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Primary Afferent Neurons Express Functional Delta Opioid Receptors in Inflamed Skin

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Abstract

Peripherally-restricted opiate compounds attenuate hyperalgesia in experimental models of inflammatory pain, but have little discernable effect on nociceptive behavior in normal animals. This suggests that activation of opioid receptors on peripheral sensory axons contributes to decreased afferent activity after injury. Previously, we reported that direct application of morphine to cutaneous receptive fields decreased mechanical and heat-evoked responses in a population of C-fiber nociceptors in inflamed skin. Consistent with reported behavioral studies, direct application of morphine had no effect on fiber activity in control skin. The aim of the present study was to determine whether mechanical responsiveness of nociceptors innervating inflamed skin was attenuated by direct activation of delta opioid receptors (DOR) on peripheral terminals. An *ex vivo* preparation of rat plantar skin and tibial nerve was used to examine effects of a selective DOR agonist, deltorphin II, on responsiveness of single fibers innervating inflamed skin. Electrical recordings were made eighteen hours after injection of complete Freund's adjuvant into the hindpaw. Deltorphin II produced an inhibition of the mechanical responsiveness of single fibers innervating inflamed skin; an effect blocked by the DOR-selective antagonist, naltrindole. The population of units responsive to deltorphin II was identified as consisting of C fiber mechanical nociceptors.

Indexing words

Opiate; peripheral; periphery; inflammation; deltorphin; electrophysiology

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Disclosures

The authors declare that they have no competing conflicts of interest.

Author Contributions

JDB participated in design and execution of experiments and writing of the manuscript.

CNH supervised studies and writing of the manuscript, and participated in design and execution of experiments.

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1. Introduction

Within skin, an intricate network of blood vessels, epithelial cells, immune cells, and sensory axons communicate through electrical and chemical signals. Opioid peptides are synthesized and released from keratinocytes as well as migrating immune cells (Slominski et al., 2011; Stein and Zollner, 2009; Wintzen et al., 2001). Mu, delta and kappa opioid receptors have been localized to various cell types (immune, keratinocytes, axons) in the skin (Bigliardi et al., 1998; Stein et al., 1990b; Coggeshall, et al., 1997). Although the receptors are present, it has been difficult to demonstrate their function in healthy tissues.

Efficacy of opioid receptor agonists is enhanced under inflammatory conditions (Stein and Zollner, 2009; Wenk et al., 2006). Peripherally-restricted opioids attenuate behavioral hyperalgesia in models of inflammatory pain, with little effect in normal animals (Stein and Zollner, 2009), suggesting that activation of opioid receptors on sensory axons decreases afferent excitability. We found that locally-applied morphine inhibits mechanical and thermal responsiveness of nociceptors innervating inflamed skin while having no effects on fibers in healthy skin (Wenk et al., 2006). It is unknown, to what degree specific mu, delta, or kappa opioid receptors on nociceptors contribute to peripheral opioid analgesia. The objective of the present study was to investigate whether mechanical responsiveness of nociceptors innervating inflamed skin was attenuated by direct activation of peripheral delta opioid receptors (DOR). We report that application of deltorphin II, a DOR-selective agonist, decreased evoked activity of nociceptors innervating inflamed skin. This effect was prevented by co-application of naltrindole, a DOR-selective antagonist. These results provide electrophysiological evidence for the existence of functional delta opioid receptors on the peripheral processes of nociceptive fibers innervating inflamed skin.

