Different phosphoinositide 3-kinase isoforms mediate carrageenan nociception and inflammation

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Abstract

Phosphoinositide 3-kinases (PI3Ks) participate in signal transduction cascades that can directly activate and sensitize nociceptors and enhance pain transmission. They also play essential roles in chemotaxis and immune cell infiltration leading to inflammation. We wished to determine which PI3K isoforms were involved in each of these processes. Lightly anesthetized rats (isoflurane) were injected subcutaneously with carrageenan in their hind paws. This was preceded by a local injection of 1% DMSO vehicle or an isoform-specific antagonist to PI3K-α (compound 15-e), -β (TGX221), -δ (Cal-101), or -γ (AS252424). We measured changes in the mechanical pain threshold and spinal c-Fos expression (4 hours after injection) as indices of nociception. Paw volume, plasma extravasation (Evans blue, 0.3 hours after injection), and neutrophil (myeloperoxidase; 1 hour after injection) and macrophage (CD11b+; 4 hour after injection) infiltration into paw tissue were the measured inflammation endpoints. Only PI3K-γ antagonist before treatment reduced the carrageenan-induced pain behavior and spinal expression of c-Fos ($P \leq 0.01$). In contrast, pretreatment with PI3K-α, -δ, and-γ antagonists reduced early indices of inflammation. Plasma extravasation PI3K-α ($P \leq 0.05$), -δ ($P \leq 0.05$), and -γ ($P \leq 0.01$), early (0-2 hour) edema -α ($P \leq 0.05$), -δ ($P \leq 0.001$), and -γ ($P \leq 0.05$), and neutrophil infiltration (all $P \leq 0.001$) were all reduced compared to vehicle pretreatment. Later (2-4 hour), edema and macrophage infiltration ($P \leq 0.05$) were reduced by only the PI3K-δ and -γ isoform antagonists, with the PI3K-δ antagonist having a greater effect on edema. PI3K-β antagonism was ineffective in all paradigms. These data indicate that pain and clinical inflammation are pharmacologically separable and may help to explain clinical conditions in which inflammation naturally wanes or goes into remission, but pain continues unabated.

Keywords: c-Fos, Edema, Macrophage, Neutrophil, Plasma extravasation, Pain

Class-1 phosphoinositide 3-kinases (PI3Ks) may be involved in both nociception, ie, pain and inflammation. Importantly, these receptor families participate in signal transduction cascades, culminating in upregulation and/or activation of both nociceptive and leucocyte chemotactic agents such as complement factor C5a, G-protein–coupled formyl receptors (FPRs), nerve growth factor, vascular endothelial growth factor, sphingosine-1-phosphate, and receptors for a variety of cytokines and chemokines.[25,40] Activation of PI3K is also associated with the neuronal plasma membrane insertion of TRPV1, ASIC1a, Nav1.8, AMPA, and voltage-gated Ca^{2+} channels.[18,28,52,55] This last action is attributed to PI3K-γ.[55] Insertion of Ca^{2+}-permeable AMPA receptors in the spinal dorsal horn is associated with the PI3K-β isoform.[35] Specific isoforms involved in all other receptor- or channel-trafficking functions are as yet undetermined. PI3Ks are known to not only sensitize and upregulate TRPV1 receptors in the dorsal root ganglia through NGF or TrkA, but, in addition, they play a role in the signal transduction pathway for MAP-kinase activation in addition.[3,60,61] They also participate in Epac-mediated eicosanoid signaling in a number of tissue types.[42,59] Within peripheral tissue, all class 1 isoforms of PI3K, except PI3K-β, are involved in inflammatory processes. Anti-inflammatory effects can be subdivided by site of action, permeability of the vascular endothelium, or chemotactic effects on infiltrating immune cells.[7,25,44,45]

Recently,[35] we observed that of the 4 class 1 PI3K isoforms, antagonism of only PI3K-γ reduced paw carrageenan-induced nociceptive behavior. We noted that similar pretreatment with antagonists to PI3K-α, -δ, or -γ, all reduced paw swelling as observed with cursory visual inspection. Pain and tissue damage resulting from inflammation or inflammatory disease constitute major medical problems. Although the 2 frequently covary,[29] it is now appreciated that they are separable. This may happen naturally during the course of a disease such as rheumatoid arthritis, in which inflammation waxes and wanes, but the pain is continuous.[2,14,53,57] Alternatively, pharmacologic agents may selectively ameliorate one condition, but not the other.[5,14,50]

This study was designed to examine the effect of isoform-specific PI3K antagonism on 2 indices of nociception (mechanical threshold and induction of spinal cord c-Fos) and 4 measures of inflammation (edema, plasma extravasation, and infiltration of neutrophils and macrophages) in the same inflammatory pain model.

1. Materials and methods

1.1. Animals

Male Holtzman rats (200-350 g; Harlan Industries, Indianapolis, IN) were housed with food and water provided ad libitum, under a 12-hour light or dark cycle. On the day of the experiment, animals habituated to the laboratory for at least 1 hour. All procedures were conducted between 09:00 hours and 16:00 hours. We attempted to blind the experimenters regarding experimental groups; however, the PI3K-γ antagonist had a distinctive yellow color. Experiments were in compliance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and the Institutional Animal Use Committee of the University of California, San Diego, approved all animal protocols.

1.2. Drug administration

After baseline testing (behavior) or at the start of the experiment, animals were anesthetized with isoflurane (5% induction; 1%-2% maintenance). A Hamilton syringe attached to calibrated poly-ethylene tubing and a 30-gauge needle were used to administer agents subcutaneously to the midplantar hind paw. Vehicle (1% DMSO) or a PI3K isoform-specific antagonist and 100 μL, 2% λ-carrageenan (Wako Pure Chemical Industries, Ltd) contained in the same tubing were injected 2 minutes apart. Class 1 PI3K antagonists used were: compound 15-e (mW 313.4; Enzo Life Sciences, Farmington, NY), alpha; TGX 221, (mW 364.4; Cayman Chemical Company, Ann Arbor, MI), beta; Cal-101 (mW 415.42; Selleck Chemicals, Houston, TX), delta; and AS252424 (mW 305.28; Cayman Chemical Company), gamma. Aliquots of antagonists in DMSO were defrosted and diluted just before use. Vehicle and all antagonist solutions were adjusted to pH of 7.2 to 7.4. Despite different reported IC50s, starting or standard doses of each antagonist were 5 nm administered in 50 μL of vehicle. Doses used were based on recent work.[13,35]

