Effect of capsazepine on the release of calcitonin gene-related peptide-like immunoreactivity (CGRP-LI) induced by low pH, capsaicin and potassium in rat soleus muscle

Paolo Santicioli, *Elena Del Bianco, *Michela Figini, †Stuart Bevan & †Carlo Alberto Maggi

Pharmacology Department, A Menarini Pharmaceuticals, Via Sette Santi 3, 50131, Florence, Italy; *Dept. of Internal Medicine, University of Florence, Florence, Italy and †Sandoz Institute for Medical Research, 5 Gower Place, London

- 1 We have determined the effect of the competitive antagonist capsazepine at the capsaicin receptor on the release of calcitonin gene-related peptide-like immunoreactivity (CGRP-LI) from rat isolated soleus muscle induced by capsaicin (1 μM), by superfusion with low pH medium (pH 5) or by KCl (80 mM).
- 2 Each one of the three stimuli tested produced a marked CGRP-LI release. Total evoked release (fmol g^{-1}) was 482 ± 69 , 169 ± 20 and 253 ± 43 for capsaicin, low pH medium and KCl, respectively.
- 3 Prior application of capsaicin (10 μM for 30 min followed by 30 min of washout) to produce capsaicin desensitization *in vitro* abolished CGRP-LI release induced by the three stimuli.
- 4 Capsazepine (1–100 μM , 45 min preincubation) inhibited the evoked CGRP-LI release. Capsaicin-induced release was significantly inhibited by 77, 92 and 96% with 10, 30 and 100 μM capsazepine, respectively. Low pH-induced release was inhibited by 78, 84, 88 and 93% with 3, 10, 30 and 100 μM capsazepine, respectively. KCl-induced release was significantly inhibited by 55 and 93% with 30 and 100 μM (but not with 10 μM) capsazepine, respectively.
- 5 These findings demonstrate that capsazepine prevents low pH- and capsaicin-induced CGRP-LI release from rat soleus muscle at concentrations which do not affect the release evoked by KCl. These findings imply a relationship between the action of low pH and activation of the capsaicin receptor. At high concentrations, capsazepine produces a nonspecific inhibitory effect on CGRP-LI release from peripheral endings of the capsaicin-sensitive primary afferent neurone.

Keywords: Calcitonin gene-related peptide (CGRP); protons; rat soleus muscle; capsaicin-sensitive primary afferents; capsazepine

Introduction

Capsaicin-sensitive primary afferent neurones are known to exert an 'efferent' function by releasing sensory neuropeptides, tachykinins and calcitonin gene-related peptide (CGRP), from their peripheral endings. Pronounced chemosensitivity is a distinguishing feature of the capsaicin-sensitive primary afferents: peripheral release of sensory neuropeptides is evoked by a number of chemical stimuli, including mediators or inflammation (bradykinin, 5-hydroxytryptamine etc.) and conditions (elevated extracellular K^+ and H^+ concentrations) which are commonly encountered during inflammation/tissue damage (Maggi, 1991a for review).

In recent years, much information has been gained on the mechanisms through which capsaicin and related molecules act on primary afferent neurones (Maggi *et al.*, 1991b; Bevan & Szolcsanyi, 1991 for reviews). The selective action of capsaicin is mediated through the stimulation of a specific 'vanilloid' receptor, identified by the use of resiniferatoxin, an ultrapotent capsaicin analogue (Szallasi & Blumberg, 1990). Stimulation of the capsaicin or 'vanilloid' receptor opens a novel type of cation channel leading to calcium and sodium entry into the sensory neurones (Bevan & Szolcsanyi, 1991). In peripheral terminals of capsaicin-sensitive afferents, the increased cation conductance is believed to underlie the capsaicin-induced depolarization and generation of afferent impulses, whilst calcium entry is the trigger for the concomitant local secretion of sensory neuropeptides (Maggi, 1991b).

