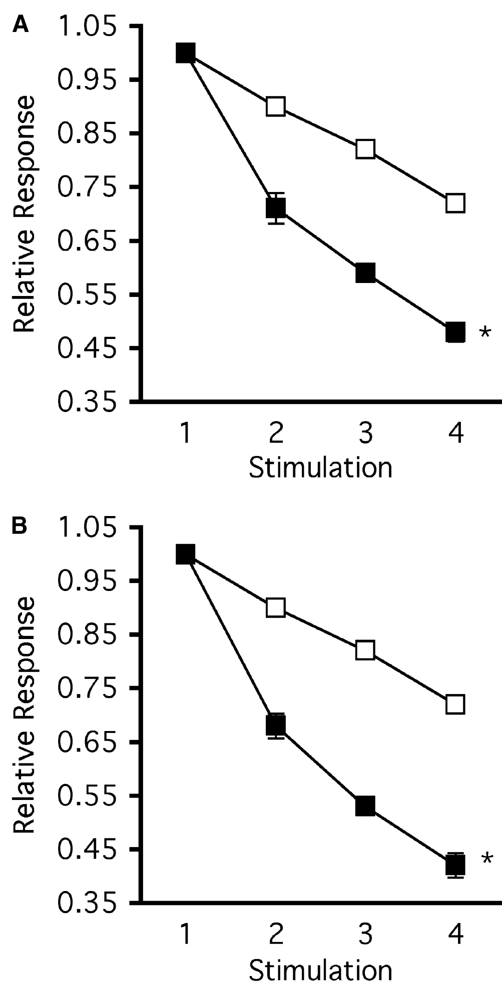

Fig. 6. Other factors affecting habituation to ATP. (A) Effect of the L-type VOCC blocker nicardipine on the habituation of PC12 cells to 300 μM ATP. Cells were stimulated with 300 μM ATP in normal Locke's solution (□, $n = 16$) or a solution containing 10 μM nicardipine (■, $n = 3$). (B) Comparison of cells desensitized to 300 μM ATP in background solutions containing either 2.2 mM Ca^{2+} (□, $n = 16$) or 0.6 mM Ba^{2+} (■, $n = 3$). An asterisk indicates a significant difference from the habituation of cells under control conditions. Error bars denote one SEM.

Results

To determine how the extent of habituation depends on the strength of stimulation, we first altered the strength of stimulation by changing the concentration of the stimulant, ATP. The cells were stimulated with three concentrations of ATP: 30 μM, which produces a release of catecholamine roughly half of the maximum release possible (Fig. 2, bar 4); 300 μM, commonly used concentration to cause maximum secretory response (Fig. 2, bar 1); and 600 μM, which gives the same secretory response as 300 μM ATP (data not shown) but might set in motion ATP-activated processes with lower sensitivity to ATP than those involved in exocytosis.

The degree of habituation observed when the cells were stimulated with 30 μM ATP (81 ± 2%, $n = 14$) was significantly less than that seen with 300 μM ATP (72 ± 1%, $n = 16$) and 600 μM ATP (71 = /– 2%, $n = 3$) (Fig. 3). There was no significant difference between the habituation produced by 300 and 600 μM ATP. Thus, initial results suggested that habituation is affected in parallel with the secretory response.

The second way stimulation intensity was modified was by changing the Mg^{2+} concentration. Mg^{2+} is known to complex with ATP [17], altering the balance of free and complexed ATP. ATP receptors differ in their relative affinity for ATP and its Mg^{2+} complex, thus Mg^{2+} lowers the ionotropic receptor's affinity for ATP, but may not similarly affect other ATP receptors [18,19]. Changing [Mg^{2+}] from 0.0 to 1.2 mM Mg^{2+} halved the initial secretory response of PC12 cells to 30 μM ATP, while an increase to 3.0 mM Mg^{2+} reduced the initial secretory response to a quarter of that seen in 0.0 mM Mg^{2+} (Fig. 2, bars 3–5). This is in agreement with the findings of several groups [18–23]. Mg^{2+} concentration had no effect on the response of the cells to a saturating concentration of 300 μM ATP (data not shown). This is also in agreement with other groups [19,22]. We therefore focused our attention on 30 μM ATP.