1.3. Behavioral paradigm

Animals were acclimated to the testing room for 2 days before the experiment and placed in test chambers for 1 hour. On the experiment day, mechanical allodynia was assessed before drug injection (baseline) and at 1, 2, 3, and 4 hours after unilateral intraplantar (IPL) hind paw carrageenan injections (100 μL; Wako Pure Chemical Industries, Ltd). Withdrawal responses were determined using calibrated von Frey filaments (Stoelting, Wood Dale, IL) with buckling forces between 0.41 to 15.1 g (4.0-148 mN) as described previously.[11,51]

1.4. Spinal cord c-Fos

A second, more objective, measure of nociceptive activation of the central nervous system was used to corroborate the behavioral results. Spinal cords of rats perfused 4 hours after carrageenan injection were removed, the L4-L5 segments cryoprotected and 30-μm transverse sections collected. Immunocytochemistry for c-Fos was performed on free-floating sections using the standard avidin–biotin peroxidase method. Tissue was rinsed in phosphate-buffered saline (PBS), incubated in 30% H_2O_2 and blocked with 10% normal goat serum in 0.3% PBS-TX (Triton X-100). Tissue was labeled using rabbit anti–c-Fos (1:20,000, Calbiochem; EMD Biosciences, La Jolla, CA, lot #D00088552) incubated overnight at room temperature, rinsed with PBS and then incubated in biotinylated goat antirabbit secondary antibody. The signal was amplified using a Vectastain ABC kit (Vector Laboratories, Burlingame, CA) and visualized with diaminobenzidine (DAB). Laminae I-III of L4 or L5 spinal segments were outlined. Cells positive for c-Fos were manually counted using Image J (NIH freeware, Bethesda, MD).

1.5. Inflammation

We used 3 independent measures of inflammation: edema, plasma extravasation, and immune cell infiltration.

1.6. Edema

To examine local effects of isoform-specific class I PI3K antagonists on carrageenan-induced edema, basal, and post-injection changes in paw volume were determined at 0.33, 0.5, 0.67, 1, 2, 3, and 4 hours after carrageenan for each hind paw using a plethysmometer (type 7150; Ugo Basile, Varese, Italy). We used 3 doses (1.7, 5.0, and 15 nm) for all antagonists except for PI3K-β (5 nm only). Antagonist was always injected into 1 paw and vehicle into the other, thus each animal served as his own control. After injections, anesthetic levels were reduced to the lowest concentration of isoflurane that resulted in immobility (approximately 1%). As previous studies have shown that anesthesia is anti-inflammatory, in that it reduces neutrophil adhesion to vascular endothelial cells, the anesthesia level was uniformly maintained at the lowest possible level.[39]

1.7. Plasma extravasation

Antagonists (50 μL, 5.0 nm) were injected 2 minutes before carrageenan. Ten minutes after carrageenan, Evans blue (EB; 50 mg/kg; MP Biomedicals, LLC) was injected through the tail vein. Rats were transcardially perfused with saline, 10 minutes after EB injection. A 5-mm² piece of glabrous skin surrounding the plantar injection site was removed and weighed. Tissue was incubated for 96 hours at room temperature in 2 mL formamide for dye extraction. Supernatants were read at 620 nm using an MRX revelation plate reader (Dynex Technologies, Chantilly, VA) and standardized to tissue weight and standard curves.

1.8. Immune cell infiltration

After injection of pretreatments and carrageenan, animals remained lightly anesthetized for 1 or 4 hours, at which time isoflurane was increased to 5% and animals were perfused first with saline and then cold 4% paraformaldehyde. The plantar skin (0.5 × 1 cm) was harvested from each hind paw and postfixed for 1 hour. After cryoprotection in sucrose, tissue was sectioned at 10 μm along the long axis and collected onto superfrost plus slides (Fisher Scientific, Waltham, MA) using a Leica CM 1800 cryostat, sequential sections were 150-μm apart. Slides were rinsed in a series of PBS washes and then blocked with 5% normal goat serum. Infiltrating neutrophils positive for myeloperoxidase (MPO) were labeled using prediluted rabbit anti-MPO polyclonal antibody (Thermo Scientific, Waltham, MA, lot #OK1780138). In other tissue, infiltrating proinflammatory macrophages were labeled using a mouse anti-CD11b monoclonal antibody (1:5000; Serotec, Raleigh, NC, lot #MCA2756A). An antilaminin polyclonal antibody (1:300, rabbit; Sigma Aldrich, St. Louis, MO, lot #L9393) was used to stain the dermal–epidermal junction and membranes of vascular endothelial cells in both sets of tissue. Secondary antibodies were goat antirabbit or goat antimouse conjugated to Alexa Fluor 594 for MPO and CD11b, respectively, and goat antirabbit antibody conjugated with Alexa Fluor 488, for the laminin (secondary antibodies 1:1000; Life Technologies, Carlsbad, CA).

Images of a minimum of 5 tissue sections, in the middle of each injection site, were captured using an Olympus BX-51 fluorescence microscope (Olympus Optical, Tokyo, Japan) and an Olympus America Magnafire SP camera. Exposure levels were kept constant. Filter sets consisted of an Omega Optical XF100 to 2 Green Bandpass Filters (Omega Optical Inc, Brattleboro, VT) for the Alexa 488 fluorphore and an Olympus UIS2 series U-MNG2 blue bandpass filter for the Alexa 594 fluorophore. A box within the dermis, just under the epidermal border, was superimposed on each captured image. Boxes were placed one-eighth of the width of the tissue section off midline and encompassed the full depth of the dermal layer. Infiltrating cells positive for MPO and CD11b were manually counted using Image J. MPO staining enclosed within the vascular endothelial cells was not included in neutrophil within-tissue counts, these cells were tabulated separately.

1.9. Statistics

Graph Pad Prism 4.0 (GPP 4.0; GraphPad Software, Inc, San Diego, CA) was used for statistical analyses and graphs. Two-way analyses of variance were used to determine a drug effect over the whole time course. T-tests were used to determine individual time effects and to compare areas under the curve for each time course of each antagonist compared to vehicle. Data were expressed as means and SEM, and a statistical significance of $P \leq 0.05$ was accepted. The Bonferroni multiple comparison test was used for post hoc testing of individual group comparisons.