Determination of the mechanism of action of capsaicin on primary afferent neurones has been facilitated by the development of pharmacological tools which selectively block capsaicin action on primary afferent neurones. Bevan *et al.*

(1991, 1992a) developed the benzazepine derivative, capsazepine, as the first example of a competitive receptor antagonist for the vanilloid receptor. Capsazepine has been shown to antagonize selectively capsaicin action at both central and peripheral endings of primary afferent neurones (Urban & Dray, 1991; Bevan *et al.*, 1992a; Belvisi *et al.*, 1992; Maggi *et al.*, 1993) and the nature of the antagonism is compatible with competitive interaction at the capsaicin receptor. The selectivity of capsazepine for the vanilloid receptor makes it an extremely useful tool to investigate the possibility that an endogenous ligand exists for this receptor. Another tool which, at certain concentrations, acts as a selective capsaicin antagonist is the dye, ruthenium red (Amann & Maggi, 1991, for review). In contrast to capsazepine, ruthenium red does not bind to the vanilloid receptor and its action is characterized by noncompetitive antagonism of capsaicin-induced primary afferent stimulation (Bevan *et al.*, 1992a; Maggi *et al.*, 1993). Although the precise mechanism of action of ruthenium red is unclear, there is evidence that it prevents the opening of the cation channel coupled to the vanilloid receptor (Dray *et al.*, 1990; Amann & Maggi, 1991 for review). According to this model, ruthenium red not only prevents the action of capsaicin on sensory neurones, but also blocks the action of other stimuli which share with the vanilloid receptor the same cation channel for sensory neurone stimulation. A notable case for this is the action of protons (low pH media) which activate the efferent function of capsaicin-sensitive afferent neurones in a ruthenium red-sensitive manner (Geppetti *et al.*, 1991; Del Bianco *et al.*, 1991; Santicioli *et al.*, 1992a). Patch clamp studies on membranes of primary afferents have demonstrated that capsaicin and protons open the same cation channel (Bevan *et al.*, 1993).

¹ Author for correspondence.

In a recent study we demonstrated that application of low pH medium produced release of CGRP-LI of sensory origin from the rat soleus muscle and that release evoked by low pH medium or capsaicin is selectively reduced by ruthenium red, the release evoked by KCl being unaffected (Santicioli *et al.*, 1992a). In the present study we aimed to investigate the effect of capsazepine on CGRP-LI release induced by these three stimuli from rat soleus muscle. Preliminary results of this study were presented at a Meeting of the British Pharmacological Society (Santicioli *et al.*, 1992b).

Methods

Male albino Wistar rats (Charles River, Italy) weighing 250–300 g were anaesthetized with urethane (1.2 g kg^{-1} , i.p.). The soleus muscle was excised from both legs and the animals killed with an excess dose of the anaesthetic. The muscles were placed in oxygenated (96% O_2 , 4% CO_2 , pH 7.4) Krebs solution of the following composition (mM): NaCl 119, NaHCO_3 25, KH_2PO_4 1.2, MgSO_4 1.5, KCl 4.7, CaCl_2 2.5 and glucose 11. In each experiment, the soleus muscles were excised from the legs of 2 rats: tissues were sliced (thickness 0.4 mm) with a MacIlwain tissue chopper, the chopped muscles pooled and divided (150–250 mg each) into 4 thermostated (37°C) 1 ml perspex chambers and superfused at rate of 0.4 ml min^{-1} with oxygenated Krebs solution containing 0.1% (w/v) bovine serum albumin (BSA) and $10 \mu\text{M}$ thiorphan. All values in the text and figures are mean $\pm$ s.e.mean of replicates of different experiments.

Thiorphan was added to inhibit endopeptidase 24.11, minimize CGRP-degradation and enhance CGRP-LI recovery in superfusates (Katayama *et al.*, 1991; Davies *et al.*, 1992). For experiments aiming to assess the effect of low pH solutions, phosphate buffer solutions at pH 7.4 and 5 were prepared and used as described in previous studies (Geppetti *et al.*, 1991; Del Bianco *et al.*, 1991; Santicioli *et al.*, 1992a). The soleus muscle was placed in phosphate buffered physiological salt solution at pH 7.4 after excision and during equilibration. In these experiments, the phosphate buffer solutions were gassed with 100% O_2 .

After a 45 min stabilization period, 5 min fractions were collected into polypropylene tubes containing acetic acid (final concentration 2 N). In some experiments, in order to induce a long lasting blockade of primary afferents, capsaicin ($10 \mu\text{M}$) was left in contact with the tissue for 30 min and then washed out for 30 min before sample collection and application of low pH medium. Controls were run in the presence of capsaicin vehicle (0.001% ethanol).

To study the effect of capsazepine on CGRP-LI release evoked by the various stimuli, the samples were incubated with the drug for 45 min before application of the stimuli. At the end of the experiment the samples from each chamber were collected and weighed.

