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Involvement of Ca^{2+}-dependent PKCs in the adaptive changes of μ-opioid pathways to sympathetic denervation in the guinea pig colon

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ABSTRACT

In the guinea pig colon, chronic sympathetic denervation entails supersensitivity to inhibitory μ-opioid agents modulating cholinergic neurons. The mechanism underlying such adaptive change has not yet been unravelled, although protein kinase C (PKC) may be involved. A previous study indirectly demonstrated that activation of μ-opioid receptors on myenteric neurons facilitates PKC activity. Such coupling may counteract the inhibitory action of μ-opioid agents on acetylcholine overflow, since PKC, per se, increases this parameter. After chronic sympathetic denervation such restraint abates, representing a possible mechanism for development of supersensitivity to μ-opioid agents. In the present study, this hypothesis was further investigated. After chronic sympathetic denervation, Ca^{2+}-dependent PKC activity was reduced in colonic myenteric plexus synaptosomes. The μ-opioid agent, DAMGO, increased Ca^{2+}-dependent PKC activity in synaptosomes obtained from normal, but not from denervated animals. In myenteric synaptosomes obtained from this experimental group, protein levels of Ca^{2+}-dependent PKC isoforms βI, βII and γ decreased, whereas α levels increased. In whole-mount preparations, the four Ca^{2+}-dependent PKC isoforms co-localized with μ-opioid receptors on subpopulations of colonic myenteric neurons. The percentage of neurons staining for PKCβII, as well as the number of μ-opioid receptor-positive neurons staining for PKCβII, decreased in denervated preparations. The same parameters related to PKCα, βI or γ remained unchanged. Overall, the present data strengthen the concept that μ-opioid receptors located on myenteric neurons are coupled to Ca^{2+}-dependent PKCs. After chronic sympathetic denervation, a reduced efficiency of this coupling may predominantly involve PKCβII, although also PKCβI and γ, but not PKCα, may be implicated.

1. Introduction

Endogenous opioid peptides and opioid drugs are known to mediate a variety of biological processes, including stress response, immunity, analgesia, motor activity and autonomic functions, such as gastrointestinal motility and secretion [1,2]. Biochemical transduction of these effects involves molecular binding of the drug to opioid receptors, which belong to the G protein-coupled receptor family. Activation of opioid receptors principally results in attenuating neuronal activity by inhibiting neurotransmitter release and changing neuronal excitability by pre- and post-synaptic mechanisms, respectively [3,4].

A number of studies have demonstrated that a functional interaction may occur between opioid receptors and other inhibitory G protein-coupled receptors, which may have importance in the development of responses after both acute and chronic exposure to opiates. One of the most studied interplay involves opioid receptor and α_2-adrenoceptor pathways. Such interaction may be antagonistic or synergistic. Several studies have demonstrated that acute activation of one receptor pathway may lead either to attenuation or potentiation of the other [5–10]. Functionally related α_2-adrenoceptor and opioid receptor pathways may be involved in adaptive changes occurring in excitable cells when a net stimulus is chronically changed. Tolerance and dependence to the effect of opiates may represent an example of adaptive sensitivity change leading to altered function not only of opioid but also of a variety of other neuronal systems, including α_2-adrenoceptors, in the central nervous system and peripherally [11,12]. Several reports have described the occurrence of changes in α_2-adrenoceptor

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sensitivity after chronic morphine treatment in the enteric nervous system (ENS) [13–15], which represents a complex and integrative neuronal network suitable for the study of neuronal plasticity [16]. Adaptive changes involving inhibitory μ-opioid receptor and α_2-adrenoceptor pathways have been documented also after chronic ablation of the sympathoadrenergic pathway innervating the guinea pig distal colon. In these experimental conditions, reduced sensitivity to α_2-adrenergic agonists and enhanced sensitivity to μ-opioid agents developed on both acetylcholine overflow and peristalsis, in order to maintain intestinal motility under an homeostatic control [17]. Indeed, there are reports in the literature suggesting that after extrinsic denervation, an intrinsic pathway may take over the function of the suppressed extrinsic input, allowing restoration of the intestinal function [16]. More generally, the occurrence of rearrangements of intrinsic enteric neuronal circuitries after extrinsic denervation may help to understand the occurrence of functional abnormalities/functional recovery in patients after injuries or surgical procedures [16].

From a molecular viewpoint, the functional interplay between opioid receptors and α_2-adrenoceptors has been suggested to depend upon several factors, including changes in transmitter release, alterations at the receptor level and in the intracellular signalling pathways coupled to receptor activation [12,18,19]. At this latter regard, a key role in the modulation of intracellular responses is played by protein kinase C (PKC). PKC is a family of at least 11 isoenzymes, some of which are highly expressed at a neuronal level [20–22]. Among the different PKC isoforms, Ca^{2+}-dependent or conventional PKCs α, βI, βII and γ, are fully activated by calcium ions and by a transient increase in diacylglycerol and/or arachidonic acid and metabolites, which are formed through various pathways after breakdown of membrane phospholipids by different phospholipases, including phospholipase C (PLC), phospholipase D (PLD) and phospholipase A_2 (PLA_2) [23].

The ability of opioid receptors to activate phosphoinositide pathways and, consequently, PKC, has been demonstrated in different experimental models [24,25]. In addition, PKC may participate in the cellular and synaptic adaptation mediating opioid dependence [26]. Recently, functional and biochemical evidence has been provided to suggest that a reduced efficiency of Ca^{2+}-dependent PKC, in the myenteric plexus of the guinea pig colon after chronic sympathetic denervation, might contribute to the development of supersensitivity to μ-opioid agonists in these experimental conditions [27].