We examined the effect of Mg^{2+} on habituation of cells to 30 μM ATP (Fig. 4A). Initial response to 30 μM ATP is twice as great in the 0 mM Mg^{2+} solution, as in the 1.2 mM Mg^{2+} solution approximately similar to the difference between 300 μM ATP and 30 μM ATP in 1.2 mM Mg^{2+}. ANOVA analysis does not indicate that differences in the habituation curves of the three [Mg^{2+}] conditions are statistically significant (0.0 mM Mg^{2+} = 75 ± 2%, $n = 3$, 1.2 mM Mg^{2+} = 81 ± 2%, $n = 14$, 3.0 mM Mg^{2+} = 83 ± 6%, $n = 3$). This suggests that habituation does not necessarily correlate with stimulus intensity, and suggests that other factors may be involved.

ATP activates not only P_2X receptors but also metabotropic P_2Y receptors on PC12 cells [24]. Work described in the introduction suggests a number of possible ways in which these P_2Y triggered pathways could affect habituation. The ATP analog 2MeSATP is a good agonist of the ionotropic receptor, but unlike ATP has little ability to activate the phospholipase C pathway [25]. 2MeSATP can therefore test the involvement of the phospholipase C pathway in the habituation of P_2X mediated exocytosis.

For these studies, we used 60 μM 2MeSATP, which produced a secretory response in 1.2 mM Mg^{2+} solution similar to that of 30 μM ATP at the same [Mg^{2+}]. Figure 2 (bars 6–8) shows the effect of altering the [Mg^{2+}] on the response of PC12 cells to 60 μM 2MeSATP. The response of the cells in a 0.0-mM Mg^{2+} solution was significantly higher than the response in a 1.2-mM Mg^{2+} solution that, in turn, was significantly higher than the response in a 3.0 mM Mg^{2+} solution. As with ATP, Mg^{2+} interferes with exocytosis elicited by 2MeSATP, presumably by interfering with the binding of 2MeSATP to P_2X and P_2Y receptors.

PC12 cells in 0.0 mM and 1.2 mM Mg^{2+} habituated to 60 μM 2MeSATP (0.0 mM Mg^{2+} = 72 ± 3%, $n = 3$, 1.2 mM Mg^{2+} = 76 ± 2%, $n = 11$) to roughly the same degree that they did to 30 μM ATP (Fig. 4B). Increasing the concentration of external Mg^{2+} from 1.2 mM to 3.0 mM, however, virtually eliminated habituation to 2MeSATP (1.02 ± 4%, $n = 3$). This clearly shows that habituation is

not a necessary consequence of stimulation. It takes more than simple activation of P_2X receptors to desensitize them. The uncoupling of secretion and habituation shown in Fig. 5 suggests that one or more metabotropic purinergic receptors involved in habituation are more sensitive to Mg^{2+} than the P_2X receptor.

An established difference between ATP and 2MeSATP is that the latter does not activate the phospholipase C pathway in PC12 cells. UTP is a specific P_2Y agonist that activates this pathway [26]. If this pathway promotes habituation to ATP in 3.0 mM [Mg^{2+}] where none is seen to 2MeSATP, UTP might restore habituation by activating that pathway.

When UTP was used as a costimulant, it caused no significant change in initial secretory response at 0 mM Mg^{2+}, but significantly decreased the effect of increasing [Mg^{2+}] on exocytosis elicited from the cells (compare Fig. 2, bars 6–8 with 10–12). UTP alone did not produce a significant amount of exocytosis in our PC12 cells, ruling out direct stimulation of P_2X receptors by UTP. A background solution containing UTP does not affect secretion in response to 2MeSATP (compare Fig. 2, bars 7 and 9), showing that UTP is not affecting secretion by sequestering Mg^{2+}, in agreement with published dissociation constants (not shown). It seems likely that the synergistic increase in secretion is due to the Ca^{2+} released by UTP from internal stores. While insufficient to trigger substantial secretion, it reduces the diffusion of Ca^{2+} entering through the ion channels, thus increasing the effective [Ca^{2+}] at the secretory sites.

At 0.0 and 1.2 mM Mg^{2+}, habituation to costimulations with 2MeSATP and UTP were not significantly greater than habituation to 2MeSATP alone (Fig. 4C) (0.0 mM $Mg^{2+} = 65 \pm 1\%$, $n = 3$, 1.2 mM $Mg^{2+} = 69 \pm 1\%$, $n = 3$). While UTP did not completely restore habituation to 2MeSATP at 3.0 mM Mg^{2+} to levels seen when ATP was the stimulant, it did significantly increase it ($78 \pm 3\%$, $n = 3$). Therefore the difference in the effect of high [Mg^{2+}] on the habituation of cells to ATP and 2MeSATP can be attributed in part to metabotropic activity stimulated via the UTP-sensitive P_2Y receptor.