The fractions were freeze-dried, reconstituted with the assay buffer (0.1 M phosphate buffer at pH 7.4 containing 0.9% NaCl, 0.01% NaN_3 and 0.1% bovine serum albumin (BSA)) and their CGRP-LI content was determined by radioimmunoassay (RIA), as described previously (Geppetti *et al.*, 1991; Del Bianco *et al.*, 1991; Santicioli *et al.*, 1992a).

Drugs used were: capsaicin and thiorphan (Sigma). Capsazepine was synthesized at the Sandoz Institute for Medical Research.

In each experiment the total evoked release (TER) of CGRP-LI produced by the various stimuli above the basal values was calculated. Statistical analysis was performed by means of two-tailed Student's *t* test for paired and unpaired data, or by analysis of variance, when applicable.

Results

Application of capsaicin ($1 \mu\text{M}$), high potassium medium

(80 mM) or superfusion with low pH medium (pH 5) produced a clearcut increase in CGRP-LI outflow (Figure 1). TER (fmol g^{-1} wet wt) produced by these three stimuli was 482 ± 69 , 169 ± 20 and 253 ± 43 for capsaicin, low pH medium and KCl, respectively.

Prior application of capsaicin totally prevented CGRP-LI outflow induced by a subsequent application of capsaicin, pH 5 buffer solution or KCl ($n = 4-6$ for each agonist, Figure 1, open columns).

Capsazepine ($1-100 \mu\text{M}$) did not produce CGRP-LI release on its own. In the presence of capsazepine, the CGRP-LI release evoked by the three stimuli was depressed in a concentration-dependent manner, but the concentrations of the drug which inhibited the evoked release were different for the three agonists (Figure 2). In particular, the capsaicin-evoked CGRP-LI release was inhibited by $10 \mu\text{M}$ capsazepine and abolished at higher concentrations. The CGRP-LI release evoked by low pH medium was significantly inhibited by $3 \mu\text{M}$ capsazepine and further depressed and abolished by

Figure 1 Calcitonin gene-related peptide-like immunoreactivity (CGRP-LI) release evoked by capsaicin ($1 \mu\text{M}$, a), superfusion with low pH medium (pH 5, b) or KCl (80 mM, c) from the rat isolated soleus muscle, in control (open columns) or capsazepine-pretreated ($10 \mu\text{M}$ for 30, 30 min, before, solid columns) preparation. In each panel, the horizontal bars indicate the period of application of the various stimuli. Each value is mean $\pm$ s.e.mean of 4–16 replicates. Significantly different from basal values: * $P < 0.05$.

Figure 2 Effect of capsazepine (1–100 μM) on calcitonin gene-related peptide-like immunoreactivity (CGRP-LI) release induced by capsaicin (1 μM) (a), low pH medium (pH 5), (b) or KCl (80 mM) (c) from rat soleus muscle. Con = control. Each value is mean ± s.e. mean of 4–8 replicates. Significantly different from controls: * $P < 0.05$.

higher concentrations. The KCl-induced release was only reduced at 30 μM and was abolished at 100 μM capsazepine.

Discussion

The rat soleus muscle receives a CGRP-containing, capsaicin-sensitive afferent innervation. In skeletal muscle, another source of CGRP exists, the peripheral terminals of somatic motoneurons (Yamamoto & Tohyama, 1989, for review). Capsaicin pretreatment, at a dose regimen which completely depletes CGRP-LI in e.g. the rat urinary bladder, only partially reduces (50–60%) CGRP-LI levels of the soleus muscle (Santicoli *et al.*, 1992a), which is consistent with dual CGRP innervation. On the other hand the available evidence indicates that only the 'sensory' source of neuronal CGRP is released from skeletal muscle by depolarizing media (Sakaguchi *et al.*, 1991). In agreement with this notion, CGRP-LI release evoked by KCl was totally prevented by *in vitro* application of high capsaicin concentrations (capsaicin desensitization). Therefore, peripheral endings of primary afferent

neurons are the only source of releasable CGRP-LI in our preparation.