2. Results

2.1 Distribution of Single Units

We have shown previously that peripheral axons of nociceptors innervating healthy skin are not sensitive to morphine (Wenk et al., 2006) or to selective delta or mu receptor agonists (Schramm and Honda, 2010), so all recordings were made from axons innervating inflamed skin. Compound action potential recordings were made from the tibial nerve at the start of experiments. Isolated single units were classified into fiber type ($A\alpha/\beta$, $A\delta$, or C) based on comparison of their conduction velocities to the range of conduction velocities of compound action potential waveforms for that experiment (Figure 1). Ranges of conduction velocities ($\pm$ standard deviation) for compound action potential waveforms were: $A\alpha/\beta$ ($n=47$) 37.6 ± 12.5 m/s to 9.5 ± 3.0 m/s; $A\delta$ ($n=44$) 11.7 ± 3.9 m/s to 6.8 ± 2.6 m/s; C ($n=18$) 0.72 ± 0.15 m/s to 0.48 ± 0.10 m/s. These averaged ranges of waveforms were used to classify single units in experiments where components of the compound action potential were not recorded. One standard deviation of the mean was added to the fastest part of each wave and one standard deviation was subtracted from the slowest part: units that conducted within 50.1 m/s and 6.5 m/s were classified as $A\alpha/\beta$ fibers; units that conducted within 15.5 m/s and 4.2 m/s were classified as $A\delta$ fibers; units that conducted within 0.87 m/s and 0.38 m/s were classified as C fibers. Units that conducted between the conduction velocity ranges of the $A\delta$ and C waves were classified as C/ $A\delta$ fibers.

During isolation of single units, emphasis was focused on slowly conducting (< 4.0 m/s) mechanical nociceptors. Forty units were included. Nineteen of these conducted within the C/A δ fiber range with a mean conduction velocity ($\pm$ standard deviation) 2.5 m/s ± 1.88 , and twenty-one conducted within the C fiber range with a mean conduction velocity 0.58 m/s ± 0.14 (Table 1). Spontaneous activity was observed in 10 of 19 (52%) C/A δ fibers and 15 of 21 (71%) C fibers (Table 1). The median mechanical threshold was 3.9 mN for C/A δ fibers and 14.2 mN for C fibers. Eighteen of 19 C/A δ fiber units (95 %) and 20 of 21 C fiber units (95%) were functionally classified as nociceptors based on the pattern of their stimulus-response relationship (Table 1).

2.2 Vehicle

Responses of C and C/A δ fibers to mechanical stimulation before and after application of vehicle were compared ($n=7$). The median baseline response to a 3.3 bar suprathreshold stimulus was 10.3 Hz (25th and 75th percentiles = 3.0 and 11.6). The median response to a second mechanical stimulation after vehicle application was 14.8 Hz (25th and 75th percentiles = 1.9 and 17.4). The pre- and post-vehicle responses were not significantly different from each other ($P>0.05$, Wilcoxon matched-pairs signed-ranks test).

2.3. Deltorphan II

As a combined population of C and C/A δ fibers, responses of deltorphan II-treated units to mechanical stimuli were significantly inhibited as compared with vehicle treated fibers ($P<0.01$; one-way ANOVA). Mean percent baseline response ($\pm$ SEM) at each concentration included: 1000 or 3000 nM ($n= 6$, 71.8 ± 25.2); 700 nM ($n= 4$, 127.9 ± 12.9); 300 nM ($n= 5$, 49.9 ± 7.8); 100 nM ($n= 4$, 95.8 ± 23.62); 10 nM ($n= 3$, 39.5 ± 21.6); 0.1 nM ($n= 5$, 93.1 ± 5.2); 0.001 nM ($n= 6$, 100.3 ± 14.3); vehicle ($n= 7$, 123.2 ± 11.6).

Previously we identified a population of morphine-sensitive primary afferent neurons as being C fiber nociceptors innervating inflamed skin (Wenk et al., 2006). In the present study, although the combined population of slowly conducting units was inhibited by deltorphan II, we also independently analyzed the effects of deltorphan II on C and C/A δ fiber nociceptor populations. Single units were functionally classified as nociceptors based on their stimulus-response profile. As a population, the mechanical responsiveness of C/A δ nociceptors was not inhibited by local application of deltorphan II ($P>0.05$, one way ANOVA; Figure 2A). Mean percent baseline response ($\pm$ SEM) at each concentration included: 1000 or 3000 nM ($n= 3$, 98.6 ± 44.0); 100 nM ($n= 3$, 113.4 ± 22.2); 0.1 nM ($n= 4$, 92.0 ± 6.6); vehicle ($n= 7$, 123.2 ± 11.6). Although there was no population effect on C/A δ nociceptors, some individual units were responsive to deltorphan II application. A representative example of a single fiber response to deltorphan II is shown in Figure 3.