Having examined the effect that costimulation with UTP had on the response and habituation of PC12 cells to 2MeSATP and ATP, we then looked at the impact of including UTP in the background solution. We hypothesized that the second messenger activity required for habituation can be triggered by UTP, so that activating the UTP pathway continuously could either increase habituation by priming the inactivating pathway or reduce habituation by desensitizing the inactivatory pathway.

Figure 2 (bars 1, 2, 7, and 9) shows that a continuous application of 100 μM UTP in the background solution had no significant effect on the initial response of cells to either 300 μM ATP or 60 μM 2MeSATP. In contrast, Fig. 5(A,B) shows that a background of 100 μM UTP significantly increased the habituation of PC12 cells to both ATP ($51 \pm 2\%$, $n = 3$) and 2MeSATP ($55 \pm 1\%$, $n = 3$) stimulations. This is a very different outcome from that observed when UTP was used as a costimulant. UTP costimulation increased secretory response, but did not affect habituation. We have suggested that UTP's effect on secretion was due to Ca^{2+} released from internal stores. It is reasonable to suggest that after 10 min of continuous UTP stimulation, the released Ca^{2+} has been sequestered and removed from the internal milieu. This would explain why UTP in the background did not increase secretion. The impact of UTP on habituation will be addressed in the discussion.

Studies by Fasolato *et al.* [21] and our laboratory (G. Balan, unpublished data) suggested that cation influx through P_2X receptor-channels during ATP stimulation is sufficient to activate VOCCs, allowing Ca^{2+} to enter the cell. More recently studies have confirmed this pathway and investigated it in detail [27]. However, several researchers [3,28–31] have demonstrated that treatment with VOCC blockers does not affect the total amount of Ca^{2+} that enters a cell during ATP stimulation.

We explored the possible role of the L-type VOCC in habituation by looking at both the initial response and the habituation of PC12 cells to ATP in the presence of the VOCC blocker nicardipine (10 μM). As with other experiments in which the background solution was altered, the cells were exposed to nicardipine for 10 min before being stimulated to ATP or 2MeSATP. This provided ample time for nicardipine to block L-type VOCC activity.

Nicardipine did not significantly affect the response of the cells in any case (data not shown), in agreement with findings quoted above but in contrast to the result of Kim's laboratory [20]. In contrast to the lack of effect of nicardipine on the initial response, Fig. 6A shows that 10 μM nicardipine increases habituation of PC12 cells to 300 μM ATP ($48\% \pm 2\%$, $n = 10$). Similar effects were observed when 30 μM ATP and 60 μM 2MeSATP were used as stimulants (data not shown). Even though Ca^{2+} influx through the L-type VOCCs appears to have little role in secretion during ATP stimulation, it does decrease habituation.

Nakazawa and collaborators [30,32] have demonstrated that high levels of $[Ca^{2+}]_{in}$ can prevent ion flow through both VOCCs and P_2X receptor-channels in PC12 cells. Others [33–35] have demonstrated that this inhibition of ion flow through VOCCs is likely due to Ca^{2+} directly binding to a cytosolic region of the channels. To assess the effects that this might have on habituation, the 2.2 mM Ca^{2+} in the external solution was replaced with 0.6 mM Ba^{2+}, which triggers exocytosis in a manner and magnitude similar to Ca^{2+}, but does not inactivate ion channels to as great a degree [13].

Figure 6B shows that replacing 2.2 mM Ca^{2+} with 0.6 mM Ba^{2+} produced a dramatic increase in the degree of habituation produced by 300 μM ATP ($42\% \pm 2\%$, $n = 3$). This supports the idea that blockage of ion channels by high $[Ca^{2+}]_{in}$ can decrease the habituation of PC12 cells to ATP.

Discussion

Although this paper represents only a beginning in the study of habituation to ATP, three important findings are clearly demonstrated. The first is that habituation does not necessarily correspond with either stimulus intensity or amount of secretion. Support for this comes from the study employing 2MeSATP in the presence of 3.0 mM Mg^{2+}. 2MeSATP (60 μM) stimulation produces a secretory

response approximating that of 30 μM ATP, and the secretion produced by both stimuli are similarly reduced by the increase in [Mg^{2+}], yet in 3.0 mM Mg^{2+} habituation to ATP is unchanged while habituation to 2MeSATP is essentially eliminated. The secretory responses are nearly identical, but habituation patterns are dramatically different. Support for this finding can also be provided by comparing the effects of UTP as a costimulant and UTP in the background solution. When UTP was used as a costimulant, it increased 2MeSATP induced secretion, but had no effect on habituation. While UTP in the background solution did not increase secretion, it produced a dramatic increase in habituation. Our data therefore shows that there is no necessary correlation between habituation and stimulus intensity or level of secretion.

