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Met-enkephalin-induced mobilization of intracellular Ca^{2+} in rat intracardiac ganglion neurones

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Abstract

The effects of Met-enkephalin on Ca^{2+}-dependent K^+ channel activity were investigated using the cell-attached patch recording technique on isolated parasympathetic neurones of rat intracardiac ganglia. Large-conductance, Ca^{2+}-dependent K^+ channels (BK_{Ca}) were examined as an assay of agonist-induced changes in the intracellular free calcium ion concentration ($[Ca^{2+}]_i$). These BK_{Ca} channels had a conductance of ˜200 pS and were charybdotoxin- and voltage-sensitive. Caffeine (5 mM), used as a control, evoked a large increase in BK_{Ca} channel activity, which was inhibited by 10 μM ryanodine. Met-enkephalin (10 μM) evoked a similar increase in BK_{Ca} channel activity, which was dependent on the presence of extracellular Ca^{2+} and inhibited by either ryanodine (10 μM) or naloxone (1 μM). In Fura-2-loaded intracardiac neurones, Met-enkephalin evoked a transient increase in $[Ca^{2+}]_i$. Met-enkephalin-induced mobilization of intracellular Ca^{2+} may play a role in neuronal excitability and firing behaviour in mammalian intracardiac ganglia.

Keywords: Parasympathetic ganglia; Intracellular Ca^{2+}; Caffeine; Ryanodine; Opioid receptor; Ca^{2+}-dependent K^+ channel

Index Terms: heart innervation

Endogenous opioid peptides have been found in the extrinsic and intrinsic innervation of the rat heart [7] and vagal transmission to the heart has been shown to be inhibited by exogenous opioid substances [11, 18].

Opioid receptor activation has been shown to evoke numerous cellular responses via G_i/G_o proteins, for example, inhibition of N-type Ca^{2+} channels, inhibition of adenylyl cyclase, activation of K^+ currents (for review, see [12]) and intracellular Ca^{2+} mobilization [2, 8]. Mu-opioid receptor activation in cultured bovine adrenal medullary chromaffin cells has also been shown to potentiate Ca^{2+}-dependent K^+ channel activation [17]. Opioid receptor-mediated increases in intracellular Ca^{2+} has been reported to arise from two mechanisms, namely, Ca^{2+} influx across the plasmalemma [4, 16] and Ca^{2+} release from inositol 1,4,5-trisphosphate (IP_3)-sensitive Ca^{2+} stores [2, 8].

In rat intracardiac neurones, activation of μ-opioid receptors has been shown to inhibit N-type Ca^{2+} channels via a *Pertussis* toxin-sensitive G-protein [1]. However, the action potential after-hyperpolarization due to a Ca^{2+}-dependent K^+ conductance(s) in these neurones was unaltered in the presence of Met-enkephalin. In the present study, the actions of Met-enkephalin and caffeine, a known intracellular Ca^{2+} mobilizing agent, on intracellular Ca^{2+} mobilization and activation of the large conductance Ca^{2+}-dependent K^+ channels (BK_{Ca}) were investigated.

Parasympathetic neurones from neonatal rat intracardiac ganglia were isolated as previously described [19]. The dissociated neurones were plated onto glass coverslips coated with laminin, incubated at 37°C in 95% O_2 – 5% CO_2 atmosphere and used for experiments within 36–72 h. At the time of experiments, the neurone-plated glass coverslips were transferred to a recording chamber (0.5 ml volume) and mounted on the stage of an inverted phase contrast microscope. Unitary current recordings were obtained using the cell-attached patch recording configuration of the patch clamp technique [6]. In the cell-attached configuration, K^+ currents in the membrane patch follow the relationship $i_K=\gamma(V_m-V_h-E_K)$. When the cells were bathed in a depolarizing external solution (V_m ˜0 mV) and V_h was held at 0 mV, the membrane potential in the patch was close to 0 mV. Unitary currents were recorded using an Axopatch 200B patch clamp amplifier (Axon Instruments, Foster City, CA), filtered at 5 kHz, and stored on digital tape using a DAT digital recorder (DTR-1204, Biologic Science Instruments, Claix, France). Records were transferred to a Pentium computer using an analogue-to-digital converter (TL-1 DMA interface) and pClamp and Axotape software (Axon Instruments). Membrane currents were filtered at either 500 Hz or 2.5 kHz and sampled at 2 or 10 kHz, respectively.