In contrast, peripheral application of deltorphan II evoked a robust inhibition of responses to noxious mechanical stimulation in the C fiber nociceptor population ($P<0.01$, one way ANOVA; Figure 2B). Mean percent baseline response ($\pm$ SEM) at each concentration included: 1000 or 3000 nM ($n= 3$, 45.1 ± 22.6); 700 nM ($n= 3$, 118.9 ± 13.0); 300 nM ($n= 3$, 46.3 ± 13.3); 10 nM ($n= 3$, 39.5 ± 21.6); 0.001 nM ($n= 3$, 121.4 ± 15.8); vehicle ($n= 7$, 123.2

± 11.6). No inhibition was observed in 700 nM group. This group was included in the statistical analysis (one way ANOVA), but omitted from the graph (Figure 2B).

2.4. Deltorphan II and naltrindole

To determine if the inhibitory effect of deltorphan II on C fiber nociceptors was opioid receptor-mediated, we examined the effects of deltorphan II applied in the presence of naltrindole. All fibers included in this analysis were functionally identified as C fiber nociceptors. Co-application of equimolar concentrations of deltorphan II and naltrindole ($n=6$) prevented the inhibitory effect observed with deltorphan II alone ($n=3$). Mean percent baseline response ($\pm$ SEM) was decreased to 46.3 ± 13.3 when deltorphan II was applied alone, compared to 87.5 ± 9.6 percent of baseline response when deltorphan II (300 nM) was co-applied with 300 nM naltrindole (* $P<0.05$, unpaired t test; Figure 4).

3. Discussion

Previously, we identified a morphine-sensitive population of nociceptors 18 hours after CFA-induced inflammation. This population of fibers conducted within the C-fiber range and displayed signs of sensitization such as enhanced levels of spontaneous activity, decreased mechanical threshold, and increased responses to suprathreshold mechanical and thermal stimuli (Wenk et al., 2006). Presently, we report the effects of a DOR-selective ligand on response properties of mechanical nociceptors in the same isolated preparation and at the same time point (18 hrs) following CFA. We did not quantitatively test for the presence of sensitization in the present study, but similar to the previous study, a large proportion of nociceptors were spontaneously active.

The inhibitory effects of deltorphan II on the excitability nociceptors was blocked by co-application with naltrindole, a DOR-selective antagonist, providing pharmacological evidence that the inhibitory effects of deltorphan II were mediated by delta opioid receptors. In the previous study, we distinguished morphine “responders” from “non-responders” based on a standardized response criterion. Such criteria were not used in the present study. All fibers tested with deltorphan II were included in a population analysis regardless of the extent to which their responses deviated from baseline. The inhibitory effect of deltorphan II was robust enough to be detected in a heterogeneous population of fibers.

The inhibitory effects of deltorphan II were predominantly on the excitability of C-fiber mechanical nociceptors. This is consistent with previous immunohistochemical observations that DOR is expressed on unmyelinated, but not myelinated peripheral axons (Coggeshall et al., 1997; Wenk and Honda, 1999). A recent study using DOReGFP reporter mice suggests that DOR is also expressed in a substantial proportion of sensory neurons with myelinated axons (Scherrer et al., 2009). However, it is unclear whether the peripheral distribution or trafficking of DOR is similar in rats to that of DOReGFP in mice.

Much evidence suggests a specific role for peripheral DOR in nociceptive processing in sensory neurons following inflammation. Opioids are expressed by infiltrating immune cells (Brack et al., 2004; Stein and Zollner, 2009), and disruption of the perineurium in nerves (Antonićević et al., 1995) during inflammation may improve access of ligand to axonal

DOR. Trafficking of DOR is sensitive to levels of ligand (Cahill et al., 2001), and inflammation induces increased trafficking of DOR from intracellular compartments of central processes of sensory neurons (Cahill et al., 2003). Moreover, coupling of DOR to inhibitory G protein-signaling molecules is more efficient following inflammation (Stein and Zollner, 2009). In trigeminal ganglion, bradykinin stimulates translocation of DOR from intracellular compartments to plasma membrane, where “functional competence” depends on activation of PKC and arachidonic acid signaling pathways (Patwardhan et al., 2005).