2. Results

2.1. Antagonist pretreatment and assessment of carrageenan-induced mechanical allodynia

Basal mechanical paw withdrawal thresholds did not differ among pretreatment groups. After IPL injection of 50 μL of 1% DMSO, IPL carrageenan (N = 10) induced a steep decrease in the withdrawal threshold (**Fig. 1**), with the sharpest decline over the first hour. Intraplantar pretreatment with PI3K-α, -β, or -δ preferring antagonists (N = 7-9) had no effect on carrageenan-induced allodynia. In marked contrast, pretreatment with AS252424, the PI3K-γ isoform-specific antagonist (N = 8) delayed allodynia onset by over 2 hours compared to vehicle. These results confirm that PI3K-γ, but not any other class 1 PI3K isoform antagonist, prevents development of paw carrageenan-induced tactile allodynia.[35]
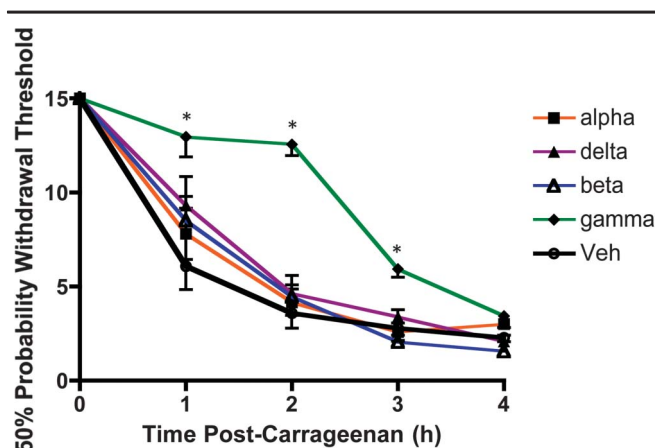

Figure 1. Only PI3K-γ antagonist inhibits mechanical allodynia. Pretreatment with only the PI3K-γ antagonist (N = 8) increases the onset time and reduces the magnitude of intraplantar carrageenan-induced mechanical allodynia. Antagonism of the PI3K-α, -β, and -δ isoforms was without effect. N = 7 to 9 per group. **$P \leq 0.01$; *$P \leq 0.05$, compared to 1% DMSO vehicle.

2.2. c-Fos

In the superficial dorsal horn of naive (anesthetized for 4 hours) animals, numbers of c-Fos–stained neurons were low (5.5 ± 1.1) and randomly distributed across grey matter. **Figure 2** depicts the c-Fos results along with representative micrographs. Vehicle injection alone resulted in a mean of 10.8 ± 0.6 stained neurons per section ($P \geq 0.05$). Combined vehicle and carrageenan injection increased the c-Fos count to 27.7 ± 2.3 ($P \leq 0.01$) compared to either naive or vehicle. Proportionately, more stained neurons were located in the medial portion of the superficial dorsal horn indicative of stimulation on the plantar paw.[30,37] Administration of PI3K-α,-β, or -δ antagonist pretreatments resulted in staining no different from that of vehicle- or carrageenan-treated animals. However, pretreatment with the γ-specific antagonist blocked the carrageenan-induced c-Fos expression such that it was no different than that of naive animals ($P \leq 0.01$) and lower than that of vehicle- or carrageenan-treated animals. In these animals, residual staining was randomly distributed.

2.3. Edema

We documented the carrageenan-induced changes in paw volume, and its amelioration by the various PI3K-isoform–specific antagonists. The basal paw volume was 1.66 ± 0.02 ccs across all groups. Carrageenan injection, preceded by 1% DMSO, resulted in increased paw volume. Most swelling occurred within the first hour, however, volume continued to increase throughout the experiment. There was virtually no difference in edema progression between paws in individual rats (data not shown). There was, however, a large difference in paw-swelling magnitude across animals, the range of final volume increase was from 0.69 to 1.52 ccs; this was unrelated to animal weight or initial paw size and large variations were seen on the same day, injected from the same batch of carrageenan. Compared to matched vehicle-treated paws, 5.0 nm of PI3K-α antagonist prevented paw swelling over the first hour; thereafter, anti-inflammatory effects slowly started to dissipate. Increasing the dose to 15 nm was marginally more effective for the first hour and resulted in less response variability. The maximal protective effect occurred at 20 minutes for both doses. Decreasing the dose to 1.7 nm resulted in edema equivalent to vehicle controls. Pretreatment with 5.0 nm TGX221, the PI3K-β antagonist, had no effect on carrageenan-induced swelling compared to contralateral vehicle-injected paw (**Fig. 3B**), lower and higher doses were not explored. The PI3K-δ antagonist, Cal-101, was the most effective pretreatment for blocking edema with marked effects for the 5.0-nm dose over the full 4-hour period. Half-log lower and higher doses were also successful in preventing paw swelling, interestingly, had both shorter durations of action and the lowest dose had a slightly slower onset. It is unknown as to why 15.0 nm was less effective than 5.0 nm, although, proinflammatory cytokines frequently have a similar bell-shaped dose–response curve and our observations may be a reflection of these downstream actions.

Pretreatment with 5 or 15 nm of AS252424, the PI3K-γ antagonist (**Fig. 3D**) successfully reduced edema development for 2 hours. The lowest dose of the PI3K-γ antagonist resulted in no change from the contralateral vehicle-injected paws. For all dose groups, mean edema seen in vehicle-injected paws (0.33, 1.0% and 3.0% DMSO) was comparable, except for the low-dose PI3K-γ antagonist-injected animals. Unexpectedly, in this group, both vehicle and antagonist-injected paws exhibited more swelling than in any other treatment group. Thus, although vehicle-injected paws were combined for presentation purposes for all other groups,
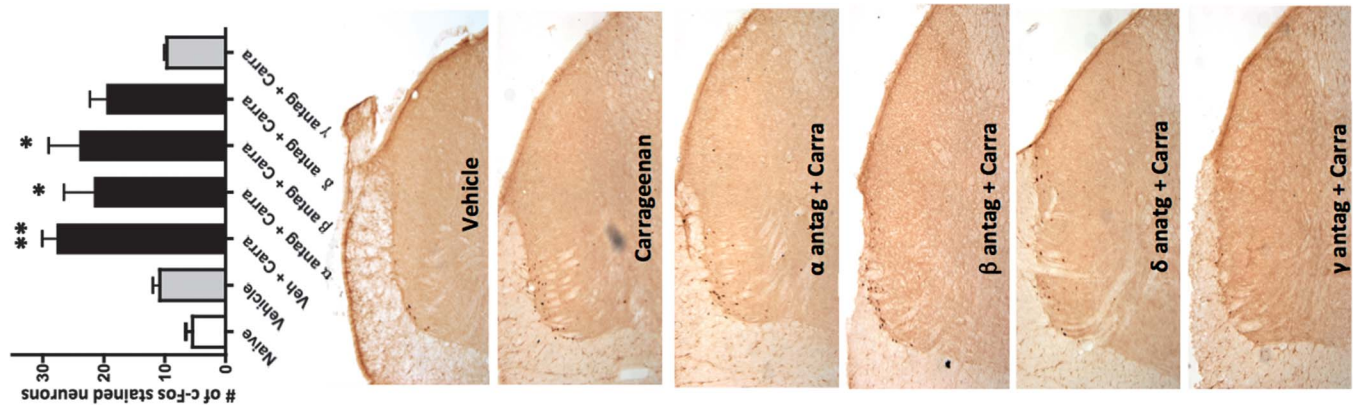
Figure 2. Only PI3K-γ antagonists blocks carrageenan-induced c-Fos. Subcutaneous pretreatment with only the PI3K-γ antagonist blocks the intraplantar carrageenan-induced somatotopic expression of c-Fos in the dorsal horn. Peripheral antagonism of the PI3K-α, -β, and -δ isoforms was no different than the intraplantar vehicle plus carrageenan. N = 3 to 8 per group; **$P \leq 0.01$; *$P \leq 0.05$, compared to vehicle.

vehicle data for the low-dose γ antagonist-injected animals are shown separately. Statistics were always performed on matched contralateral paws of the group being analyzed.

