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Protein Kinase C Enhances Serotonergic and Non-Serotonergic Synaptic Transmission

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ABSTRACT

Serotonin (5-HT) is an excitatory neurotransmitter found in the CNS that regulates brain activities such as sleep, mood, and learning. Protein kinase C (PKC) is a G-protein-activated second-messenger enzyme that is responsible for the closing of potassium (K+) channels and the subsequent broadening of action potentials resulting in the release of neurotransmitters. While several previous studies have examined the role of PKC in vertebrate serotonergic transmission, practically no research has been conducted involving the functioning of 5-HT and PKC in invertebrate synaptic transmission. Thus, we devised an experiment utilizing the crayfish neuromuscular junction and intracellular recording techniques to determine the role of PKC in invertebrate serotonergic synaptic transmission. Through the application of 5-HT, the PKC inhibitor chelerythrine, and the 5-HT(2) receptor antagonist ketanserin, we found chelerythrine considerably reduced the heightened responses resulting from serotonin. This suggests that PKC plays a role in excitatory serotonergic synaptic transmission, and further results indicate PKC is also active in enhancement of non-serotonergic EPSPs.

INTRODUCTION

Serotonin (5-HT) is a pervasive neurotransmitter found in the CNS that plays a profound regulatory role in several key functions of the brain, including sleep, pleasure, mood, and learning. Moreover, 5-HT is an excitatory neurotransmitter, which induces membrane depolarization and/or action potentials. The excitatory response elicited by 5-HT may be attributed to the inhibition of outward potassium current conductance via phosphorylation—a phenomenon that involves activation of the G-protein-stimulated second-messenger enzyme protein kinase C (PKC). Since phosphorylation closes K+ channels, the cell repolarizes more slowly, causing action potentials to be longer in duration. In terms of synaptic transmission, the extended amount of time that the cell is depolarized causes voltage-gated calcium channels to remain open longer, triggering more vesicles to spill neurotransmitters into the synaptic cleft (Zhang, Fearon, Zhong, Nurse 2003). Additionally, PKC may also possess the ability to promote the movement of vesicles from reserve pools to releasable pools, increasing neurotransmitter release (Matthews 1998). Previous studies involving chemical manipulation have provided evidence of PKC's role in synaptic transmission. A 1999 study conducted by Inoue and colleagues demonstrated that 5-HT-induced excitatory responses were eliminated following application of the PKC inhibitor chelerythrine (Inoue *et al* 1999). Likewise, the PKC activator OAG mimicked the depolarizing effects of 5-HT (Zhang, Fearon, Zhong, Nurse 2003).

Like 5-HT, PKC is a vital subject of investigation in that it has been shown to contribute to long-term potentiation—a process that enhances the strength of synaptic transmission, for instance, in the hippocampus, an area of the brain that plays a fundamental role in learning and memory (Matthews, 1998). The combined action of 5-HT and PKC could perhaps even contribute to brain plasticity and the formation of memories.

While previous experimentation examining the functions of PKC in serotonergic synaptic transmission have focused primarily on vertebrates, our study attempted to clarify the role of PKC in invertebrate serotonergic synaptic transmission, specifically by studying the fast extensor muscles at the crayfish (species *Procambaris clarkii*) neuromuscular junction (NMJ). The crayfish NMJ served as an ideal preparation because the comparatively uncomplicated nature of the crayfish muscle system allowed us to observe the functioning of the invertebrate nervous system at the synaptic level. Here, the amplitude, elicitation, and control of EPSPs are relatively simple to measure.

Furthermore, our study aimed specifically to enhance knowledge of the connection between 5-HT and PKC in invertebrates. Whereas earlier studies have been conducted observing the effects of PKC on EPSPs while utilizing crayfish as the experimental preparation, these studies involved the excitatory effects of neuropeptides on transmission as mediated by PKC (Noronha, Mercier

1995). Indeed, 5-HT has received little attention compared to dopamine and other neurotransmitters.

Our experiment aimed to alleviate the dearth of information concerning the role of PKC in invertebrate serotonergic synaptic transmission by determining whether the excitatory effects of 5-HT are a result of PKC activation. We hypothesized that activating potassium channels by means of inhibiting PKC will reverse the effects of serotonin, decreasing both the duration and amplitude of action potentials and EPSPs in serotonergic synaptic transmission. Our findings demonstrate that this is indeed the case; application of chelerythrine to inhibit PKC led to a significant decrease in the magnitude of EPSPs created by the presence of serotonin.