There is substantial evidence suggesting a major involvement of mu and kappa opioid receptors in peripheral analgesia (Machelska et al., 1999; Stein and Zollner, 2009), but these receptors are also critical in skin homeostasis, inflammatory processes, and some peripheral chronic pruritic skin diseases. (Bigliardi-Qi et al., 2007; Yamamoto and Sugimoto, 2010). DOR also appears to be important in skin homeostatic processes including differentiation and wound healing (Bigliardi-Qi et al., 2006).

Our data suggest that delta opioid receptors contribute to the receptor-mediated effects of morphine previously reported. In the present study, activation of peripheral delta opioid receptors mimicked the inhibitory effect of morphine on single unit responses to mechanical stimulation of the cutaneous receptive fields (Wenk et al., 2006). Others have shown that DOR-selective ligands increase nociceptive thresholds in the paw pressure test in CFA-inflamed hindpaws (Stein et al., 1989; Antonijevic et al., 1995; Zhao et al., 1998).

Interestingly, inflammation-induced translocation of DOR from intracellular compartments to the plasma membrane was reported absent in MOR-knockout mice (Morinville et al., 2004). Others have reported that DOR-mediated hyperalgesia is dependent on MOR expression or enhanced by pretreatment with morphine (Gendron et al., 2007). A recent study demonstrated that co-administration of opiates enhanced opioid receptor-mediated inhibition of CGRP release from normal peripheral nerve when morphine or DAMGO were preceded by deltorphin II (Schramm and Honda, 2010). These data suggest that enhanced DOR function is not necessarily dependent on MOR. It remains unclear, however, to what extent MOR and DOR contribute to enhanced opioid function in the presence of multiple agonists.

The present findings support the hypothesis that the effects of peripherally delivered opiate compounds under conditions of inflammation are mediated, at least in part, by activation of delta opioid receptors. Furthermore, these data suggest potential clinical utility of peripherally restricted DOR-agonists for the treatment of inflammatory pain.

4. Materials and Methods

Animals and Induction of Inflammation

Adult male Sprague-Dawley rats (250–400 grams; $n=31$) were used in experiments approved by the Institutional Animal Care and Use Committee at the University of Minnesota. Rats in the inflamed group were deeply anesthetized with isoflurane and received a unilateral subcutaneous injection of CFA (100 μ l, emulsified 1:1 with sterile

saline) into the plantar surface of the hindpaw, eighteen to nineteen hours prior to experimentation.

Isolated Glabrous Skin-nerve Preparation

Rats were deeply anesthetized with a combination of ketamine, acepromazine and xylazine (6.8/0.09/0.45 mg/kg). The glabrous skin of the hindpaw was dissected and excised together with the attached tibial nerve and the medial and lateral branches of the plantar nerve. The skin-nerve preparation was immediately transferred to a chamber that was continuously perfused (18 ml per minute) with warmed ($30\pm 2^{\circ}\text{C}$) oxygen-saturated synthetic interstitial fluid (SIF; Koltzenburg et al. 1997) containing (in mM) 123 NaCl; 3.5 KCl, 0.7 MgSO_4 , 2.0 CaCl_2 , 9.5 Na gluconate, 1.7 NaH_2PO_4 , 5.5 glucose, 7.5 sucrose, 10.0 Hepes (pH 7.45 ± 0.05 , 290 ± 0.05 mOsm). The preparation was placed corium side up and anchored with insect pins. The preparation was further dissected until the skin and nerve were cleared of all tendons, muscles, and vasculature. The cut end of the tibial nerve was threaded through a small aperture into a recording chamber and placed on the surface of a mirrored dissection platform. The recording chamber was filled with buffer below and oil above the mirror.

General Electrophysiological Procedures

A compound action potential (neurogram) was recorded at the beginning of experiments. A monopolar microelectrode (insulated except at tip) was placed on the main trunk of the medial or lateral plantar nerve for electrical stimulation. Filaments divided from the main tibial nerve were lifted onto a fine gold electrode for extracellular recording. The recording electrode was suspended in oil and referenced to the bath with a silver/silver chloride electrode. Stimulating current was delivered with increasing intensity until each waveform component of the compound action potential ($\text{A}\alpha\beta$, $\text{A}\delta$, and C) could be evoked. The rate of conduction calculated for each waveform was expressed as meters per second, and subsequently-recorded single fibers were classified according to the range of conduction velocities of neurogram waveforms. When a particular waveform could not be evoked for a given experiment, single units were classified according to the mean conduction rate recorded for waveforms from compound action potentials recorded in all experiments. Electrical signals were differentially amplified (DAM80, World Precision Instruments, Austin, TX), filtered, and routed in parallel to an oscilloscope and computerized data acquisition system (see below).