2.4. Plasma extravasation

We measured plasma extravasation to determine if IPL PI3K antagonism-blocked plasma protein leakage from the vasculature. Pretreatment with 5.0-nm PI3K-α, -δ, or -γ antagonists, all resulted in up to a 50% decrease in carrageenan-induced plasma extravasation compared to the vehicle (**Fig. 4**). Unlike the other isoform antagonists, pretreatment with PI3K-β antagonist was ineffective in blocking leakage of EB. Because of reports of synergy between PI3K-δ and PI3K-γ,[56] we repeated this experiment using a pretreatment cocktail containing a half-dose of each antagonist (2.5 nm Cal-101 and 2.5 nm AS252424). Dual inhibition of the PI3K-δ and -γ antagonists reduced mean plasma extravasation by more than 93%.

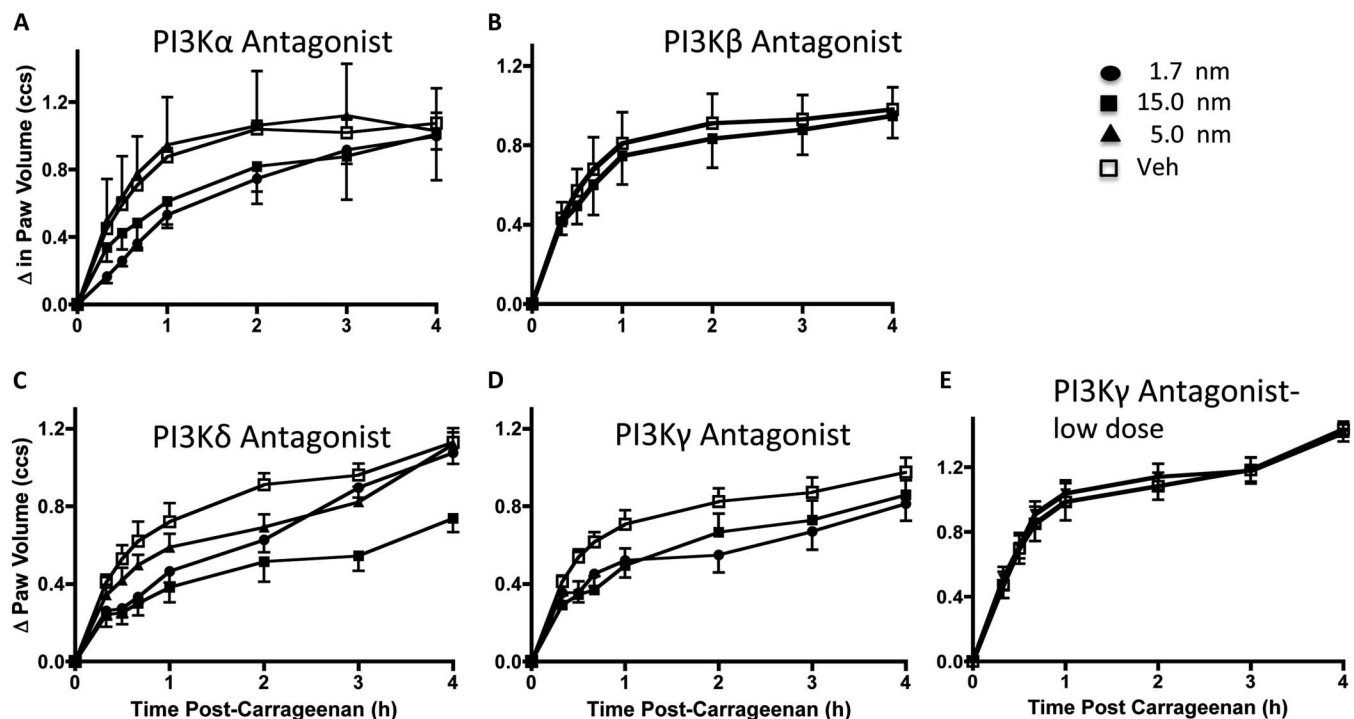## PI3K antagonists inhibit edema: isoform specific and time dependent

Figure 3. Multiple PI3K antagonists inhibit carrageenan-induced edema. (A) Edema was reduced in the animals treated with the 1.5- and 0.5-mM PI3K-α antagonist compared to vehicle injected contralateral paws. Analysis of variance $P \leq 0.001$ post hoc testing revealed significant differences for both injected groups throughout the first 2 hours after carrageenan $P \leq 0.05$. Peripheral injection of the lowest dose of the antagonist, 1.7 nm, was not effective. (B) Intraplantar pretreatment with the 5.0-nm dose of the PI3K-β had no effect on edema. (C) Edema was reduced in the animals treated with 1.7-, 5.0- and 15.0-nm PI3K-δ antagonist compared to vehicle injected contralateral paws. Analysis of variance $P \leq 0.001$. Only the middle dose was effective for the entire 4-hour period. (D) The 2 higher doses of the PI3K-γ antagonist modestly reduced the carrageenan-induced edema compared to vehicle pretreatment $P \leq 0.05$. (E) The lowest dose of PI3K-γ antagonist was without effect on carrageenan-induced edema when given as a pretreatment. N = 6 to 9 per group.
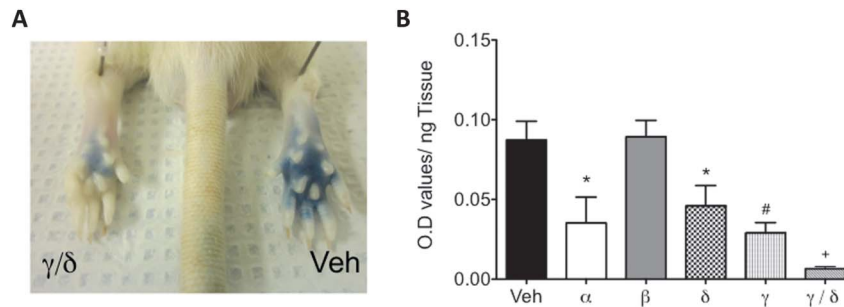
Figure 4. Multiple PI3K antagonists block plasma extravasation. (A) Photograph illustrating carrageenan-induced leakage of Evans blue and visible edema after vehicle pretreatment with vehicle on the right paw. The left paw was injected with a pretreatment cocktail of 2.5 nm each of PI3K -δ and -γ antagonists. (B) Bar graph indicating that pretreatment with antagonists to PI3K-α, -δ, and -γ inhibits carrageenan-induced plasma extravasation. *$P \leq 0.05$, #$P \leq 0.01$, +$P \leq 0.001$.

