Effects of 12, 13 dibutyrate (PDBu) on resting and capsaicin-stimulated glutamate release in adult rat dorsal root neurons in culture.

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Abstract

This investigation explored the role of PKC as an activator of VR1 through the use of capsaicin, a natural ligand for VR1, and the PKC activating phorbol ester 12,13 dibutyrate (PDBu) . Direct effects of 30nM, 300nM, and 3000nM capsaicin and 0nM, 0.3nM, 1.0nM, 3.0nm, 10nM, and 30nM PDBu on glutamate release from adult rat dorsal root ganglia (DRGs) were examined. Based on those observations, the concentration of capsaicin causing the least glutamate release and the concentrations of PDBu that did not elicit significant glutamate release were selected to study effects of capsaicin on same cell type acutely sensitized with the selected concentrations of PDBu. It was found that glutamate released from combinations of 30nM capsaicin and 1.0nM PDBu ($p<0.036$) as well as 30nM capsaicin and 3.0nM PDBu ($p<0.01$) were statistically significant from the stimulation control—which was 30nM capsaicin alone.

Introduction

Because they activate protein kinase C (PKC) in vitro (Parker 1998), phorbol esters are often used to investigate PKC function in cells. PKC refers to a family of eleven serine-threonine kinase isoforms with unique substrate specificities and roles in cell regulation and differentiation (Blobe et al. 1996). For instance, PKC is known to translocate from the cytosol to the plasma membrane for activation by diacylglycerol (DAG), an endogenous substrate. Phorbol esters compete with DAG for its binding site on PKC. It has been demonstrated that the C1 domains of classical and novel PKC isoforms bind diacylglycerol and phorbol esters with high affinity, after acute (10 minutes) exposure to phorbol esters (Wang et al. 2001). Chronic exposure (24-48 hours) leads to PKC isotype selective proteolysis and down-regulation (Kramer & Simon 1999).

Small diameter peripheral neurons, Aδ and C-fibers, have been recognized as the peripheral nerve types carrying protopathic information—pain, temperature, and crude touch. When introduced into the body, capsaicin, an exogenous substance found in chili peppers, specifically binds vanilloid receptors of type 1 (VR1), which are expressed in Aδ and C-fibers (Huang 2002). VR1 is involved in transmitting noxious chemical and heat stimuli from the periphery to the central nervous system. One direct role of PKC in VR1 activation has been recently proposed. PKC-α was required for acute activation of VR1 with

phorbol 12, 13 dibutyrate, (PDBu), a small and somewhat hydrophilic phorbol ester, in embryonic dorsal root ganglia (DRG) cells and in NIH 3T3 and HeLa cells transfected to express a VR1 (Olah et al. 2002).

Demonstrating that PDBu is capable of enhancing resting and capsaicin-stimulated glutamate release in adult rat sensory neurons lends further insight into the mechanism of PKC-induced neuron sensitization in human pain transmission and perception. PDBu has been shown to enhance resting and capsaicin-stimulated calcitonin gene-related peptide (CGRP) and substance P (SP) release in embryonic rat DRG (Barber & Vasco, 1996).

Although glutamate and its receptors have been recognized as important in the neurotransmission of acute pain and the onset of chronic pain (Varney & Gereau 2002), there has not been extensive study of the regulation of glutamate release from sensory neurons. It is known that glutamate is stored in synaptic vesicles at nerve terminals and is immediately released upon sensory neuron stimulation. However, a direct connection between PKC and VR1 activation and known mechanisms of glutamate release has not been established.

Since the adult rat peripheral nervous system (PNS) is without the developmental consideration present in an embryonic model, DRG cells of adult rats were used. By virtue of PDBu activity as a PKC agonist, it was hypothesized that capsaicin stimulation of glutamate release would be enhanced by PDBu. It was found that treating the cultured cells with increasing capsaicin or PDBu elicited a concentration-dependent increase in glutamate release. Given alone, 30nM capsaicin and 0.3nM, 1.0nM, and 3.0nM PDBu did not induce glutamate release from cultured DRG cells. However, treating the cells with 30nM capsaicin after acute sensitization with 1.0nM or 3.0nM PDBu caused significantly higher glutamate release than treatment with 30nM capsaicin alone.