Isolation and Characterization of Single Units

Initially, small bundles of nerve fibers were teased from the nerve trunk and placed on the recording electrode to observe activity from multiple axons. The corium surface of the skin was then lightly probed with a blunt glass rod to identify the general area innervated by the bundle. Next, electrical search stimuli were delivered to the nerve trunk through a microelectrode to elicit single fiber activity as progressively smaller filaments were isolated and placed on the recording electrode. Once single unit activity could be isolated, a second, roving, stimulating electrode was progressively traced along the plantar nerve branches until the receptive field could be electrically identified. Conduction velocity of individual axons was determined by electrical stimulation of the center of the receptive field and expressed as meters per second.

Spontaneous Activity

Un-evoked neuronal activity was recorded for at least ten seconds before each mechanical stimulus. Spontaneous activity was defined as firing > 0.1 Hz in the absence of any intentional stimulus.

Mechanical Response Properties

Mechanical threshold for each single unit was determined using a series of calibrated von Frey nylon monofilaments delivered to the corium surface of the skin in an increasing order of force from 0.7 mN to 78.37 mN. Forces less than 0.7 mN were not delivered because these thinner filaments could not be reliably placed through the SIF flowing through the chamber. Mechanical threshold was defined as the lowest force that consistently evoked an action potential response 100% of the time. Stimulus-response relationships were determined for each unit using calibrated mechanical probes (tip diameter 2.5 mm) that delivered fixed pressures of 0.5, 1.8 and 3.3 bars. The 3.3 bar stimulus was considered suprathreshold for most nociceptors, and nociceptors were further defined as units whose firing rate increased monotonically with increasing pressure of stimulation. High threshold units that responded only to pressures greater than approximately 3.3 bars were also defined as nociceptors. Probes were positioned in a micromanipulator and lowered onto the receptive field for five seconds. The stimulation onset and offset times were signaled to the online data acquisition program.

Preparation of Drugs

Stock solutions of deltorphin II (100 μ M; Phoenix Pharmaceuticals, Belmont, CA) were made in water and stored at 4°C. Working concentrations of the ligand were diluted in SIF, as needed. Naltrindole hydrochloride (Tocris, Ellisville, MO) was reconstituted in water to a stock solution of 100 μ M and was stored at -20°C. Each drug solution and vehicle was warmed to room temperature and saturated with oxygen prior to use.

Drug Application and Receptive Field Stimulation

A small cylinder (8 mm diameter) was sealed over the receptive field with petroleum jelly, filled with SIF, and served as a reservoir for drug delivery. SIF was then replaced with deltorphin II, deltorphin II and naltrindole, or vehicle (SIF) for two minutes. Compounds were only tested on units innervating inflamed skin, and each fiber was tested with a single concentration of compound or vehicle. The number of spikes occurring in the first five seconds of each mechanical stimulus were counted and are reported as spikes per second firing rate (Hz). Baseline responses to a suprathreshold stimulus were recorded before drug delivery while determining the mechanical response properties of each fiber. Units were re-tested immediately after drug removal. Recovery following drug application was defined as at least a 50% return towards baseline firing rate, and units that did not recover were excluded from this study. Data are reported as the mean percent of baseline firing ((post-drug response/baseline response) $\times 100$) $\pm$ standard error (SEM) for each unit unless otherwise noted.

Data Collection and Analysis

Compound action potential, teased fiber recordings, and stimulus delivery times were collected using a data acquisition program written on the LabView platform (version 5.1; National Instruments, Austin, TX). Data analysis and spike discrimination were performed online and offline. Prism (GraphPad, La Jolla, CA) was used for statistical analysis and generation of graphs. Data were analyzed using paired t-test, unpaired t-test or one-way ANOVA, as noted. Data are expressed as means $\pm$ SEM, except as noted.