The second significant finding is that there is a role for multiple purinergic receptor types in the habituation process. This is shown most clearly in the lack of habituation of cells to multiple stimulation with 2MeSATP in the presence of 3.0 mM Mg^{2+}, in contrast to the habituation to ATP observed at the same [Mg^{2+}] and an equivalent level of secretion. The fact that the combination of UTP and 2MeSATP causes habituation intermediate between ATP alone and 2MeSATP indicates that the UTP-sensitive P_2Y purinergic receptor likely plays a role but is not the only metabotropic purinergic receptor involved in habituation. If it were, we would expect complete recovery of habituation, instead of partial recovery. The UTP-sensitive P_2Y receptor activates phospholipase C, leading to release of Ca^{2+} from subcellular stores and activation of protein kinase C. Other purinergic metabotropic receptors can activate other second messenger pathways. Due to the complexity of purinergic signaling pathways, it may be very difficult to determine the exact pathway leading to habituation until more specific antagonists become available.

The third important finding is that factors that modify Ca^{2+} influx affect the habituation process, as shown by increased habituation when L-type VOCCs are blocked by nicardipine. Ca^{2+} regulation of the habituation process is also demonstrated by increased habituation when Ba^{2+} is used in place of Ca^{2+} to support secretion. These conclusions are in accord with previous work showing inactivation of VOCCs and ATP gated channels by Ca^{2+} [30,32] and with recent work showing a Ca^{2+} effect on habituation of P_2X channels using patch clamp methods [13].

To explain how blocking L-type VOCCs could increase habituation, we make four postulations. We first postulate that habituation is due to the desensitization of P_2X receptors. This is reasonable given previous findings [8–10,14]. Second, we postulate that P_2X channels must be in the open, active, state for desensitization to occur. The need is shown in the experiments where UTP was present in the background solution prior to and during habituation. It is important to note that background UTP does not affect the initial response to ATP, only the subsequent ones, i.e. the habituation process. This clearly shows that while the cell is primed for habituation, the process requires activation of the P_2X receptor. Third, we postulate that inactivation of P_2X receptors due to direct Ca^{2+} binding, as described by Nakazawa and Hess [32], is more rapidly reversible than the longer term desensitization triggered by the P2Y pathway. Finally, we postulate that the Ca^{2+} block protects these receptor-channels from the longer term desensitization.

During ATP stimulation, Ca^{2+} will enter the cell through both the P2X receptors and any VOCCs on the cell membrane. Internal [Ca^{2+}] will rise rapidly, therefore Ca^{2+} blockage and protection of the P_2X channel will be rapid, allowing little opportunity for P_2Y-dependent desensitization to occur. If the L-type channels are blocked, Ca^{2+} will enter the cell more slowly and take longer to reach channel-inactivating concentrations. This will allow a greater window of opportunity for the desensitization of P_2X receptor. With or without L-type channels, Ca^{2+} influx will continue until $[Ca^{2+}]_{in}$ reaches levels which block first the VOCCs and then the P_2X receptor-channels. Blocking VOCCs can therefore increase the likelihood of P_2X desensitization without affecting total Ca^{2+} influx.

Our explanation allows us to account for the increase in habituation observed when Ca^{2+} is replaced with Ba^{2+}. A higher internal concentration of Ba^{2+} is required to inactivate the P_2X receptor-channels [13,30]. This will extend the time that these channels are active, and therefore vulnerable to the desensitization processes.

This interpretation also allows a potential explanation of the activity of VOCC blockers on the response of the cells to ATP stimulation. Variation between strains of PC12 cells will likely include differences in ion channel densities. In strains where the density of P_2X receptors is sufficient to trigger maximum exocytosis, VOCCs will merely contribute to the rate of Ca^{2+} influx, not the final [Ca^{2+}]. In strains where P_2X receptor density is smaller, VOCCs may have a greater effect.

Finally, our explanation of the mechanics of ATP habituation also allows us to explain a finding of Cheever and Koshland [8] in which they found that desensitizing PC12 cells to depolarization did not desensitize them to ATP, but did increase the rate at which they desensitized to ATP. When they desensitized their cells to depolarization, they inactivated the voltage-operated channels. According to our explanation, this loss of VOCC activity would not decrease the response to ATP, but it would increase the amount of time that the P_2X receptor-channels remained open during stimulation. This longer time would result in a greater opportunity for the habituation process to take place, and therefore a greater degree of observed habituation.