2.5. Immune cell infiltration

2.5.1. Neutrophils

Presumptive neutrophils containing MPO-stained granules were virtually absent in paw tissue from naive rats. Intraplantar injection of 150-μL vehicle resulted in a modest increase in MPO staining within the delineated area of interest. Although a relatively small number of microglia and Schwann cells are positive for MPO in some pathological circumstances,[21] we believe that the majority of labeled cells were neutrophils. Carrageenan preceded by vehicle, elicited diapedesis of more MPO-positive cells into tissue than vehicle alone (**Fig. 5A and E**). The graphs indicate means of tissue in or near the center and several millimeters lateral to the injection site. The carrageenan-induced increase was equally well prevented by pretreatment with the -α, -β, or -γ antagonists; there was a tendency for these pretreated tissues to contain fewer neutrophils than seen with injection of vehicle alone.

Only rare presumptive neutrophils were observed clearly within blood vessel lumena after perfusion, these were not counted. Most profiles were extravascular, however, in injured tissue a small number appeared to be clustered within vascular endothelial cells. As MPO stained only neutrophilic granules, our data do not indicate whether other parts of the immune cell breached cell walls, as is commonly seen during transcellular diapedesis[9,10] or if in fact they were contained within the endothelial cell. However, in many cases, optimized confocal microscopy using the orthogonal sectioning tool revealed that granules were restricted to vascular endothelial cells (**Fig. 6**). We chose to tabulate these "endothelial neutrophils" separately from those with clearly extravascular MPO. Although absolute counts were lower, the pattern was the same as that seen in extravascular tissue with the notable exception of PI3K-γ antagonist pretreatment (**Fig. 5B**). Pretreatments that blocked edema also reduced the number of extravascular neutrophils to below vehicle control levels, however, only pretreatment with the PI3K-α and -δ antagonists reduced the accumulation of endothelial neutrophils in parallel. Despite reduction in extravascular neutrophils after PI3K-γ antagonist pretreatment, endothelial neutrophils in the same tissue showed a marked increase comparable or higher to that seen in animals with vehicle and carrageenan. The percentage of the total number of stained neutrophils confined to endothelial cells increased to 35% (**Table 1**; **Fig. 5J**). We have also observed this PI3K-γ antagonist induced clustering of infiltrating immune cells in the endothelial walls 1 hour after injection using H&E staining (data not shown).

2.5.2. Macrophages

Similar to neutrophils, naive animals had extremely low counts of CD11b-positive macrophages; most tissue sections had none (**Fig. 7**). One hour after carrageenan injection, there were still many sections or animal that displayed no CD11b-positive cells and only tissue sections within 300 μm of the needle track showed activity. Four hours after carrageenan, there was a massive influx of stained cells throughout the entire examined area. At this time, pretreatment with the PI3K-α and -β antagonists had no effect on macrophage infiltration (**Fig. 7F and G**). However, pretreatment with the PI3K-δ and -γ antagonists were both equally effective in reducing macrophage numbers. The pattern of inhibitor action was identical between the 2 tissue compartments and the PI3K-γ antagonist did not have a differential effect as seen for neutrophils.

3. Discussion

Intraplantar carrageenan elicits nociception and edema, dermal neutrophil accumulation, neurogenic inflammation, and release of proinflammatory cytokines, which in turn, activate signal transduction cascades.[15,17,43,51] Our data confirm many previous observations that PI3Ks in the peripheral nerve and paw tissue are involved in both nociception and tissue inflammation arising from IPL carrageenan. One novel aspect of our study is that the 4 class 1 PI3K isoforms are differentially involved in these processes. This is not surprising as the isoforms frequently have distinct, nonredundant functions in single cells and individual isoforms vary in function in different cells or tissues.[41] Direct actions of individual kinase isoforms on nociceptors, vascular endothelium, resident skin cells eg, keratinocytes and circulating immune cells are likely. Given the many parallel and redundant pathways that contribute to pain and inflammation in vivo, PI3K isoforms probably have actions at more than 1 locus. What was surprising to us was how much easier it was to block clinical signs of inflammation than nociception.