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Highlights

- Nociceptors in inflamed skin were tested for functional delta opioid receptors.
- Excitability of unmyelinated mechanical nociceptors was decreased by deltorphin II.
- Inhibition by deltorphin II was prevented by co-application of naltrindole.

Figure 1. Basis for classification of single units by conduction velocity

Representative example of a compound action potential recording. Single units were classified into fiber types based on the conduction velocity range of compound action potential waveforms recorded for each preparation. Units with conduction velocities between the fastest part of the C wave and the slowest part of the $A\delta$ wave were assigned to a fourth group, C/ $A\delta$. The average range of conduction velocities for each waveform was as follows: $A\alpha/\beta$ ($n=47$) 37.6 ± 12.5 m/s to 9.5 ± 3.0 m/s; $A\delta$ ($n=44$) 11.7 ± 3.9 m/s to 6.8 ± 2.6 m/s; C ($n=18$) 0.72 ± 0.15 m/s to 0.48 ± 0.10 m/s.

Figure 2. Concentration-response relationship for deltorphin II

A. As a population, the mechanical responsiveness of C/A δ nociceptors was not inhibited by local application of deltorphin II ($P > 0.05$, one way ANOVA). Mean percent baseline response ($\pm$ SEM) at each concentration included: 1000 or 3000 nM ($n = 3$, 98.6 ± 44.0); 100 nM ($n = 3$, 113.4 ± 22.2); 0.1 nM ($n = 4$, 94.5 ± 6.5); vehicle ($n = 7$, 123.2 ± 11.6). **B.** Deltorphin II inhibited the mechanical responsiveness of individual C fiber nociceptors ($P < 0.01$, one way ANOVA; Figure 4B). Mean percent baseline response ($\pm$ SEM) at each concentration included: 1000 or 3000 nM ($n = 3$, 45.1 ± 22.6); 700 nM ($n = 3$, 118.9 ± 13.0); 300 nM ($n = 3$, 46.3 ± 13.3); 10 nM ($n = 3$, 39.5 ± 21.6); 0.001 nM ($n = 3$, 121.4 ± 15.8); vehicle ($n = 7$, 123.2 ± 11.6).

Figure 3. Example of peripheral drug testing protocol for deltorphin II

A: An electrically evoked action potential of a C/A δ fiber with a conduction velocity of 2.37 m/s. Location of the mechanical receptive field on the plantar surface of the hindpaw is indicated by a black dot. **B:** This unit's firing rate increased monotonically with increasing pressure of stimulation, and was therefore classified as a mechanical nociceptor. **C and D:** Response of the same unit to a 3.3 bar stimulus (10 seconds each) before, immediately following and after washout of deltorphin II. In **D**, lower traces indicate timing of stimuli, and insets contain superimpositions of all sorted spikes included in analysis.

Figure 4. Effects of deltorphin II antagonized by co-application with naltrindole

A population of fibers treated with 300 nM deltorphin II ($n=3$) was compared to the population treated with 300 nM deltorphin II plus 300 nM naltrindole ($n=6$). Mean percent baseline response ($\pm$ SEM) was decreased to 46.3 ± 13.3 when deltorphin II was applied alone, compared to 87.5 ± 9.6 percent of baseline response when deltorphin II (300 nM) was co-applied with 300 nM naltrindole (* $P<0.05$, unpaired t test).

Table 1

Distribution of single units

Forty units were included, nineteen of these conducted within the C/Aδ fiber range with a mean conduction velocity ($\pm$ SD) $2.5 \text{ m/s} \pm 1.88$, and twenty-one conducted within the C fiber range with a mean conduction velocity $0.58 \text{ m/s} \pm 0.14$. The median mechanical threshold was 3.9 mN for C/Aδ fibers and 14.2 mN for C fibers. Eighteen of 19 C/Aδ fiber units (95 %) and 20 of 21 C fiber units (95%) were functionally classified as nociceptors based on the pattern of their stimulus-response relationship. Fifty-two percent of C/Aδ fibers and 71 percent of C fibers were spontaneously active.

Fiber Type	n	CV (m/s)	Mechanical Threshold (mN)	# of Nociceptors	# Spontaneously Active
C/Aδ	19	2.5 ± 1.88	3.9	18 (95%)	10 (52%)
C	21	0.58 ± 0.14	14.2	20 (95%)	15 (71%)