In summary, we have provided evidence that habituation of PC12 cells to ATP is a process separate from the secretory process and that it involves P_2Y receptor pathways. We have also produced a model that allows for the contribution of VOCCs to Ca^{2+} influx and a role in habituation during ATP stimulation without affecting the secretion that this stimulation produces.

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References

1. Burnstock, G. (1997) The past, present and future of purine nucleotides as signaling molecules. *Neuropharmacology* **36**, 1127–1139.

2. Greene, L.A. & Rein, G. (1977) Release of (^{3}H)norepinephrine from a clonal line of pheochromocytoma cells (PC12) by nicotinic cholinergic stimulation. *Brain Res.* **138**, 521–528.
3. Inoue, K., Nakazawa, K., Fujimori, K. & Takanaka, A. (1989) Extracellular adenosine 5′-triphosphate-evoked norepinephrine secretion not relating to voltage-gated Ca channels in pheochromocytoma PC12 cells. *Neurosci. Lett.* **106**, 294–299.
4. Greene, L.A. & Rein, G. (1977) Release, storage and uptake of catecholamines by a clonal cell line of nerve growth factor (NGF) responsive pheo-chromocytoma cells. *Brain Res.* **129**, 247–263.
5. Fredholm, B.B., Abbracchio, M.P., Burnstock, G., Daly, J.W., Harden, T.K., Jacobson, K.A., Leff, P. & Williams, M. (1994) Nomenclature and classification of purinoceptors. *Pharmacol. Rev.* **46**, 143–156.
6. Nakazawa, K., Fujimori, K., Takanaka, A. & Inoue, K. (1990) An ATP-activated conductance in pheochromocytoma cells and its suppression by extracellular calcium. *J. Physiol.* **428**, 257–272.
7. Nakazawa, K., Fujimori, K., Takanaka, A. & Inoue, K. (1991) Comparison of adenosine triphosphate- and nicotine-activated inward currents in rat phaeochromocytoma cells. *J. Physiol.* **434**, 647–660.
8. Cheever, L. & Koshland, D.E. Jr (1994) Habituation of neurosecretory responses to extracellular ATP in PC12 cells. *J. Neurosci.* **14**, 4831–4838.
9. Chow, Y.W. & Wang, H.L. (1998) Functional modulation of P_2X_2 receptors by cyclic AMP-dependent protein kinase. *J. Neurochem.* **70**, 2606–2612.
10. Cheever, L. & Koshland, D.E. Jr (1992) Retention of habituation in PC12 cells. *Proc. Natl Acad. Sci. USA* **89**, 10084–10088.
11. Ding, S. & Sachs, F. (1999) Single channel properties of P_2X_2 purinoceptors. *J. Gen. Physiol.* **113**, 695–720.
12. North, R.A. (2002) Molecular physiology of P2X receptors. *Physiol. Rev.* **82**, 1013–1067.
13. Ding, S. & Sachs, F. (2000) Inactivation of P_2X_2 purinoceptors by divalent cations. *J. Physiol.* **522**, 199–214.
14. Chen, C. & Bobbin, R.P. (1998) P_2X receptors in cochlear Deiters' cells. *Br. J. Pharmacol.* **124**, 337–344.
15. Boue-Grabot, E., Archambault, V. & Seguela, P. (2000) A protein kinase C site highly conserved in P_2X subunits controls the desensitization kinetics of $P_2X(2)$ ATP-gated channels. *J. Biol. Chem.* **275**, 10190–10195.
16. Brandle, U., Spielmanns, P., Osteroth, R., Sim, J., Surprenant, A., Buell, G., Ruppersberg, J.P., Plinkert, P.K., Zenner, H.P. & Glowatzki, E. (1984) Desensitization of the $P_2X(2)$ receptor controlled by alternative splicing. *FEBS Lett.* **404**, 294–298.
17. Pecoraro, V.L., Hermes, J.D. & Cleland, W.W. (1984) Stability constants of Mg^{2+} and Cd^{2+} complexes of adenine nucleotides and thionucleotides and rate constants for formation and dissociation of MgATP and MgADP. *Biochemistry* **23**, 5262–5271.