The activity of BK_{Ca} channels was monitored as an assay of agonist-induced changes in intracellular free Ca^{2+} concentration ($[Ca^{2+}]_i$) at the cytoplasmic face of the plasma membrane [10]. The neurones were superfused with physiological salt solution (PSS) composed of (mM): 140 NaCl, 3 KCl, 2.5 $CaCl_2$, 0.6 $MgCl_2$, 7.7 glucose and 10 HEPES-NaOH, pH 7.2. In a series of experiments, external NaCl was isotonically replaced by KCl. The patch pipette contained a high K^+ solution of the following composition (mM): 140 KCl, 2.5 $CaCl_2$, 0.6 $MgCl_2$, 7.7 glucose and 10 HEPES-KOH, pH 7.2. The osmolality of the solutions was in the range 285–295 mmol/kg. The following drugs were used at the final concentrations stated: methionine enkephalin acetate (Met^5-enkephalin) and caffeine (Sigma, St. Louis, MO), naloxone hydrochloride (RBI, Natick, MA), ryanodine (Calbiochem, San Diego, CA) and charybdotoxin (Auspep, Parkville, Victoria, Australia). Experiments were carried out at room temperature (22°C).

Microfluorometric measurements were carried out on Fura-2 loaded neurones using standard ratiometric techniques. Isolated neurones, incubated for 1 h in PSS containing 5 μM Fura-2/AM, were illuminated with 340 and 380 nm light, and a Hatamatsu R928 photomultiplier tube collected the emission fluorescence (510 nm band pass filter) through a variable aperture which was set around the cell image. The output signal was digitized using a PTI interface (Photon Technology International, South Brunswick, NJ) and sampled at 5 Hz using Felix 1.1 software and a Pentium computer. Changes in $[Ca^{2+}]_i$ were calculated as previously described [5]. Data are expressed as the mean±SEM of *n*, the number of observations.

Ca^{2+}-dependent K^+ channels were studied in dissociated neurones from rat intracardiac ganglia in the cell-attached membrane patch recording configuration. In the presence of PSS, depolarization of the membrane by>20 mV evoked unitary outward currents, whose amplitude and open probability increased with further depolarization. The single channel conductance determined from the slope conductance of unitary current-voltage (I–V) relations was ˜200 pS (*n*=3). Changing the external K^+ concentration shifted the reversal potential of the I–V relationship as predicted by the Nernst equation for a K^+-selective electrode (unpublished data). In four out of four patches, charybdotoxin (100 nM), added to the patch pipette solution, abolished the activity of the large conductance channels (data not shown). The large K^+-selective single channel conductance and charybdotoxin- and voltage-sensitivity suggest that these channels are BK_{Ca} channels.

The effects of caffeine were investigated to determine the presence of a caffeine-induced Ca^{2+} release from intracellular stores in rat intracardiac neurones. In all patches (*n*=11), 5 mM caffeine, applied to the superfusing solution, induced a large increase in BK_{Ca} channel activity in neurones held at 0 mV (Fig. 1A). The caffeine-induced increase in outward current was reversed after prolonged washout (>10 min) (not shown). Ryanodine (10 μM), a plant alkaloid that binds to the ryanodine receptor on the endoplasmic reticulum (ER) and reduces ER Ca^{2+} permeability at micromolar concentrations [15], inhibited the caffeine-induced activation of BK_{Ca} channels in all (seven/ seven) patches studied (Fig. 1B). In all membrane patches studied (*n*=7), bath application of ryanodine alone for (15 min had no effect on BK_{Ca} channel activity in these neurones.

