Published in final edited form as:

Neurosci Lett. 2010 August 16; 480(2): 106–111. doi:10.1016/j.neulet.2010.06.015.

G-protein-independent modulation of P-type calcium channels by μ -opioids in Purkinje neurons of rat

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Abstract

P-type calcium channels play a key role in the synaptic transmission between mammalian central neurons since a major part of calcium entering pre-synaptic terminals is delivered via these channels. Using conventional whole-cell patch clamp techniques we have studied the effect of μ -opioids on P-type calcium channels in acutely isolated Purkinje neurons from rat cerebellum. The selective μ -opioid agonist DAMGO (10 nM) produced a small, but consistent facilitation of current through P-type calcium channels ($10 \pm 1\%$, $n=27$, $p<0.001$). The effect of DAMGO was rapid (less than 10 sec) and fully reversible. This effect was both concentration and voltage-dependent. The EC_{50} for the effect of DAMGO was 1.3 ± 0.4 nM and the saturating concentration was 100 nM. The endogenous selective agonist of μ -opioid receptors, endomorphin-1 demonstrated similar action. Intracellular perfusion of Purkinje neurons with GTP γ S (0.5 mM) or GDP β S (0.5 mM), as well as strong depolarizing pre-pulses (+50 mV), did not eliminate facilitatory action of DAMGO on P-channels indicating that this effect is not mediated by G-proteins. Furthermore, the effect of DAMGO was preserved in the presence of a non-specific inhibitor of PKA and PKC, (H7, 10 μ M) inside the cell. DAMGO-induced facilitation of P-current was almost completely abolished by the selective μ -opioid antagonist CTOP (100 nM). These observations indicate that μ -type opioid receptors modulate P-type calcium channels in Purkinje neurons via G-protein-independent mechanism.

Keywords

μ -opioid receptors; agonist; antagonist; G-protein-independent facilitation

Introduction

Calcium influx through voltage-dependent calcium channels (VDCCs) mediates a plethora of cellular responses, such as transmitter release, gene expression, morphological differentiation and activation of calcium-dependent enzymes. Virtually all types of calcium channels are subject to modulatory actions of opioid receptors. Opioids regulate the activity of high-threshold L-, N-, P/Q-, R- [11] and low-threshold T-type [1] VDCCs through activation of μ -, κ -, δ - and nociceptin/orphanin FQ (N/OFQ) opioid receptors. The “classical” inhibitory effects of opioids on high-threshold VDCCs are mediated by activation of PTX-sensitive GTP-binding G_i and G_o proteins [11]. A prominent example

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associated with activation of opioid receptors is a reduction in calcium-dependent transmitter release via G-protein-mediated inhibition of N- and P/Q-type calcium channels [3].

A much smaller, but gradually increasing bulk of evidence indicates that opioids can produce G-protein-independent modulation of ion channels through activation of μ -, κ -, δ - and nociceptin/orphanin FQ (N/OFQ) opioid receptors [1,9,10,19]. Thus, opioids can facilitate transmitter release by modulating N-type calcium channels [9,10]. The latter effect does not involve activation of heterotrimeric G_i/G_o -proteins. The molecular mechanism(s) by which opioids exert this effect remains unclear.

The ability of κ -opioid agonists to inhibit P-type calcium channels in Purkinje neurons has been reported [8]. These authors demonstrated that P-current can be biphasically inhibited by a synthetic κ -opioid agonist U50488 through a G-protein mediated mechanism by activation of κ -opioid receptors (high affinity binding) and via a direct action on the P-channels (low affinity binding). Here we demonstrate that μ -opioid agonists acting in low nanomolar concentrations can facilitate P-current in a G-protein-independent manner.

Materials and methods

All experimental procedures were performed in accordance with the European Communities Council Directive of 24 November 1986 (86/609/EEC) [18]. All efforts were made to minimize animal suffering and to reduce the number of animals used. Isolation of cerebellar Purkinje neurons has been described in detail elsewhere [16]. Briefly, the cerebellum was cut into 300 to 400- μ m-thick slices and incubated with 2.4 mg/ml of proteinase XXIII from *Aspergillus oryzae* (Sigma, Germany) for 30–35 min at 22°C. After rinsing off the enzyme, single cells were isolated by triturating the pieces of tissue through several fire-polished pipettes with openings of 0.5–0.1 mm. Purkinje neurons were identified by their characteristic shape and partially preserved dendritic arborization.