Additionally, this study utilizes a novel paradigm, developed by Dr. Judith Richter in the Pharmacology and Toxicology department at Indiana University-Purdue University at Indianapolis, for the assay of glutamate released in low concentrations from adult rat DRG cells in culture.

Materials and Methods

Materials

Forty to forty-five-day-old, male Sprague-Dawley rats were purchased from Harlan-Sprague Dawley (Indianapolis, IN, U.S.A.). Costar twenty-four well culture plates were bought from Fisher Scientific (Chicago, IL, U.S.A.). Other cell culture supplies were procured from Invitrogen/Gibco (Carlsbad, CA, U.S.A.). Nerve growth factor was acquired from Harlan Bioproducts for Science (Indianapolis, IN, U.S.A.).

Phorbol 12,13,-dibutyrate (PDBu) and 8-methyl-n-vanillyl-6-nonenamide (Capsaicin) were obtained from Sigma Chemical Company (St. Louis, MO, U.S.A.).

Dibasic sodium phosphate was from Mallinckrodt Baker (Phillipsburg, NJ, U.S.A.), and chromatographic grade o-pthalaldehyde (OPA) and OPA diluent were acquired from Pickering Laboratories (Mountain View, CA, U.S.A.). Millipore Ultrafree Biomax centrifugation filters (10K cutoff) with tubes and Optima Grade Methanol were bought from Fisher Scientific (Chicago, IL, U.S.A.). All other chemicals were obtained from Sigma/Aldrich/Fluka (St. Louis, MO, U.S.A.).

Capsaicin and phorbol ester were initially dissolved in 1-methyl-2-pyrrolidinone (MPL) and diluted to appropriate concentrations with AB buffer (explained below) for release experiments. Resultant solutions contained MPL concentrations of 0.03% or less, which affected neither neurotransmitter release nor the assay of glutamate (glu) or calcitonin gene related peptide (CGRP).

The procedures used in this exercise were approved by the Animal Care and Use Committee at Indiana University School of Medicine (Indianapolis, IN, U.S.A.).

Maintaining sensory neurons in culture

Sensory neurons were harvested and cultured according to standard procedures (Southhall & Vasco, 2001), with a few modifications. In detail, the following procedure was used.

Adult rats were first euthanized in a syrofoam CO_2 chamber. Their vertebral columns, from the upper brainstem to the cauda equina, were manually removed. Spinal cords were ejected from the columns by forcing sterilized Puck's solution into the caudal end of the columns with a syringe. The columns were then placed in HEPES buffer, containing 25nM HEPES, 135mM NaCl, 3.5mM KCl, 2.5mM $CaCl_2$, 1mM

$MgCl_2$, 3.3mM D-glucose, 0.001mM phosphoramidon and 0.1% bovine serum albumin at pH 7.4 and 37°C, which was supplemented with 1.6μg/ml Fungizone® to inhibit fungal contamination in the remainder of the preparation.

The columns were removed from the supplemented buffer, and Mid-line cuts along the length of the dorsal and ventral surfaces of the columns were then made to reveal the DRGs in their recesses. Using a Nikon model SMZ645 light microscope at 8 times magnification, the DRGs were mechanically extracted from their recesses and collected in a culture dish filled with sterilized Puck's solution on ice. In the culture dish the DRG afferent and efferent nerve processes were removed with a scalpel.