3.1. Nociception

Only antagonism of PI3Kγ reduced carrageenan-induced pain behavior and expression of spinal c-Fos. Despite the fact that PI3K-α and -δ antagonists injected into the paw significantly reduced edema, plasma extravasation and infiltration of immune cells, indicating their probable effect on vascular endothelium and the immune system, they did not alter pain behavior or spinal expression of c-Fos. Although PI3K-γ antagonism also reduced the same signs of inflammation, it is likely that antihyperalgesic
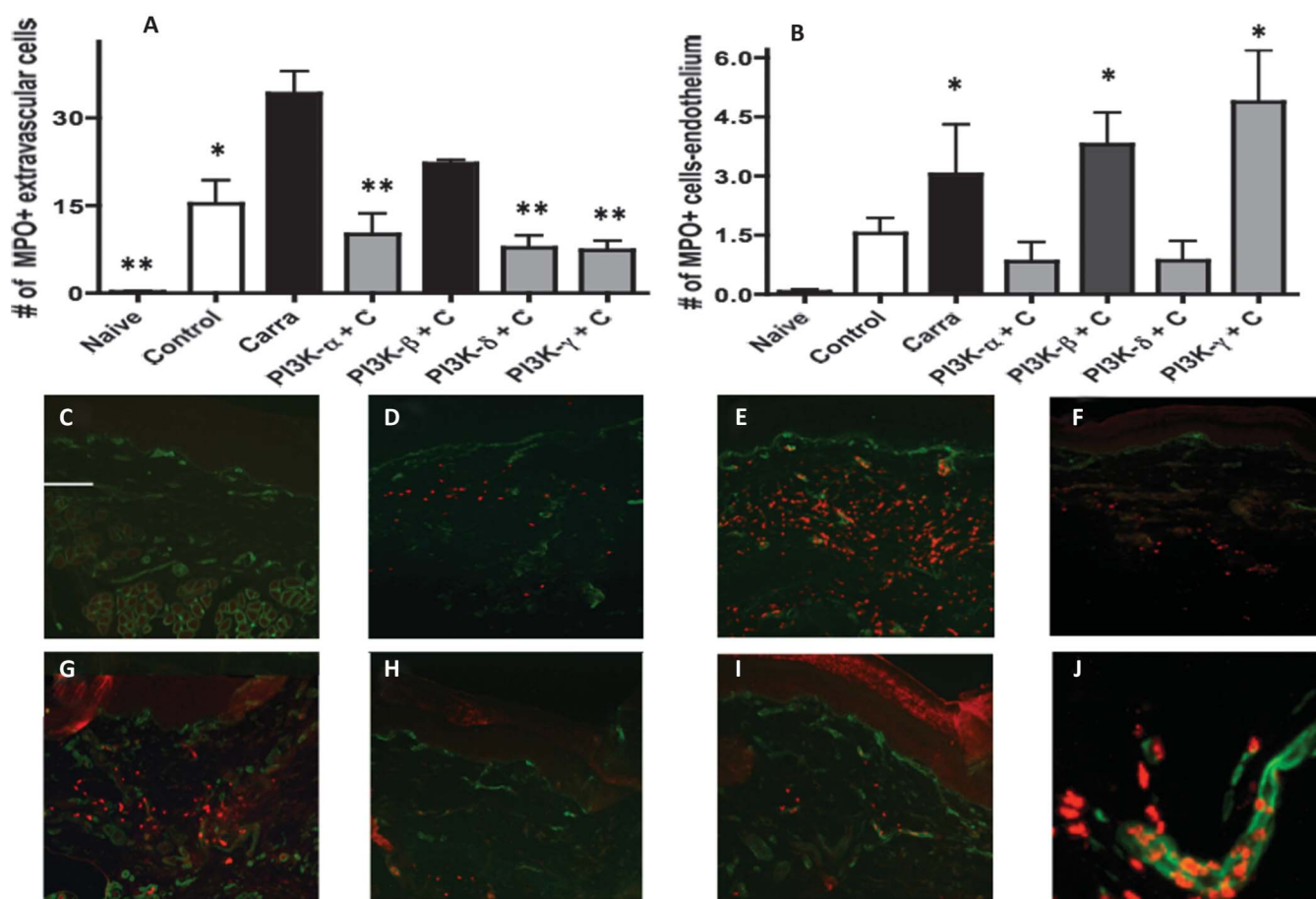
Figure 5. Multiple PI3K antagonists block neutrophyl infiltration. (A) Intraplantar carrageenan elicits neutrophil infiltration into the tissue 1 hour after injection. This is blocked by individual antagonism of PI3K-α, -δ, and -γ, but not PI3K-β. **$P \leq 0.001$, *$P \leq 0.01$, compared to vehicle + carrageenan. N = 3 to 7 rats per group. (B) The same pattern is seen for small clusters of myeloperoxidase (MPO)-stained granules contained within vascular endothelial cells with the exception that PI3Kγ antagonism increases, rather than decreases, the number of "endothelial neutrophils." *$P \leq 0.05$, compared to PI3K-γ antagonist + carrageenan. (C-I) Representative sections stained for laminin (green) and MPO (red) (C). Naive (D). Control or vehicle (E). Carrageenan injected with vehicle as pretreatment, (F). Carrageenan injected with PI3K-α antagonist as pretreatment, (G). Carrageenan-injected animals with PI3K-β antagonist as pretreatment, H. Carrageenan injected with PI3K-δ antagonist as pretreatment, and (I). Carrageenan injected with PI3K-γ antagonist as pretreatment, (J). Enlargement of blood vessel with clustered neutrophils. (C-I) Calibration bars = 100 μm.

effects of PI3K-γ antagonism are mediated by direct prevention of primary afferent sensitization. PI3K-γ seems to be selectively expressed in nociceptive (IB4 or TRPV1 receptor containing) primary afferent neurons.[16,31,35] Although PI3K-α is also seen on primary afferent neurons, it appears to have no particular affinity for nociceptive ones.[35] Intradermal injection of the PI3K-γ catalytic subunit quickly induces mechanical hyperalgesia.[38] Intraplantar injection of receptor tyrosine kinase (RTK) activators, such as ephrinB1-Fc, activates both MAP-kinase and PI3K pathways within primary afferent neurons[8] and induces a dose-dependent increase in PI3K-γ expression in skin and a PI3K sensitive hyperalgesia.[22] PI3K-γ has traditionally been thought to be downstream of G-coupled protein receptors; this has been definitively shown for μ-opiate receptors on nociceptive primary afferent neurons.[31] Relevant receptor classes for primary afferent PI3K-γ activation after carrageenan remain undetermined. However, given that Epac can mediate prostaglandin signaling leading to peripheral nociceptor sensitization[19,54] and that PI3K is downstream of cAMP and Epac transduction pathways in other systems,[42,59] one can speculate that locally released prostaglandins or other cAMP activating agents, activate nociceptor

PI3K-γ in turn. PI3K-γ–induced hyperalgesia is blocked not only by PI3K antagonists, but also by MEK inhibitors, implying crosstalk between the 2 signal transduction pathways.[38,61] Activation of FPRs on nociceptive (Nav 1.8 containing) afferent neurons produces mechanical hyperalgesia that is unrelated to edema.[12] Interestingly, PI3K is downstream of FPRs in several cell types,[1] and PI3K-γ is downstream of bacterial activation in others,[24] although this has not yet been demonstrated in nociceptive neurons, we postulate a downstream role for PI3K-γ in these cells.[25]

