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ENHANCED VASCULAR PERMEABILITY IN RAT SKIN INDUCED BY SENSORY NERVE STIMULATION: EVALUATION OF THE TIME COURSE AND APPROPRIATE STIMULATION PARAMETERS

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Abstract—Activation of nociceptors causes them to secrete neuropeptides. The binding of these peptides to receptors on blood vessels causes vasodilation and increased vascular permeability that allows loss of proteins and fluid (plasma extravasation, PE); this contributes to inflammation. This study defines the relationship between electrical activation of nociceptors and PE and evaluates the time course of this response in the skin of rats. We measured the time course and extent of PE by digital imaging of changes in skin reflectance caused by leakage of Evans Blue (EB) dye infused in the circulatory system before stimulation. Stimulation of the exclusively sensory saphenous nerve caused the skin to become dark blue within 2 min due to accumulation of EB. While PE is usually measured after 5–15 min of electrical stimulation, we found that stimulation for only 1 min at 4 Hz produced maximum PE. This response was dependent on the number of electrical stimuli at least for 4 Hz and 8 Hz stimulation rates. Since accumulation of EB in the skin is only slowly reversible, to determine the duration of enhanced vascular permeability we administered EB at various times *after* electrical stimulation of the saphenous nerve. PE was only observed when EB was infused within 5 min of electrical stimulation but could still be observed 50 min after capsaicin (1%, 25 μl) injection into the hind paw. These findings indicate that enhanced vascular permeability evoked by electrical stimulation persists only briefly after release of neuropeptides from nociceptors in the skin. Therefore, treatment of inflammation by blockade of neuropeptide release and receptors may be more effective than treatments aimed at epithelial gaps. We propose, in models of stimulation-induced inflammation, the use of a short stimulus train.

Key words: neurogenic inflammation, plasma extravasation, nociceptors, pain, vascular epithelium.

Many pathological conditions associated with inflammation are exacerbated by the peripheral release of vasoactive agents that enhance vascular permeability. The endothelium, which forms the interface between blood and tissues, controls the extravasation of macromolecules and prevents the loss of blood constituents, such as albumin and other plasma proteins. The neuropeptide, substance P (SP), plays a significant role in altering endothelial permeability. Following activation of nociceptors, SP is released and binds to the neurokinin-1 (NK-1) receptor on blood vessels (Andrews et al., 1989), eventually causing the formation of small gaps that allow plasma extravasation (PE) (McDonald et al., 1999; Baluk et al., 1997). SP also acts on mast cells and leukocytes, inducing the release of inflammatory substances such as histamine, serotonin and prostaglandins, which play a role in prolonging leakage (Jorizzo et al., 1983; Hartung et al., 1988; Le Filliatre et al., 2001; Schmelz and Petersen, 2001). Primary afferent terminals also contain calcitonin gene-related peptide (CGRP) (Gamse and Saria, 1985; Bartfai et al., 1988), which, when released following activation, causes vasodilation and potentiates the effects of SP. CGRP does not produce PE by itself at physiological concentrations (Brain et al., 1985; Louis et al., 1989; Gamse and Saria, 1985). Together, the actions of SP and CGRP provoke an inflammatory response known as neurogenic inflammation (NI).

In the currently accepted model of NI, a noxious stimulus activates the nociceptive afferents thereby causing depolarization and release of SP and CGRP (Bayliss, 1901; Jancso et al., 1967; Lembeck and Holzer, 1979). Although, a variety of substances are stored in primary afferent terminals that can contribute to NI, SP and CGRP are the principal mediators of NI (Garret et al., 1991; Lembeck et al., 1992; Xu et al., 1992; Escott and Brain 1993; Wiesenfeld-Hallin and Xu, 1993; Lynn, 1996; Gonzalez et al., 2005). Experimentally, NI can be elicited by electrical stimulation of primary afferent nerves at intensities sufficient to activate nociceptive fibers (Szolcsanyi, 1988) or by application of a variety of inflammatory irritants, such as capsaicin or mustard oil (Szolcsanyi, 1996; Fiorentino et al., 1999) which activate nociceptors. While high stimulus intensities and low frequencies (1–10 Hz) are the best parameters to induce NI (Szolcsanyi, 1996; Pinter et al., 1997; Towler and Brain, 1998), there is no consensus regarding the appropriate duration and frequency of the stimulus train. Previous studies used stimulus train durations of 5–15 min and 1–8 Hz to elicit PE (Lembeck and Holzer, 1979; Escott and Brain, 1993; Le Filliatre et al., 2001; Gonzalez et al., 2005). In the development of treatments for conditions involving NI, measurements of PE may be valuable. However, potential drug effects could be difficult to detect if the stimulation evoking the PE is supramaximal. Therefore, we sought to determine stimulation parameters causing submaximal PE by investigating

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Abbreviations: CGRP, calcitonin gene-related peptide; DRR, dorsal root reflex; EB, Evans Blue; NI, neurogenic inflammation; NK-1, neurokinin-1; PE, plasma extravasation; PI, pixel intensity; ROI, region of interest; SP, substance P.

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the effects of train length on saphenous nerve stimulation-induced PE.

The duration and maximal extent of PE depend on many factors, such as the continued presence of inflammatory mediators, the driving force for the movement of proteins out of blood vessels (i.e. blood pressure and concentration gradient), time course of activation and internalization of G-protein-coupled receptors (GPCR) and the kinetics of gap formation and closure in the endothelium. Depending on the type of stimulus used to induce PE, the resulting enhanced vascular permeability can be a transient process involving rapid opening and closing of endothelial gaps or the event can be prolonged (van Hinsbergh and van Nieuw Amerongen, 2002).