Trimmed DRGs were then incubated in a culture dish with 3 mL of F-12 media containing 1 mg ml^{-1} collagenase 1A for two hours at 37 °C, in two separate intervals. After the two hour incubation, the DRGs and incubation fluid were transferred to a 20mL conical tube, in a sterile atmosphere. Following one minute centrifugation, the enzymatic supernatant removed. The cellular pellet was re-suspended in F-12 medium supplemented with 250 ng ml^{-1} 7S-nerve growth factor and 50mg/ml Normocin O™. Cells were then mechanically dissociated from the DRGs using fire polished Pasteur pipettes until a uniform suspension remained. Approximately 22,500 cells per well were plated onto 24-well, polystyrene culture plates, formerly coated with poly-D-lysine and laminin. The cells were maintained in an atmosphere of 3% CO_2/97% air at 37°C for nine days. The incubation medium containing F-12 with nerve growth factor and Normocin O™ was exchanged, on the second (48h), third (72h), sixth (144h), and eighth (192h) days after plating. Release studies were performed on the ninth day.

Glu release experiments from sensory neurons in culture

Sensory neurons were washed, within their culture wells, three times with HEPES buffer. After washing, 0.5mL of buffer was added to each well and the cultures incubated for one hour at 37°C. The cells were again washed three times with HEPES buffer. At this point, buffer with or without added drugs was applied in 0.5 mL aliquots and incubated for 10 minutes at 37°C, and four or more such incubations were performed. Generally, a control (basal) incubation was followed by a second, ten-minute incubation in HEPES buffer or HEPES buffer containing various concentrations of PDBu. Subsequently, cells were incubated for a third ten-minute interval in HEPES buffer containing various capsaicin and, or, PDBu

concentrations, often in combinations, to stimulate glu release under a sensitized state. Cells were then incubated for a final ten-minute interval in HEPES buffer without stimulating agents.

Assay of glutamate released from sensory neurons

This procedure was performed according to Richter et al. (in preparation). Specifically, 20μL of 1M perchloric acid containing 12.5μM homoserine, an internal standard, was added to 200μL of the second, third, and fourth groups of buffers collected from experimentation. Homoserine in perchloric acid was also added to 200μL aliquots of a set of glutamate standards (0, 5, 10, 20, and 40 pmoles glu/20 μL). The solutions were mixed, transferred to centrifugation filters, and centrifuged at 10,000g for approximately 15 minutes at 4°C.

20μL of the filtrate in autosampler tubes were then placed into an autosampler at 4°C. There, 15μL of daily prepared derivative forming reagent, made by a four-fold dilution of a weekly prepared stock solution (27mg o-Phthaldialdehyde (OPA), 7.5μL β-mercaptoethanol, 1mL methanol and 9mL OPA diluent) in OPA diluent, was injected into one sample. One minute later, 27μL of the sample/reagent mixture was automatically injected onto a reverse phase, 3 micron, 4.6 mm x 8 cm, C-18 column (model HR-80 from ESA, Inc., Chelmsford, MA, U.S.A.). Solvent composition remained constant throughout analysis with a mobile phase of 0.1M sodium phosphate at pH 6.75 with 24% methanol pumped at 1.2mL/min.

Amino acids were electrochemically detected by a dual electrode ESA Coulochem II system. First, an electrochemical cell oxidized impurities in the sample at 300mV. A second cell oxidized the remaining amino acid derivatives at 600mV.

ESA System software, installed on an HP Pentium IV computer, generated chromatographs and standard curves for experiment samples. Glutamate and homoserine eluted at approximately three and ten minutes respectively. Furthermore, ESA software calculated experimental glutamate concentrations within a sample, based upon homoserine levels and the glutamate standard curve.

Statistical Analysis

Data are presented as mean ± SEM. Values more than two times the standard deviation away from the mean were excluded. Each set of experiments was repeated using two or more different culture preparations to account for variability among different cultures. One-way ANOVA tests, with Dunnett or Bonferoni post hoc tests, comparing basal release to stimulated release in direct effect studies of capsaicin and PDBu, were performed with Graph Pad Prism software (San Diego, CA). A one-way ANOVA and LSD post hoc test was performed on transformed data, using Statistica software (Statsoft, Inc., Tulsa, OK, U.S.A.), for analysis of combined PDBu and capsaicin stimulation studies. In all cases significance was set at $p \leq 0.05$.