MATERIALS AND METHODS

Preparation

Crayfish were submersed in ice for 20 minutes as a means of decreasing their metabolic processes. The tail of the crayfish was then cut off at the base, where the thorax and abdomen meet (Taggart and Torner, 2002). The dorsal portion of the tail was then removed, leaving the ventral abdominal flexor muscle exposed. The ventral portion of the abdominal muscle was then removed and discarded, leaving the fast extensor muscle exposed. The preparation was then covered in a standard crayfish Ringers' solution. The preparation was then pinned ventral side up to a Petri dish coated with Sylgard^{TM} (Taggart and Torner, 2002).

Solutions

Preparations were submerged in a standard Crayfish Ringers' solution composed of: 205.0 mM NaCl, 5.4 mM KCl, 13.5 mM CaCl, 2.6 mM $MgCl_2$, and 10.0 mM Tris. A stock solution of serotonin creatine sulfate complex from Sigma Chemical Supply Company was diluted to its final concentration (30 μM) by adding it to the standard Crayfish Ringers' solution prior to application. Chelerythrine chloride (Sigma Chemical Supply Company), a PKC inhibitor, was prepared as a stock solution and diluted to its final concentration (50 nM) the day of application. The serotonin (5-HT2) receptor antagonist, ketanserin tartrate salt (Sigma Chemical Supply Company) in a stock solution was diluted to its final concentration (25 μM) prior to application.

Recordings

Microelectrodes with a resistance of 4 MΩ-20 MΩ were made by pulling glass capillary tubes using a Pul-1 by World Precision Instruments. Microelectrodes were then filled with a 3 M KCl solution. Microelectrode holders were also filled with 3 M KCl. Microelectrodes were then inserted in anterior side of the corresponding segment of the medial fast extensor muscle in order to record EPSPs (Taggart and Torner, 2002). Recording microelectrodes were connected to Mac Lab Scope v3.6.3 software.

Suction electrodes were utilized to electrically stimulate a motor nerve as a means of eliciting an excitatory post-synaptic potential (EPSP). Suction electrodes were made by breaking the tips off of microelectrodes and either fire polishing the tips, or sanding the tips. The method used to make the suction electrodes was dependent upon the diameter of the nerve. Suction electrodes were filled with the standard crayfish Ringers' Solution by drawing the solution into the suction electrode from the solution that the preparation was submersed in. This was done prior to drawing the motor nerve into the suction electrode. Motor nerves were stimulated using a Grass electric stimulator.

Experimental Design

Baseline readings were taken of EPSPs in the fast extensor muscle of the Crayfish tail under normal conditions with the standard crayfish Ringer's solution. Baseline readings were used as a comparison for establishing the excitatory effects of serotonin on EPSPs. Once baseline readings were taken, serotonin creatine sulfate complex (30 μM) was added to the standard Crayfish Ringers' solution and applied to the preparation. EPSP readings were recorded after five minutes of application to observe the effect of serotonin on synaptic transmission. Next, chelerythrine chloride (50 nM), a protein kinase C inhibitor, was added to a crayfish Ringer's solution containing serotonin creatine sulfate complex (30 μM). Readings were taken ten minutes after initial application. Subsequently, ketanserin tartrate salt (25 μM), a serotonin (5-HT2) receptor antagonist, was applied to the preparation. EPSP readings were once again taken after five minutes of application. A wash procedure was then conducted twice in which the preparation was bathed in the standard crayfish Ringer's solution for five minutes each time. EPSP recordings were then taken to ensure that observations are a result of experimental manipulation (via chemical application) rather than degradation of the preparation.

Data Analysis

To determine the effects of the experimental chemicals utilized, the percentage difference in EPSPs between baseline readings and experimental conditions were compared. Percent differences were averaged for each condition for each crayfish. Then, the percent differences were averaged together, and paired t-testes were

conducted to determine the statistical significance. The standard error was calculated for each condition, and displayed as error bars on the bar graph.