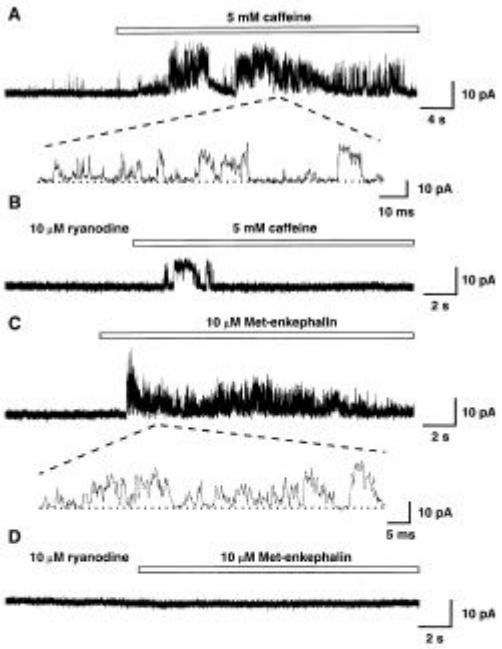

Fig. 1. Activation of BK_{Ca} channels by caffeine and Met-enkephalin in rat intracardiac neurones. Membrane currents recorded from cell-attached patches in the presence of high K^+ pipette solution. Membrane patch was depolarized by applying a pipette potential of –50 mV. (A) Bath application of caffeine (5 mM) evoked an increase in BK_{Ca} channel activity. Inset. Unitary currents recorded on an expanded time scale. (B) Effect of caffeine (5 mM) in the presence of ryanodine (10 μM) applied throughout the trace. (C) Bath application of Met-enkephalin (10 μM) produced an increase in BK_{Ca} channel activity. Inset. Unitary currents recorded on an expanded time scale. (D) Effect of Met-enkephalin (10 μM) in the presence of ryanodine (10 μM) applied continuously throughout the recording.

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To investigate whether opioid receptor stimulation could mobilize intracellular Ca^{2+} and activate BK_{Ca} channels in rat intracardiac neurones, Met-enkephalin was added to the superfusing solution. In 21 of 25 patches, 10 μM Met-enkephalin evoked BK_{Ca} channel activity in cell-attached patches held at 0 mV (Fig. 1C). Prolonged washout (>60 min) was required to reverse the increase in BK_{Ca} channel activity. In all neurones examined, desensitization of the response to Met-enkephalin occurred upon subsequent application of Met-enkephalin to the same cell or to other neurones in the dish (Fig. 3). In four out of four patches, 10 μM ryanodine inhibited the increase in BK_{Ca} channel activity induced by Met-enkephalin (Fig. 1D) suggesting that ryanodine-sensitive Ca^{2+} stores may be the source of Ca^{2+} for the activation of BK_{Ca} channels by Met-enkephalin.

To determine if the Met-enkephalin-induced increase in $[Ca^{2+}]_i$ was dependent on Ca^{2+} influx, the effects of Ca^{2+}-free external solutions and the inorganic Ca^{2+} channel blocker, cadmium (Cd^{2+}) were studied on BK_{Ca} channel activity. In the presence of Ca^{2+}-free solution (1 mM EGTA), 10 μM Met-enkephalin failed to evoke a significant increase in BK_{Ca} channel activity (n=4; Fig. 2A). However, the activation of BK_{Ca} channels by Met-enkephalin was observed when extracellular Ca^{2+} (2.5 mM) was restored (Fig. 2B). In the presence of an isotonic K^+ external solution which depolarized the neurone to ˜0 mV, Met-enkephalin failed to evoke an increase in BK_{Ca} channel activity (n=5; not shown). Bath application of 100 μM Cd^{2+}, to block Ca^{2+} influx through voltage-gated Ca^{2+} channels, also inhibited the Met-enkephalin-induced increase in BK_{Ca} channel activity (n=3; not shown). Taken together, these results suggest that Met-enkephalin-induced mobilization of intracellular Ca^{2+} is dependent on Ca^{2+} influx through plasmalemmal Ca^{2+} channels.