18. Reichsman, F., Santos, S. & Westhead, E.W. (1995) Two distinct ATP receptors activate calcium entry and internal calcium release in bovine chromaffin cells. *J. Neurochem.* **65**, 2080–2086.
19. Rhoads, A.R., Parui, R., Vu, N.D., Cadogan, R. & Wagner, P.D. (1993) ATP-induced secretion in PC12 cells and photoaffinity labeling of receptors. *J. Neurochem.* **61**, 1657–1666.
20. Choi, S.Y. & Kim, K.T. (1996) Characterization of Na^+ influx mediated by ATP(4-)-activated P2 purinoceptors in PC12 cells. *Br. J. Pharmacol.* **118**, 935–940.
21. Fasolato, C., Pizzo, P. & Pozzan, T. (1990) Receptor-mediated calcium influx in PC12 cells. ATP and bradykinin activate two independent pathways. *J. Biol. Chem.* **265**, 20351–20355.
22. Kim, W.K. & Rabin, R.A. (1994) Characterization of the purinergic P2 receptors in PC12 cells: evidence for a novel subtype. *J. Biol. Chem.* **269**, 6471–6477.
23. Trezise, D.J., Bell, N.J., Kennedy, I. & Humphrey, P.P. (1994) Effects of divalent cations on the potency of ATP and related agonists in the rat isolated vagus nerve: implications for P2 purinoceptor classification. *Br. J. Pharmacol.* **113**, 463–470.
24. Unterberger, U., Moskvina, E., Scholze, T., Freissmuth, M. & Boehm, S. (2002) Inhibition of adenylyl cyclase by neuronal P_2Y receptors. *Br. J. Pharmacol.* **135**, 673–684.
25. Nikodijevic, B., Sei, Y., Shin, Y. & Daly, J.W. (1994) Effects of ATP and UTP in pheochromocytoma PC12 cells: evidence for the presence of three P2 receptors, only one of which subserves stimulation of norepinephrine release. *Cell. Mol. Neurobiol.* **14**, 27–47.
26. Koizumi, S., Nakazawa, K. & Inoue, K. (1995) Inhibition by Zn^{2+} of uridine 5′-triphosphate-induced Ca^{2+}-influx but not Ca^{2+}-mobilization in rat phaeochromocytoma cells. *Br. J. Pharmacol.* **115**, 1502–1508.
27. Hur, E.M., Park, T.J. & Kim, K.T. (2001) Coupling of 1-type voltage-sensitive calcium channels to $P_2X(2)$ purinoceptors in PC-12 cells. *Am. J. Physiol. Cell. Physiol.* **280**, C1121–C1129.
28. Grohovaz, F., Zacchetti, D., Clementi, E., Lorenzon, P., Meldolesi, J. & Fumagalli, G. (1991) $[Ca^{2+}]_i$ imaging in PC12 cells: multiple response patterns to receptor activation reveal new aspects of transmembrane signaling. *J. Cell Biol.* **113**, 1341–1350.
29. Michel, A.D., Grahames, C.B. & Humphrey, P.P. (1996) Functional characterization of P2 purinoceptors in PC12 cells by measurement of radiolabelled calcium influx. *Naunyn Schmiedebergs Arch. Pharmacol.* **354**, 562–571.
30. Nakazawa, K. & Inoue, K. (1992) Roles of Ca^{2+} influx through ATP-activated channels in catecholamine release from pheochromocytoma PC12 cells. *J. Neurophysiol.* **68**, 2026–2032.
31. Raha, S., de Souza, L.R. & Reed, J.K. (1993) Intracellular signalling by nucleotide receptors in PC12 pheochromocytoma cells. *J. Cell. Physiol.* **154**, 623–630.
32. Nakazawa, K. & Hess, P. (1993) Block by calcium of ATP-activated channels in pheochromocytoma cells. *J. Gen. Physiol.* **101**, 377–392.
33. de Leon, M., Wang, Y., Jones, L., Perez-Reyes, E., Wei, X., Soong, T.W., Snutch, T.P. & Yue, D.T. (1995) Essential Ca^{2+}-binding motif for Ca^{2+}-sensitive inactivation of 1-type Ca^{2+} channels. *Science* **270**, 1502–1506.
34. Haack, J.A. & Rosenberg, R.L. (1994) Calcium-dependent inactivation of 1-type calcium channels in planar lipid bilayers. *Biophys. J.* **66**, 1051–1060.
35. Imredy, J.P. & Yue, D.T. (1994) Mechanism of Ca^{2+}-sensitive inactivation of 1-type Ca^{2+} channels. *Neuron* **12**, 1301–1318.

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