RESULTS

Through each condition of the experiment, several EPSPs were recorded to determine each drug's effect. Amplitudes of baseline EPSPs had a large amount of variability; to remove this factor, results are expressed as a percentage of the amplitude of the baseline EPSPs. The neurotransmitter serotonin was the first drug applied, and an excitatory reaction was expected. Figure 1 shows the increase of the serotonin response as relative to the baseline readings. Serotonin responses were nearly 150% that of baseline. Although this increase lacks statistical significance when compared to baseline, it is generally accepted that serotonin creates an excitatory response (Inoue et. al. 1999). However, during two separate experiments, serotonin responses were slightly smaller in magnitude than baseline.

Next, chelerythrine, a protein kinase C inhibitor, was added to the preparation. Chelerythrine caused a significant decrease in the amplitude of EPSPs (Fig. 1), being about 75% of baseline. A 5-HT(2) receptor antagonist, ketanserin, was the next chemical added to the solution. Contrary to the hypothesis, responses in the presence of ketanserin were, on average, higher than those with chelerythrine (Fig. 1). The bar graph depicts an average of all experiments, but there were in fact no exceptions to ketanserin responses being greater than chelerythrine. A typical set of data from one preparation is displayed in Figure 2. Ketanserin EPSPs were also larger in magnitude than baseline at 113%.

Finally, a wash procedure was conducted. Surprisingly, in the presence of a standard Ringer's solution, responses were considerably larger than initial recordings (Fig. 1). The average response following the wash was 175% that of baseline. One preparation elicited unusually small baseline responses and wash responses were 334% in comparison. There are several reasons that could account for the unexpected results obtained with ketanserin and following the wash procedure.

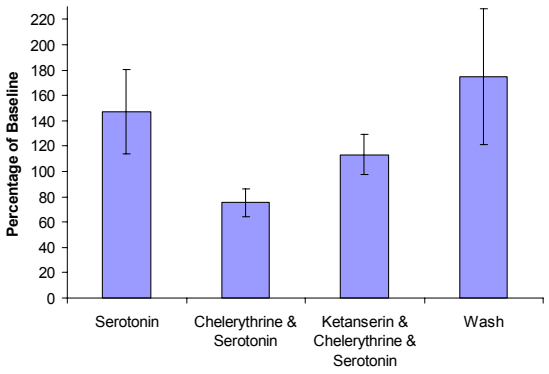

Figure 1
Each bar represents the average EPSP response after the addition of each chemical; results are displayed as a percentage of the average baseline response. Error bars show standard error values (see results) in positive and negative directions. Calculated values are as follows: Serotonin: 147.1% (n = 7, p = .15); Chelerythrine: 75.4% (n = 7, p = .03); Ketanserin: 113.2% (n = 5, p = .26); Wash: 174.8% (n = 4, p = .07).

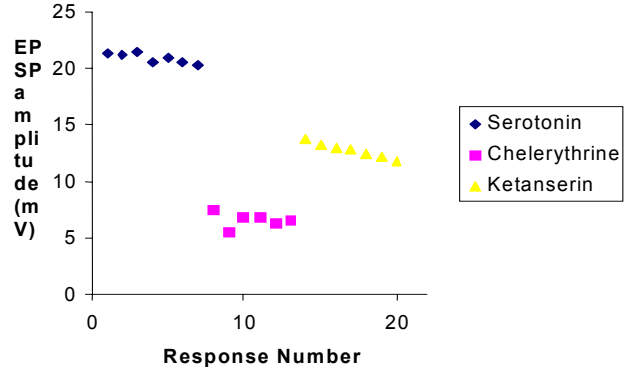

Fig. 2
Typical data points obtained during one experiment; the different series display which chemical was being added to elicit the corresponding EPSPs. Each point represents one recorded response, they are shown in terms of amplitude (number of millivolts difference between the highest and lowest points of the EPSP). Note the definite responses created by each drug, and the relatively low amount of variability within each condition.

DISCUSSION

Our hypothesis was to determine the role protein kinase C (PKC) plays in serotonin's excitatory functions. After applying serotonin, the PKC inhibitor was applied to observe whether the resulting increase in EPSP magnitude would be reduced. The chelerythrine reduced EPSPs to 75% of the original baseline (see results section). Therefore, the increase caused by serotonin was completely abolished. This coincides with our hypothesis that PKC plays an active role in facilitating the excitatory actions of serotonin. In addition, the decrease in magnitude beyond baseline indicates that PKC also influences synaptic transmission at sites that are not dependent upon serotonin. An endogenous amount of PKC that enhances normal EPSPs was most likely inhibited, leading to responses below baseline. Another possibility is that serotonin was already present during the baseline readings and was contributing to these responses.