Data and Results

To study the direct, concentration-related effects of capsaicin on glutamate release, culture wells were incubated in 0.5mL HEPES buffer containing 30nM, 300nM, or 3000nM capsaicin for 10 minutes at 37°C. This followed two ten-minute incubations in 0.5mL of buffer alone. Cells were then incubated a final 0.5mL of buffer for ten minutes. Incubation fluid was collected at the end of each interval.

Glutamate assayed from the second, third, and fourth collections demonstrated a concentration-dependent relationship between the increasing capsaicin concentrations applied and resultant glutamate release, as indicated (Figure 1.) A one-way ANOVA on the capsaicin stimulation results using GraphPad Prism showed a significant effect of capsaicin concentration ($p<0.0072$). There was an approximate 50% increase in the stimulated glutamate release with exponential increases in the given capsaicin concentrations. Because it stimulated the least glutamate release and no apparent increase over basal glutamate release, 30nM capsaicin was chosen to stimulate glutamate release in the final sensitization, or combined experiments.

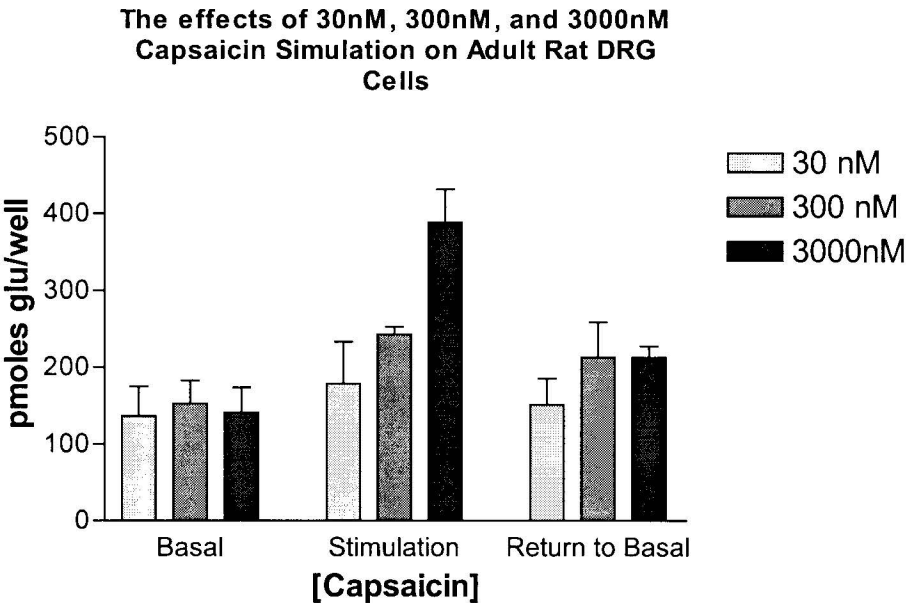

Figure 1. Amount of glutamate released from direct capsaicin treatment

Culture wells were incubated for four ten-minute intervals in solutions of HEPES buffer, HEPES buffer (Basal), HEPES buffer containing the indicated capsaicin concentrations, or HEPES buffer (Return to Basal), successively. Glutamate released into buffer was assayed as described in Methods and Materials and is expressed as pmoles of glutamate per well. The second, third, and fourth collections were assayed. These values depicted as means ± SEM, n=5-6.

In evaluating the direct concentration effect of PDBu on glutamate release, culture wells were incubated in 0.5mL HEPES buffer containing 0nM, 0.3nM, 1.0nM, 3.0nM, 10nM, or 30nM PDBu in three successive ten minute intervals at 37°C, following one ten-minute incubation in 0.5mL of buffer alone. Again, incubation fluids were collected at the end of each ten-minute incubation interval. Glutamate released from the second third and fourth intervals are represented in Table 1.