The activation of BK_{Ca} channels by Met-enkephalin was inhibited by the opioid receptor antagonist, naloxone. In the presence of 1 μM naloxone, Met-enkephalin activation of BK_{Ca} channels was reversibly inhibited (n=5) (Fig. 2C), suggesting that Met-enkephalin mobilizes intracellular Ca^{2+} by activating cell-surface opioid receptors and not from a non-specific effect of Met-enkephalin.

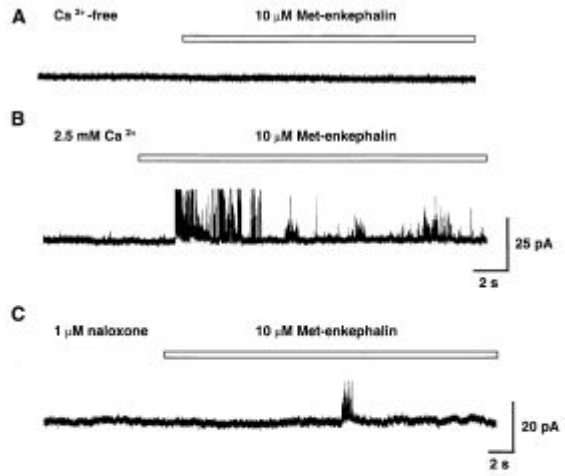

Fig. 2. Ca^{2+}-dependence of Met-enkephalin activation of BK_{Ca} channels in rat intracardiac neurones. Membrane currents recorded from cell-attached patches in the presence of high K^+ pipette solution. (A) Bath application of Met-enkephalin (10 μM) in the presence of a Ca^{2+}-free (1 mM EGTA) external solution had no detectable effect on BK_{Ca} channel activity. (B) Effect of Met-enkephalin (10 μM) in the same cell in the presence of 2.5 mM Ca^{2+} external solution. (C) Effect of Met-enkephalin (10 μM) in the presence of naloxone (1 μM).

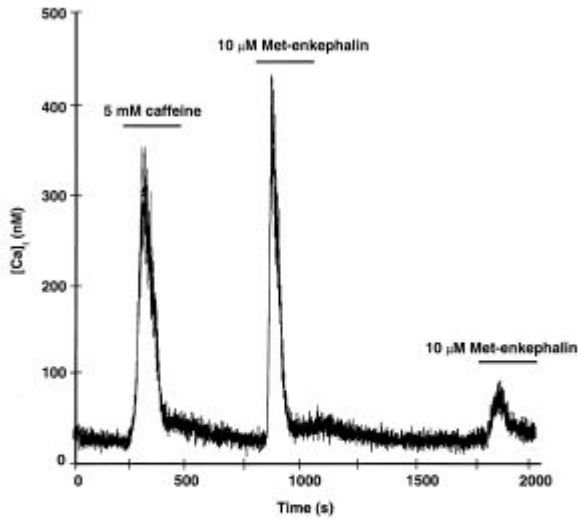

Fig. 3. Effect of caffeine and Met-enkephalin on intracellular Ca^{2+} levels in rat intracardiac neurones. Fura-2 loaded neurones were perfused with extracellular solutions containing either caffeine (5 mM) or Met-enkephalin (10 μM) at the indicated times. The response to repeated application of Met-enkephalin was attenuated even after 10 min.

Microfluorometric measurements were carried out to directly measure the Met-enkephalin-induced increase in $[Ca^{2+}]_i$ in Fura-2 loaded neurones. In isolated rat intracardiac neurones, resting $[Ca^{2+}]_i$, was 55±8 nM (n=9). Bath application of 10 μM Met-enkephalin induced a biphasic increase in $[Ca^{2+}]_i$ with an initial rapid,

transient rise followed by a sustained, slowly decaying phase (Fig. 3). The mean peak increase in $[Ca^{2+}]_i$ ($\Delta[Ca^{2+}]_i$), evoked by Met-enkephalin was 284±113 nM (n=7). In the presence of Ca^{2+}-free external solution containing 1 mM EGTA, Met-enkephalin failed to evoke an increase in $[Ca^{2+}]_i$, (n=3; not shown). Subsequent application of Met-enkephalin evoked an attenuated Ca^{2+} response even after >10 min washout (Fig. 3).