Separation of nociception and inflammatory signs has been reported previously. In the K/Bx/N serum transfer model of rheumatoid arthritis, pain behavior develops before and outlasts clinical signs of arthritis.[14] Interestingly, pharmacology of acute pain behavior and inflammation is different than that of late inflammatory pain behavior in this model, implying that acute pain is dependent on joint swelling, although the more chronic pain is independent. Woolf and colleagues,[12] using a bacterial infection model, demonstrated that induction of nociception was not coincident with edema, infiltration of neutrophils or macrophages, or of tissue levels of proinflammatory cytokines, but rather was
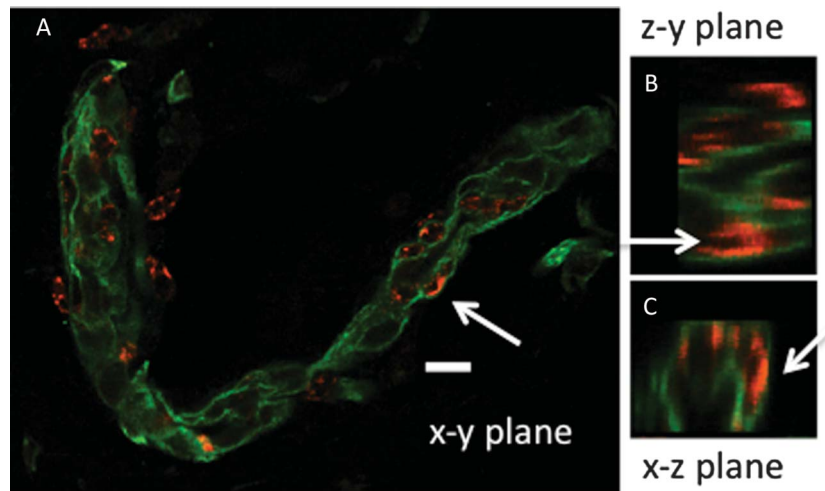
Figure 6. PI3K-γ antagonist increases the appearance of MPO granules in endothelial cells. (A) Confocal image of collapsed blood vessel stained for laminin (green) and myeloperoxidase (MPO; red). Arrow indicates MPO staining and vascular endothelium cell walls. In the x-y plane, calibration bar = 10 μm. The orthogonal sectioning tool allowed us to capture x-z (C) and z-y (B) planes from an optimized z-stack contained centered on the MPO-stained element. The MPO staining appears to be confined within the vascular endothelial cell.

linked to bacterial load. Similar to our model, blockade of immune system activation including neutrophil infiltration, did not ameliorate the hyperalgesia. Some inflammatory pain models have also demonstrated the lack of relationship between polymorphonuclear leukocytes and nociception and have indeed postulated that immune cells contribute more to analgesia than to hyperalgesia.[46,47] However, the literature is contradictory. Other studies using the paw carrageenan model have demonstrated the dependence of pain behavior on neutrophil infiltration,[17] and still others on activation of the proinflammatory cytokine cascade.[15]

3.2. Inflammation

The various PI3K isoforms participate in vascular endothelial permeability and vasodilatation as they regulate endothelial nitric oxide synthase, cell shape and stiffness by alterations in the actin cytoskeleton, and adhesion and recruitment of immune cells.[26,40,48] PI3K activation enhances transcellular diapedesis of neutrophils[33] although the relevant isoforms are unknown. Kumar et al.,[33] recently showed that paracellular, but not transcellular diapedesis is affected by individual suppression of all of the class I PI3K isoforms. PI3Ks also mediate the mast cell adenosine A3 response.[34] Although PI3K-α is best known for its role in metabolic regulation,[23] it clearly is a player in inflammation-induced changes in vascular integrity. Antagonism or depletion of PI3K-α was more effective than that of PI3K-β, -δ, or -γ in preventing tumor necrosis factor (TNF)-induced decreases in barrier function and thus, permeability increases, in human vascular endothelial cells.[6,7] This appears to be due to changes in

cell-to-cell junctional morphology influenced by phosphorylation of vascular endothelial cadherin. Bond strength between endothelial cells governs plasma extravasation and edema and paracellular diapedesis of white blood cells, our results indicating a strong, albeit transient, effect of PI3Kα inhibition on this parameters. Lack of effect of the PI3Kα antagonist on macrophage infiltration could be due to a lack of drug efficacy at this later time. Alternatively, if one assumes that TNF is the major effecter in carrageenan-induced inflammation,[15] this may represent a multi-phased response as TNF-induced PI3Kα mediated activity in endothelial cells in vitro has an early peak, 10 to 30 minutes after injection and then falls off before a second phase of Akt phosphorylation and junctional changes.[6] We could have missed this last phase. Interestingly, PI3K-α antagonism has no chemotactic effect on macrophage cells in vitro.[41]

PI3K-δ and -γ are best known for their influences on inflammation[23] and are postulated to work separately and together. These 2 isoforms have also been reported to work together to control endothelial E-selectin and thus, to regulate neutrophil rolling and migration.[40,44,45] It has been proposed that immune cell infiltration in response to many chemotactic responses uses both isoforms in a combined biphasic response with the first phase requiring PI3K-γ, and the second phase being PI3K-γ independent, but PI3K-δ dependent.[4,36] In other studies, these 2 isoforms appear to have synergistic actions.[32,56] We observed an equal inhibition of neutrophil and macrophage infiltration produced by pretreatment with individual PI3K-δ and -γ antagonists, although PI3K-δ inhibition produced the more consistent reduction in edema across the whole time course. Interestingly, the combination of low-dose PI3K-δ and -γ antagonists was

	Table 1						
Naive	**Vehicle**	**Carrageenan + Vehicle**	**Carrageenan + PI3K-α**	**Carrageenan + PI3K-β**	**Carrageenan + PI3K-δ**	**Carrageenan + PI3K-λ**	
12.5 ± 12*	10.17 ± 4.7†	7.35 ± 2.5†	10.7 ± 5.6†	15.48 ± 1.9*	11.66 ± 4.8*	35.35 ± 3.9	
3/4	1/6	1/6	3/6	0/4	2/5	0/7	