Table 1. Amount of glutamate released from direct PDBu treatment

Duration of treatment	Control *(pmoles/well)*	0.3nM PDBu *(pmoles/well)*	1.0nM PDBu *(pmoles/well)*	3.0nM PDBu *(pmoles/well)*	10nM PDBu *(pmoles/well)*	30nM PDBu *(pmoles/well)*
10 minutes	113.963 ± 19.933	337.466 ± 213.435	201.720 ± 29.330	332.841 ± 157.645	211.613 ± 22.809	281.423 ± 37.664
20 minutes	157.758 ± 60.933	175.506 ± 75.906	175.671 ± 15.317	235.673 ± 47.603	290.153 ± 23.537	254.946 ± 37.481
30 minutes	161.090 ± 52.888	117.384 ± 27.517	253.629 ± 121.817	189.267 ± 51.648	265.971 ± 33.887	239.033 ± 32.373

Culture wells were incubated for four ten-minute intervals in solutions of HEPES buffer, followed by three applications of HEPES buffer containing the indicated PDBu concentrations, successively. Glutamate released into buffer was collected and assayed after each interval as described in Methods and Materials. The second, third, and fourth collections, containing glutamate released after 10, 20, and 30 minute durations of PDBu treatment, were assayed. The values indicated are means ± SEM, n=6-7.

A two-way ANOVA on the glutamate measurements in Table 1, using GraphPad Prism, indicated significance ($p<0.05$) with respect to PDBu concentration ($p<0.0001$) and no significance with respect to time ($p<0.608$). Based on this, the glutamate measurements were collapsed with respect to time and a one-way ANOVA with Dunnets's post hoc Multiple Comparison Test was performed with the concentration of PDBu as variable. These results show that 10nM and 30nM PDBu caused significant ($p<0.001$ for both concentrations) glutamate release from the adult DRG cells. This result is depicted in Figure 2. Since 0.3nM, 1.0nM, and 3.0nM PDBu did not stimulate significant glutamate release, these concentrations were selected for "sensitizing" the cells in the final aspect of this study.

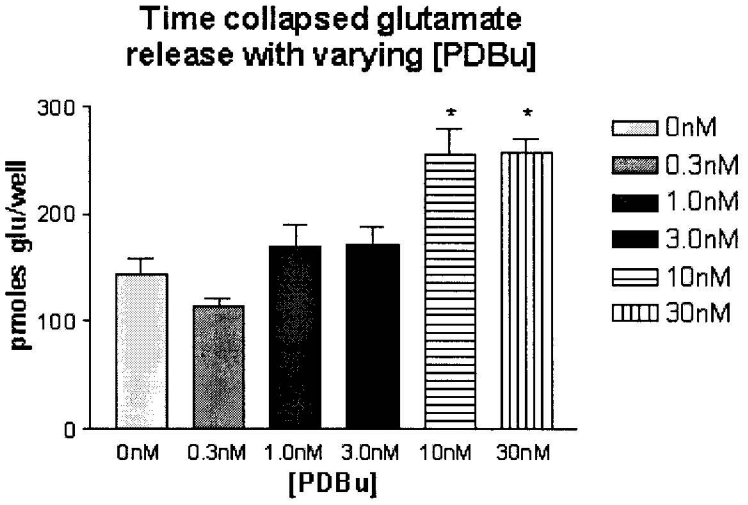

Figure 2. Amount of glutamate released from time collapsed direct PDBu effects
Time effects were not deemed significant in a two-way ANOVA of the data in Table 1, treating PDBu concentration and time as variable. Thus, the data was collapsed with respect to time and analyzed in a one-way ANOVA and Dunnets's Multiple Comparison Test, with PDBu concentration as variable. This process indicated significant glutamate release due to treatment with 10nM ($p<0.0001$) or 30nM ($p<0.0001$) PDBu; * indicates capsaicin-stimulated release significantly different from the 0nM PDBu control.