To shed more light on the possible link between PKC and the development of adaptive changes to μ-opioid agonists in the myenteric plexus of the guinea pig colon after chronic sympathetic denervation, in the present study, we evaluated the effect of the μ-opioid agonist, [D-Ala2,*N*-Me-Phe4,Gly-ol5]-enkephalin (DAMGO), on Ca^{2+}-dependent PKC activity in myenteric plexus synaptosomes. In the same experimental model, the abundance of the different Ca^{2+}-dependent PKC isoforms has been investigated by Western blotting. Finally, the possible co-localization of Ca^{2+}-dependent PKCs with μ-opioid receptors has been explored by immunohistochemistry on colonic whole-mounts preparations obtained from normal and denervated animals.

2. Methods

2.1. Animals

Male Dunkin-Hartley guinea pigs (Harlan Italy, Correzzana, Monza Italy) weighing between 300 and 350 g were housed in groups of four under controlled environmental conditions (temperature 22 ± 2 °C; relative humidity 60–70%) with free access to a standard diet and water, and were maintained at a regular 12/12-h light/dark cycle. Animals were sacrificed by decapitation and the colon was rapidly excised and rinsed with an ice-cold Tyrode's solution (composition [mM]: 137 NaCl; 2.68 KCl; 1.8 $CaCl_2 \cdot 2H_2O$; 2 $MgCl_2$; 0.47 NaH_2PO_4; 11.9 $NaHCO_3$; 5.6 glucose). Principles of good laboratory animal care were followed and animal experimentation was in compliance with specific national (DL 116 GU suppl. 40, 18 febbraio 1992; Circolare no. 8 GU 14 luglio 1994) and international laws and regulations (EEC Council Directive 86/609, OJL 358,1, December 12 1987).

2.2. Chronic sympathetic denervation

Chronic sympathetic denervation was obtained by surgical removal of the inferior mesenteric ganglion and freezing of the periarterial plexus at least 6 days before the experiments as described by Mazzanti et al. [28], with modifications. Briefly, animals were anesthetized with sevofluorane (Sevorane®, Abbott, Aprilia, Italy, 1.5–4.0% with oxygen). The intestine was exteriorized by a midline laparatomy and kept on warm 0.9% sterile saline-soaked cotton gauze to reduce dehydration and cooling. The terminal part of the colon was exposed and the inferior mesenteric ganglion was identified and removed surgically by means of fine forceps. Successively, a segment of the periarterial plexus (2–3 mm) along the inferior mesenteric artery just below the ganglion, was frozen with N_2O for 3 min. The abdominal wall was then sutured and the animals were sacrificed at least 6 days after the surgical intervention. Antibiotic prophylaxis was performed by injecting ampicillin 100 mg/kg of body weight (Amplital®, Pfizer Italia, Latina, Italy) daily for the first 4 days after surgery.

2.3. Myenteric plexus enriched synaptosomal preparation

Enriched synaptosomal fractions of myenteric plexus neurons were obtained from colonic preparations consisting of the external longitudinal muscle layer segments with attached myenteric plexus (LM/MP) after successive centrifugations as already described [29]. Briefly, LM/MP preparations pooled from 3 to 5 animals were homogenized in ice-cold 3-*N*-morpholinopropanesulfonic acid (MOPS)–sucrose isolation buffer [containing 25 mM MOPS, 10 mM $MgCl_2$, 8%, w/v sucrose, 1 mM phenylmethylsulfonyl fluoride (PMSF) and 25 μg/ml leupeptin, pH 7.4]. The crude homogenate was centrifuged in two steps at 800 × *g* for 10 min. The supernatants were collected and centrifuged again at 3500 × *g* for 10 min. The supernatant was then filtered (pore size 11 μm; Millipore, Billerica, MA) and centrifuged at high speed (120,000 × *g*) for 60 min. The resulting pellet was re-suspended and centrifuged again at 10,000 × *g* for 10 min to obtain an enriched synaptosomal pellet. Some synaptosomal preparations were obtained to measure Ca^{2+}-dependent PKC activity. In this case, LM/MP preparations were perfused at a rate of 1 ml min^{-1} with Tyrode's solution, gassed with O_2–CO_2 (95%–5%) and maintained at 36.5 °C in 3 ml organ baths. LM/MPs were allowed an equilibration period of 40 min, then either DAMGO (0.1 μM) and/or [H-D-Phe-Cys-Tyr-D-Trp-Arg-Thr-Pen-Thr-NH_2] (CTAP) (1 μM) were added to the superfusion medium for 20 min. The concentration of 0.1 μM DAMGO was chosen as it corresponds to the maximal dose of agonist inhibiting acetylcholine overflow from the guinea pig colon, obtained from both normal and denervated animals, as previously described [17]. After exposure to test drugs, LM/MPs were collected to obtain enriched synaptosomal membranes.

The enriched myenteric synaptosomal fraction was successively suspended in a protein extraction reagent, T-PER (Pierce, Rockford, Il) containing a commercial protease inhibitor cocktail (Complete®, Roche, Manheim, Germany), incubated on ice for 15 min, sonicated and centrifuged at 7000 × *g* for 5 min. Aliquots of the sample were used for protein assay by means of Bradford's micromethod [30]. The remaining was processed for either Ca^{2+}-