The first row indicates the percentage of MPO-stained cells found in the entire area of interest that are within the vascular endothelium. The ratio in the second row indicates the number of animals of the total, in which the mean value of MPO-stained cells in the vascular endothelium was 0.
Analysis of variance ≤ 0.01.
* $P \leq 0.05$.
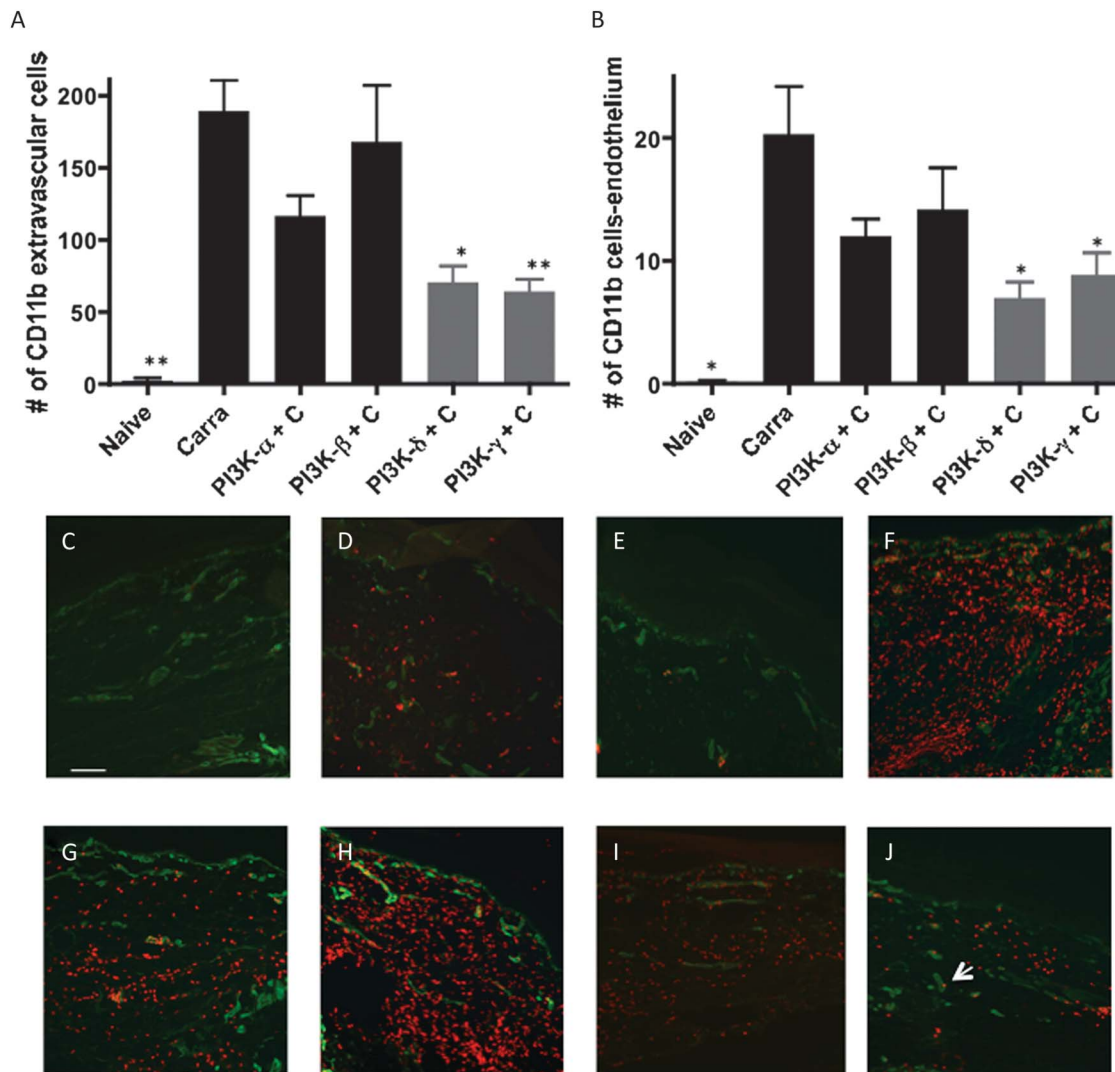† $P \leq 0.01$ compared to carrageenan + PI3Kγ.

Figure 7. PI3K-δ and -γ antagonists block macrophage infiltration. (A) Intraplantar carrageenan elicits macrophage infiltration into the tissue 4 hours after injection. This is reduced by pretreatment with antagonists to PI3K-δ and -γ, but not PI3K-α and -β. **$P \leq 0.001$, *$P \leq 0.01$, compared to vehicle + carrageenan. N = 4 to 6 rats per group. (B) Intraplantar carrageenan elicits macrophages to associate with the vascular endothelium. This is reduced by pretreatment with antagonists to PI3K-δ and -γ, but not PI3K-α and PI3K-β in the same pattern as seen for tissue infiltration. *$P \leq 0.01$, compared to vehicle + carrageenan. (C-I) Representative tissue sections stained for laminin (green) and CD11b (red) harvested 1 or 4 hours after injection. (C) Naive tissue; (D) Tissue parallel to and near needle entry harvested 1 hour after carrageenan + vehicle injection; (E) Tissue 2-mm away from needle path, harvested 1 hour after injection; (F) Carrageenan injection with vehicle pretreatment harvested 4 hours after injection; (G) Carrageenan-injected animals with PI3Kα antagonist pretreatment; (H) Carrageenan-injected animals with PI3K-β antagonist pretreatment; (I) Carrageenan-injected animals with PI3K-δ antagonist pretreatment; (J) Carrageenan-injected animals with PI3K-γ antagonist pretreatment. Arrow indicates macrophage on blood vessel.

synergistic in reducing carrageenan-induced plasma extravasation during the first phase of inflammation. Combination of PI3K-δ and -γ antagonists was inconsistent in edema studies, and these experiments were not pursued.

The PI3Kδ isoform is found in both vascular endothelium and neutrophils.[44] Its antagonism substantially reduces TNF-neutrophil adhesion to vasculature endothelium. In contrast, exposure of neutrophils to antagonist, before the experiment had no effect on neutrophil or vasculature interactions implying that the relevant kinase is on the endothelium.[44] A postulated role for PI3K-δ is selectin-mediated neutrophil tethering to endothelial cells.[44]

PI3K-γ is also found in the vascular endothelium and immune cells. In several models, its inhibition blocks immune cell infiltration, neutrophils in particular, and PI3K-γ is involved in the immune response to chemokines[20] and to complement factor

C5a and FPRs.[25] Neutrophils from PI3K-γ–deficient animals exhibit normal rolling, slightly attenuated adhesion and loss of diapedesis. This profile is due to the lack of PI3K-γ in neutrophils, rather than tissue,[36] although endothelial PI3K-γ also contributes to TNF activation of various MAP-kinase pathways.[45] Lack of PI3K-γ in the endothelium reduces selectin-mediated neutrophil attachment, and thus, increases the rolling speed.[45,49] Although, PI3K-γ antagonism or loss has consistently been shown to reduce paw edema after carrageenan injection,[20,27,58] its ability to block neutrophil infiltration in response to this pleiotropic stimulus has been called into question.[25,36] However, our data indicate that under our conditions PI3K-γ antagonism is sufficient to block carrageenan-induced neutrophil infiltration. In summary, PI3K-γ plays a role in peripheral nociception distinct from any modulating action that it has on immune cell infiltration or vascular permeability. Given the unique placement of this PI3K isoform in

nociceptive primary afferent fibers, we believe that antinociception induced by administration of the PI3K-γ antagonist represents a direct activation of PI3K-γ within nociceptive neurons, either within a sensitizing signal transduction cascade or in translocation of receptors or channels into or out of the plasma membrane. PI3K-α, -δ, and -γ all participate in different aspects of chemotaxis and endothelial permeability with different time courses and sites of action. Pain relief and maintained suppression of inflammation will require blockade of more than 1 group I PI3K isoform.

Conflict of interest statement

The authors have no conflicts of interest to declare.

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