Mobilization of intracellular Ca^{2+} by caffeine and Met-enkephalin was demonstrated in rat intracardiac neurones using the activation of large conductance, charybdotoxin-sensitive BK_{Ca} channels as an assay of changes in submembrane $[Ca^{2+}]_i$. Caffeine (5 mM) evoked a large increase in BK_{Ca} channel activity, which was inhibited by ryanodine (10 μM) suggesting that caffeine released Ca^{2+} from intracellular ryanodine-sensitive Ca^{2+} stores. Similarly, Met-enkephalin (10 μM) produced an increase in BK_{Ca} channel activity, which was dependent on extracellular Ca^{2+} and inhibited by ryanodine (10 μM), Cd^{2+} (100 μM) and K^+-induced membrane depolarization. Naloxone (1 μM) inhibited the Met-enkephalin-induced increase in BK_{Ca} activity consistent with the activation of μ-opioid receptors in these neurones [1]. Fura-2 fluorescence measurements confirm that Met-enkephalin (10 μM) and caffeine evoke a mean increase in $[Ca^{2+}]_i$ of ˜300 nM sufficient to activate BK_{Ca} channels in excised, inside-out membrane patches (unpublished data).

The inhibition of caffeine- and Met-enkephalin-induced increases in $[Ca^{2+}]_i$ by ryanodine suggests that ryanodine-sensitive Ca^{2+} stores are present in rat intracardiac neurones and that Ca^{2+}-induced Ca^{2+} release (CICR) mechanisms may contribute to the action potential after-hyperpolarization [3]. Recent immunocytochemical and autoradiographic studies have detected a widespread and heterogeneous distribution of ryanodine receptors (RyR) throughout the nervous system (for review, see [9]) suggesting that intraneuronal Ca^{2+} stores and CICR may play an important role in neuronal Ca^{2+} dynamics. Although all three isoforms of the ryanodine receptor (RyR1–3) have been found in the CNS (RyR2 is the most prevalent), the function(s) of RyRs in neurones remains relatively unknown.

The cellular effects of opioids, including Met-enkephalin, have been shown to be mediated via the IP_3 signal transduction pathway [13]. Mu-opioid activation of SH-SY5Y cells has also been reported to stimulate Ca^{2+} influx via L-type Ca^{2+} channels [14]. Furthermore, opioids have been shown to mobilize Ca^{2+} from intracellular stores in SH-SY5Y cells but require ongoing muscarinic receptor activation [2].

In conclusion, opioid receptor activation produces opposing actions on Ca^{2+} mobilization in rat intracardiac neurones; inhibition of Ca^{2+} entry through ω-conotoxin GVIA-sensitive Ca^{2+} channels [1] and stimulation of Ca^{2+} release from ryanodine-sensitive intracellular Ca^{2+} stores.