The next step was applying ketanserin, a drug that blocks 5-HT(2) receptors. The thought was that applying a receptor antagonist should either decrease responses as compared to chelerythrine, or have no effect. If ketanserin further reduced magnitude of the response, then a conclusion could have been drawn that PKC plays only a partial role in creating serotonin's excitatory effects. On the other hand, if the receptor antagonist had no effect then this would point to PKC being totally responsible for serotonin's influence. While a decreased response was expected, EPSPs with ketanserin were actually greater in magnitude than during chelerythrine application. There are a number of explanations for why this is contrary to predictions. Ketanserin contains a chemical, DMSO, which increases cell permeability. As a result, the effects of serotonin (which is still present in this step) could have been intensified. Further research could be conducted with DMSO present throughout every step to remove this variable; if this were the case serotonin and chelerythrine responses may also increase to the point where ketanserin responses would be smaller in comparison. Another explanation could be that ketanserin acts as a non-specific antagonist or was not functioning properly in the preparation. The drug may interact with other systems to produce this response or trigger other actions that could potentially interfere. Other variables may also be factors, such as the amount of time for the drug to take full effect, or interactions with the other chemicals present. Figure 2 shows that the EPSPs recorded during ketanserin application steadily declined, possibly displaying the drug in the process of taking effect. Error is also a potential here considering the ketanserin responses were not shown to be statistically valid (see results).

Final observations were made during the wash procedure. This step was conducted to ensure that our results were based on experimental manipulation and not degradation of the specimen. The EPSPs elicited following the wash procedure were unexpectedly high, again there could be several factors underlying this. Reconsidering DMSO, if this chemical permanently alters cell permeability then normal EPSPs would increase in magnitude as ions could be transported faster, causing the cell do depolarize to a greater degree. Chemicals failing to diffuse out of the cell are another possibility, if serotonin were still present in spite of the wash process it could potentially continue to heighten responses. Time is another factor that becomes more essential towards the closing of the experiment as the preparation begins to die. Cell death can cause unpredictable responses and the wash results could be attributed to this. Again, the responses recorded following the wash failed to show statistical significance, so error is still a possible explanation.

Overall, experimental error and variability significantly influenced the results of this experiment. On every preparation several nerves were available for stimulation and most experiments used different nerves and muscles cells to record responses. This leads to a problem in that some cells may be affected by drugs to a different degree than another (e.g. measuring serotonin's effect on a non-serotonergic synapse). Another factor is human error, where lack of a perfectly reproducible technique to measure responses leads to complication. Such as a nerve not having a complete seal with the suction electrode, causing EPSPs to be lower in magnitude than what is truly possible. This is especially crucial when measuring baseline responses. Considering that results from each condition of the experiment are compared to this initial reading, if the baseline EPSP is invalid, then the following steps will be skewed similarly. An example of this was when one preparation elicited a lower baseline than normal, and consequently the wash EPSPs were over three times the size of the baseline. This creates an extremely wide range of magnitudes in which these extreme values alters averages and also increases the amount of error, therefore rendering the results less valid. However, this becomes an increasingly minor problem as a greater amount of experiments are conducted. Lastly, working with a living system has inherent difficulties. There is a considerable amount of variation between specimens in addition to experimental manipulations, possibly causing damage or death to the cells.

According to the hypothesis and supported by observations, protein kinase C definitely plays a significant role in the excitatory effects of serotonin. Not only that, but results indicate that endogenous PKC also

enhances normal synaptic transmission. Further investigation could be conducted to examine PKC's role in general synaptic transmission, not merely serotonergic. The results obtained concerning ketanserin were the most unexpected and provide the best grounds for further research. Studies concerning the most proper time to record responses after application, how influential DMSO is to cell permeability, or how strong ketanserin's affinity to 5-HT(2) receptors are a few that could be conducted. To decrease the amount of variability present in this experiment, simpler investigations could be carried out involving one or two chemicals to better understand the singular role of each drug. In addition, potential interactions between serotonin, chelerythrine and ketanserin could be looked into to find whether unexpected results arise when they are mixed. In the end, this study needed simplicity and a greater degree of control over the potential confounding variables, as each step in the procedure presents another chance for error.

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