Vascular permeability is often evaluated by measuring extravasation of Evans Blue (EB) dye which binds to the plasma protein albumin that leaks out of blood vessels following induction of NI and turns the tissue dark blue. Spectrophotometric analysis of EB content in tissues and staining of endothelial gaps at various time points after inducing PE have been used to describe the duration of vascular permeability increases in rat trachea due to systemic injection of SP or stimulation of the vagus nerve (McDonald, 1994; Baluk et al., 1997; McDonald et al., 1999). Evaluation of PE and/or edema in the skin is a commonly used method of studying NI and PE and is frequently used in conjunction with behavioral and electrophysiological data when studying nociceptor activity (Chen et al., 2002; Gonzalez et al., 2005; Ambalavanar et al., 2006; Valencia-de Ita et al., 2006). However, little is known about the time course of saphenous nerve induced-PE evoked by electrical or chemical (e.g. capsaicin) stimulation. Therefore, we investigated the time course of enhanced vascular permeability in skin that is caused by electrical and chemical activation of nociceptors. We used digitized image analysis, a non-invasive technique that allows PE to be measured continuously (Gonzalez et al., 2005). This knowledge is of interest in terms of better understanding the mechanisms underlying PE and vascular permeability and may facilitate the use of PE measurements to detect drug effects on NI.

EXPERIMENTAL PROCEDURES

Animals and surgical procedures

Experiments were performed under protocols approved by the University of Toronto Animal Care Committee. All procedures were in strict accordance with the Canada Animal Research Act on the ethical use of animals. To minimize animal suffering, proper anesthesia was used and the minimum number of rats was used per experiment. Male Sprague–Dawley rats (Charles River Laboratories, Montreal, QC, Canada) weighing 250–400 g were housed in pairs in constant humidity, 20 °C with 12-h light/dark cycles. Anesthesia was induced with an i.p. injection of sodium pentobarbital (50 mg/kg). An adequate anesthetic level was evaluated at regular intervals throughout the experimental procedure by absence of pain reflexes; when necessary, an additional injection (5 mg/kg) of pentobarbitone was applied i.p. Two hours before antidromic nerve stimulation the rats were injected with 10 mg/kg i.p. of guanethidine to inhibit vasoconstriction due to activation of sympathetic efferents in order to simplify interpretation of the results.

Both hind limbs were shaved with an electrical shaver, and the hair of the dorsal part of the paws completely removed by depilatory cream (Andrea® Faulding Healthcare P/L, Melbourne, Victoria, Australia). An endotracheal tube was inserted to facilitate spontaneous breathing and a catheter was inserted into the tail vein for infusion of EB. Rectal temperature was held constant at 37–38 °C by a heating pad that was feedback controlled. Heart rate was monitored during the experiments. At the end of experiments, animals were killed by injection of T-61 (0.3 ml/mg) (Hoechst-Roussel Canada Ltd., Montreal, Quebec), a fast acting killing agent that contains 200 mg/ml embutramide, 50 mg/ml mebozonium iodide and 5 mg/ml tetracaine hydrochloride.

Induction of PE

To stimulate nociceptors both saphenous nerves were dissected free in the thigh, cut proximally and suspended over bipolar platinum electrodes connected to a constant current stimulus isolation unit (World Precision Instruments, model A360, Sarasota, FL, USA) and a stimulator (World Precision Instruments, model A310). The skin flaps of the wound were raised and paraffin oil applied to cover fully the nerve. Constant current stimulation pulses (3 mA, 0.5 ms) were delivered at 4 Hz or 8 Hz for 15 s, 30 s, 1 min or 10 min. Different animals (six to eight animals in each group) were used for each set of stimulation parameters tested. In separate experiments, 1% capsaicin (Calbiochem®, San Diego, CA, USA) was used to induce PE. Twenty-five microliters of 1% capsaicin (in 93% saline and 7% Tween-80) was injected s.c. in the dorsal region of the paw.

Evaluation of PE

Digitized image analysis was used to evaluate PE. Four minutes to 5 min before electrical stimulation, 50 mg/kg of EB dye (10 mg/ml) was injected i.v. To monitor the time course of the change in reflectance in the skin, we used a monochrome CCD video camera (Cohu 4915 with ER-2523D1 remote control, resolution: 768×494, Cohu Inc., San Diego, CA, USA), with a 28–70 mm zoom lens (Tamron, Commack, NY, USA). The camera was connected to a frame grabber (ATI All-in-Wonder, model Radeon 7500, Markham, ON, Canada). The gain and black level of the camera were set by a manual remote control and were not changed during an experiment. The camera gamma was set to 1 and automatic gain control was set to OFF. Illumination with white light was provided via a circular fiber optic ring held 15 cm above the leg. The images were digitized at regular intervals and saved as uncompressed AVI files using the program Studiosurveillance (www.studio86designs.co.uk). Images were recorded every 20 s, beginning 1 min before i.v. injection of EB. After EB injection, images were recorded for 4 min to obtain a baseline and for up to 45 min after electrical or chemical stimulation. Stimulation caused the skin to become dark blue. The resulting change in light reflectance due to saphenous nerve stimulation was measured in a rectangular (15×35 mm) region of interest (ROI) in the dorsomedial part of the paw (area innervated by the saphenous nerve). The size of the ROI was constant for each experiment in which PE was evoked by saphenous nerve stimulation. For s.c. injections of capsaicin a circular area of skin (about 10 mm diameter centered on the injection site) was used as the ROI to measure reflectance and the size of this ROI was also constant throughout each experiment. The change in pixel intensity (PI) was evaluated using an eight-bit gray scale of 0–255. To facilitate presentation of data, the gray scale of the images was inverted using the program ImageJ (inverted PI=255−measured PI). Details about our digitized image analysis have been previously reported by Gonzalez et al. (2005).