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References

1. D.J. Adams and C. Trequattrini, Opioid receptor-mediated inhibition of ω-conotoxin GVIA-sensitive Ca^{2+} channel currents in rat intracardiac neurons. *J. Neurophysiol.* **79** (1998), pp. 753–762.
2. M. Connor and G. Henderson, δ- and μ-opioid receptor mobilization of intracellular calcium in SH-SY5Y human neuroblastoma cells. *Br. J. Pharmacol.* **117** (1996), pp. 333–340.
3. P.J. Davies, D.R. Ireland and E.M. McLachlan, Sources of Ca^{2+} for different Ca^{2+}-activated K^+ conductances in neurones of the rat superior cervical ganglion. *J. Physiol.* **495** (1996), pp. 353–366.
4. P.S. Eriksson, M. Nilsson, M. Wagberg, E. Hansson and L. Ronnback, Kappa-opioid receptors on astrocytes stimulate L-type Ca^{2+} channels. *Neuroscience* **54** (1993), pp. 401–407.
5. G. Grynkiewicz, M. Poenie and R.Y. Tsien, A new generation of Ca^{2+} indicators with greatly improved fluorescence properties. *J. Biol. Chem.* **260** (1985), pp. 3440–3450.
6. O.P. Hamill, A. Marty, E. Neher, B. Sakmann and F.J. Sigworth, Improved patch-clamp techniques for high-resolution current recording from cells and cell-free membrane patches. *Pflügers Arch.* **391** (1981), pp. 85–100.
7. J. Hughes, H.W. Kosterlitz and T.W. Smith, Distribution of methionine-enkephalin and leucine enkephalin in the brain and peripheral tissues. *Br. J. Pharmacol.* **62** (1977), pp. 639–647.
8. W. Jin, N.M. Lee, H.H. Loh and S.A. Thayer, Opioids mobilize calcium from inositol 1,4,5-trisphosphate-sensitive stores in NG108-15 cells. *J. Neurosci.* **14** (1994), pp. 1920–1929.
9. P. Kostyuk and A. Verkhratsky, Calcium stores in neurons and glia. *Neuroscience* **63** (1994), pp. 381–404.
10. A. Muñoz, L. Garcia and A. Guerrero-Hernández, In situ characterization of the Ca^{2+} sensitivity of large conductance Ca^{2+}-activated K^+ channels: Implications for their use as near-membrane Ca^{2+} indicators in smooth muscle cells. *Biophys. J.* **75** (1998), pp. 1774–1782.

11. T. Musha, E. Sataoh, H. Koyanagawa, T. Kimura and S. Satoh, Effects of opioid agonists on sympathetic and parasympathetic transmission to the dog heart. *J. Pharmacol. Exp. Ther.* **250** (1989), pp. 1087–1096.
12. R.A. North, Opioid actions on membrane ion channels. *Handbook Exp. Pharmacol.* **104** (1993), pp. 773–797.
13. D. Smart, G. Smith and D.G. Lambert, mu-Opioid receptor stimulation of inositol (1,4,5)trisphosphate formation via a pertussis toxin-sensitive G protein. *J. Neurochem.* **62** (1994), pp. 1009–1014.
14. D. Smart and D.G. Lambert, Desensitization of the mu-opioid activation of phospholipase C in SH-SY5Y cells: the role of protein kinases C and A and Ca(2+)-activated K^+ currents. *Br. J. Pharmacol.* **116** (1995), pp. 2655–2660.
15. J.L. Sutko, J.A. Airey, W. Welch and L. Ruest, The pharmacology of ryanodine and related compounds. *Pharmacol. Rev.* **49** (1997), pp. 53–98.
16. T. Tang, J.G. Kiang and B.M. Cox, Opioids acting through delta receptors elicit a transient increase in the intracellular free calcium concentration in dorsal root ganglion-neuroblastoma hybrid ND8-47 cells. *J. Pharmacol. Exp. Ther.* **270** (1994), pp. 40–46.
17. W.A. Twitchell and S.G. Rane, Nucleotide-independent modulation of Ca^{2+}-dependent K^+ channels current by a μ-type opioid receptor. *Mol. Pharmacol.* **46** (1994), pp. 793–798.
18. R. Weitzell, P. Illes and K. Starke, Inhibition via μ- and δ-receptors of vagal transmission in rabbit isolated heart. *Naunyn-Schmiedeberg's Arch. Pharmacol.* **328** (1984), pp. 186–190.
19. Z.J. Xu and D.J. Adams, Resting membrane potential and potassium currents in cultured parasympathetic neurones from rat intracardiac ganglia. *J. Physiol.* **456** (1992), pp. 405–424.