To investigate the combined effects of PDBu sensitization and capsaicin stimulation, the concentrations of PDBu that did not stimulate glutamate release alone and the capsaicin concentration causing the least amount of glutamate release, were identified from the previous experiments. Thus, culture wells were incubated in HEPES buffer; HEPES buffer containing 0nM, 0.3nM, 1.0nM, or 3.0nM PDBu; HEPES buffer containing 30nM capsaicin and 0nM, 0.3nM, 1.0nM, or 3.0nM PDBu; and then HEPES buffer with no stimulants in four 10-minute intervals at 37°C, successively. Incubation fluid was

collected at the end of each interval. Glutamate released from the second third and fourth intervals are represented as Figure 3.

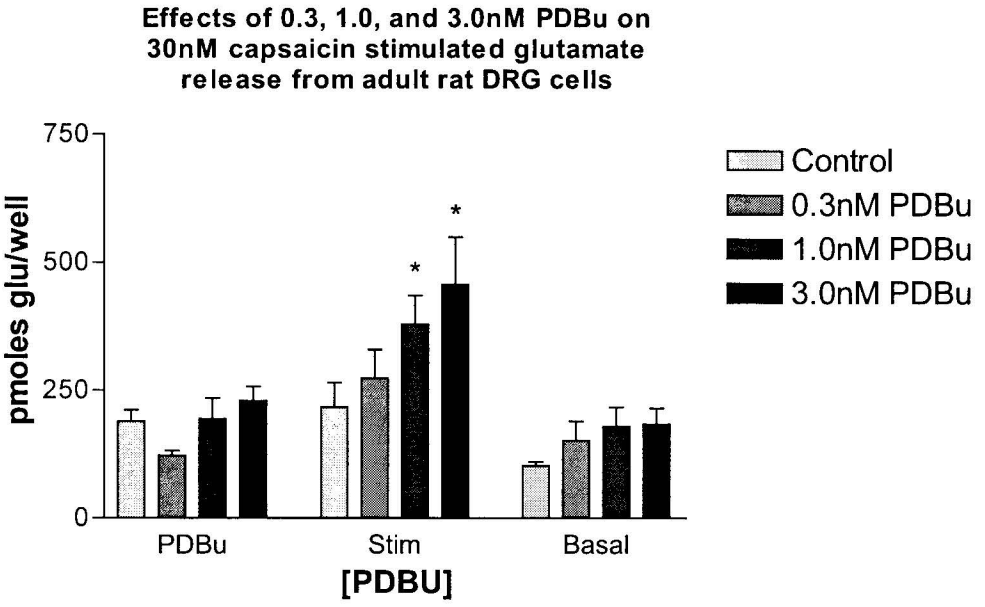

Figure 3. Amount of Glutamate Released from combined capsaicin and PDBu treatment
Culture wells were incubated for four ten-minute intervals in solutions of HEPES buffer, HEPES buffer with 0nM, 0.3nM, 1.0nM, or 3.0nM PDBu (PDBu); HEPES buffer with 30nM capsaicin and 0nM, 0.3nM, 1.0nM, or 3.0nM PDBu (Stim); and HEPES buffer (Basal), successively. Solutions were collected after each interval as described in Methods and Materials. The second, third, and fourth collections were assayed and are expressed as pmoles of glutamate per well. Values are depicted as means ± SEM, n=6-7; * indicates glutamate release significantly different from the 30nM capsaicin with 0nM PDBu stimulation control.

Using Stat Soft Statistica, the data depicted in Figure 3 was transformed to eliminate correlations between the means and SEM. Then, a one-way ANOVA followed by the LSD post hoc test was performed on the results from the stimulation interval only. This process indicated that glutamate released from combinations of 30nM capsaicin and 1.0nM PDBu ($p<0.036$) as well as 30nM capsaicin and 3.0nM PDBu ($p<0.01$) were statistically significant from the stimulation control—which was HEPES buffer with 30nM capsaicin alone.