The main finding of this study is that the competitive vanilloid receptor antagonist, capsazepine, not only prevents the capsaicin-evoked CGRP-LI outflow, but also significantly inhibits that induced by low pH. Up to 10 μM, the action of capsazepine is, in some way, selective, because no inhibition of KCl-induced CGRP-LI release was observed. At higher concentrations (30–100 μM) capsazepine possesses a non-specific inhibitory action on the neurosecretory process, as indicated by inhibition of the response to KCl. Capsazepine is a competitive antagonist of the vanilloid receptor (Bevan *et al.*, 1992a) and its failure to antagonize the capsaicin- (1 μM) induced CGRP-LI release at concentrations below 10 μM is likely to be due to the competitive nature of interaction. The magnitude of response to low pH is smaller than that to capsaicin and this may explain the greater efficacy of capsazepine. In preliminary experiments (data not shown) we attempted to use lower capsaicin concentrations but the evoked CGRP-LI release was too variable for pharmacological analysis of the inhibitory effect of capsazepine.

A major finding of this study is that capsazepine antagonized CGRP-LI release evoked by low pH medium at a concentration of 3 μM while the response to KCl, which is quantitatively comparable to that evoked by low pH, was only inhibited at 30 μM capsazepine. While this work was in progress, Franco-Cereceda & Lundberg (1992) showed that CGRP-LI release induced by low pH (pH 5) or lactic acid (5 mM) from guinea-pig isolated, perfused heart was inhibited by 10 μM capsazepine. In a subsequent study, Lou & Lundberg (1992) showed that acid-induced nasal irritation and bronchoconstriction in guinea-pigs and CGRP-LI release from guinea-pig perfused lung was inhibited by capsazepine. Our findings in rat soleus agree with the reports of Franco-Cereceda & Lundberg (1992) and Lou & Lundberg (1992) in that capsazepine inhibits release of sensory neuropeptides induced by low pH at concentrations which do not affect afferent stimulation by other stimuli.

These findings are difficult to reconcile with the data presented by Bevan *et al.* (1992b) showing that neither the amplitude of the proton-activated current, nor the acid-induced ⁸⁶Rb⁺ efflux from dorsal root ganglion (DRG) neurones in culture or acid-induced depolarization of rat vagal nerve fibres are affected by capsazepine at concentrations, that are fully effective in blocking the corresponding responses activated by capsaicin. Likewise, peripheral nociceptor activation by low pH medium in a rat tail-spinal cord preparation is unaffected by capsazepine at concentrations which block the response to capsaicin (Dray *et al.*, 1992). On this basis, the hypothesis that occupancy of the vanilloid receptor by capsazepine might interfere with stimulation of the coupled cation channel by protons seems untenable.

Our findings and those of Franco-Cereceda & Lundberg (1992) and Lou & Lundberg (1992) have a common point in that the interaction of capsazepine with the effect of low pH has been investigated at the level of peptide release from peripheral endings of capsaicin-sensitive primary afferent neurones.

On this basis it would be premature to draw a parallel between the proton-activated cation current on DRG neurone membranes (Bevan *et al.*, 1992b; 1993) and the low pH-induced neuropeptide release from peripheral endings of primary afferent neurones. In particular, the possibility cannot be excluded that, when applying low pH media to peripheral tissues, a mediator is generated from one or more of the multiple cell types which are present which, in turn, may act on primary afferents. In other words, the action of low pH on sensory neuropeptide release in the periphery could have been stimulated by the generation of some intermediate factor(s) rather than by a direct action of protons on sensory nerve terminals. This implies that if such a factor(s) exists, it could be an endogenous ligand for the vanilloid receptor, and thus account for blockade of the response to low pH media

by both capsazepine (Franco-Cereceda & Lundberg, 1992; Lou & Lundberg, 1992 and present findings) and ruthenium red (Geppetti *et al.*, 1991; Del Bianco *et al.*, 1991; Santicioli *et al.*, 1992a). On the other hand, although KCl-induced CGRP-LI release was not affected by capsazepine at concentrations that inhibit the effect of low pH, the possibility cannot be entirely ruled out that the neurosecretory process is not-specifically inhibited by capsazepine. In particular, there is evidence that KCl-evoked CGRP-LI release from

peripheral endings of primary afferent neurones in rats involves the activation of ω -conotoxin-sensitive calcium channels (Maggi & Giuliani, 1991) which may be a target for the nonspecific effect of capsazepine on evoked CGRP-LI release. The present findings indicate that at concentrations of 30 μ M or higher, capsazepine exerts a generalized depressant action on evoked peptide release and therefore caution against an uncontrolled use of this compound as a selective capsaicin receptor antagonist.

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