A comparison of absolute glutamate released from the culture wells incubated in buffer with capsaicin, PDBu, and combined capsaicin and PDBu lends an additional interpretation. On average the

22,500 cells in each well released 178.41 ± 54.5 pmoles of glutamate when incubated in buffer with 30nM capsaicin only. The wells incubated in buffer with 1.0nM PDBu and buffer with 3.0nM PDBu released 210.29 ± 55.4 and 252.59 ± 85.6 pmoles of glutamate per well on average, respectively. In the combined treatments, wells incubated with buffer and 30nM capsaicin and 1.0nM PDBu released 379.31 ± 56.1 pmoles of glutamate per well and those incubated in buffer and 30nM capsaicin and 3.0nM PDBu released 456.49 ± 93.0 pmoles of glutamate per well. Compared to wells incubated with buffer and respective concentrations of PDBu alone, wells incubated in buffer with combined capsaicin and 1.0nM or 3.0nM PDBu released 80.4 ± 26.5% or 80.7 ± 35.5% more glutamate. Furthermore, the wells incubated in buffer and 30nM capsaicin and 1.0nMPDBu and 30nM capsaicin and 3.0nM PDBu released 112.6 ± 31% and 155.9 ± 41.3% greater glutamate than cells incubated in buffer and 30nM capsaicin alone.

Discussion

The scope of this experiment posits that combined PDBu and capsaicin are capable of stimulating greater glutamate release than either substance would elicit alone. This observation is particularly supported by the effects of cells sensitized with 1.0nM or 3.0nM PDBu and stimulated with 30nM capsaicin.

Significant increases in glutamate release due combined effects, or sensitization, in this study add further perspective to the question of the mechanism of PKC and VR1 interaction. These acute findings support a hypothetical mechanism whereby PDBu diffuses across the plasma membrane, allowing PDBu to activate PKC. Furthermore, activated PKC could enhance VR1 directly, rendering VR1 more sensitive to capsaicin. Verifying PDBu's action as a PKC activator in this result necessitates further study in this experimental model. In addition to utilizing a diffusible PKC inhibitor with PDBu and capsaicin in buffer, observing the results of a non-PKC-activating, diffusible phorbol would help identify PDBu function. A decline in glutamate release, when using the PKC inhibitor, would support the finding that PKC enhances the VR1 response to capsaicin. Analogously, decreased glutamate release with the non-PKC-activating phorbol would support the observation that PDBu acutely sensitizes the cell, via PKC activation, to enhance glutamate release—as proposed in this study.

This experiment was designed to identify concentrations of capsaicin and PDBu that gave no stimulation and to observe the combined effects of those concentrations in buffer rather than test higher concentrations. In acknowledging that capsaicin stimulatory effects reach a maximum (Richter, unpublished observations) and appreciating the possibility that PDBu stimulation might also have a ceiling, it was reasoned that any combined effect would be more clearly apparent in the current milieu. Furthermore, the need to eliminate any undesirable effects of PDBu at higher concentrations, beyond its recognized role as a PKC agonist, was made less likely.

An unexpected phenomenon occurred in testing the direct effects of PDBu and in the combined studies. Namely, the glutamate released from wells incubated in buffer with 0.3nM PDBu was consistently depressed, relative to the glutamate released from buffer containing neither capsaicin, nor PDBu. This phenomenon may be the reason why the combination of buffer with 30nM capsaicin and 0.3nM PDBu did not produce statistically significant results. It has been demonstrated that phorbol derivatives have been capable of activating TRPV4, a heat sensitive ion channel belonging to the same family as VR1 (TRPV1) (Guler et al. 2002). Considering that the HEPES buffer in this experiment is kept on ice and heated, possibly not to 37°C as intended, begs the question of whether or not VR1 is also sensitive to temperature.

One can also appreciate that glutamate is rapidly cleared from the synaptic cleft by cellular re-uptake mechanisms. This too may have a significant effect in determining the actual amount of glutamate released due to stimulation. Thus, it would beneficial to observe the combined PDBu and capsaicin experiments in the presence of a glutamate re-uptake inhibitor, such as β-threo-benzyloxy-aspartate (Richter, unpublished observations).

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