Currents through VDCCs were recorded at room temperature (20–22°C) in a whole cell configuration of the patch-clamp technique [6], using an A-M Systems 2400 patch-clamp amplifier (BioMedical, Carlsborg, WA) connected to a 100-kHz Lab Master DMA board (Scientific Solutions, Solon, OH) in a personal IBM computer. Patch pipettes were pulled from borosilicate glass tubes (Sutter Instruments, Novato, CA) on a P97 Flaming/Brown micropipette puller (Sutter Instruments, Novato, CA). Pipettes had the resistances of 2–4 M Ω , when filled with the intracellular solution containing (in mM): 70 tris(hydroxymethyl)aminomethane (Tris)-phosphate, 40 tetraethylammonium chloride (TEA-Cl), 20 Tris-Cl, 5 ethyleneglycol-bis(β -aminoethylether) N,N,N',N'-tetraacetic acid (EGTA), 5 adenosine 5'-triphosphate, (ATP, magnesium salt), and 0.5 guanosine 5'-triphosphate, (GTP, Tris salt), adjusted to pH 7.3 with Tris-OH. Liquid junction potentials were compensated. Only the cells with negligible leaks (<50 pA) were used, thus current records were not leak-subtracted.

High-threshold P-type calcium current was measured at a holding potential of -70 mV to ensure complete inactivation of the low-threshold T-type calcium current present in Purkinje neurons [16]. Under these experimental conditions, the whole cell current was completely blocked by ω -Aga IVA (200 nM) [15]. To avoid Ca^{2+} -dependent inactivation processes, Ba^{2+} was chosen as a charge carrier through calcium channels. The external control solution contained (in mM): 40 TEA-Cl, 100 choline-Cl, 2 $BaCl_2$, 2 $MgCl_2$, and 20 Tris-Cl. Drug-containing solutions were applied by a “concentration clamp” technique using a “jumping table” set-up (Pharma Robot, Kiev, Ukraine). The currents were digitized every 140 μ s and

filtered at 3 kHz. Data were analyzed using jumping table software (Pharma Robot) running on IBM-PC computer.

All curve fitting and statistics were done with Microcal Origin software (Microcal, Northampton, MA, USA). The amplitude of currents was measured from baseline to peak

value. Effects of drugs were measured as the mean ratio $\frac{I - I_{con}}{I_{con}}$, where I is the amplitude of current under the action of drug and I_{con} is the amplitude of current in the control saline. The smooth curve describing the concentration-response relationships is the least squared fit of

the data points by the Hill equation $\frac{I - I_{con}}{I_{con}} = \frac{a \cdot [A]^n}{[A]^n + EC_{50}^n}$, where $[A]$ is the concentration of the drug, EC_{50} is the half-maximal effect concentration of the drug, n is the Hill coefficient and a is the maximal effect of the drug. Cumulative data were expressed as means $\pm$ SE (number of experiments). Statistical evaluation of results was performed using Student's *t*-test or one-way analysis of variance (ANOVA) followed by the Tukey–Kramer test, when more than two groups were compared. The level of significance was set at $P < 0.05$.

All chemicals were purchased from Sigma (St. Louis, MO).

Results

Extracellular application of the selective μ -opioid receptor agonist, DAMGO [13] in concentrations between 1 nM and 1000 nM induced facilitation of the P-current amplitude in 76% of the neurons ($n=37$), whereas in 24% of the neurons ($n=12$) DAMGO was of no effect at any concentration.

In the sensitive neurons application of 10 nM DAMGO resulted in a rapid (developing in less than 10 sec) increase of P-current ($10 \pm 1\%$, $n=27$, $p < 0.001$, Fig. 1A). This effect did not change during 2 minutes of drug application and immediately reversed upon the return to control solution. Subsequent application of DAMGO produced practically the same effect ($9 \pm 3\%$, $n=6$, $p < 0.05$) demonstrating a lack of desensitization.

The facilitation of P-current by DAMGO was concentration-dependent (Fig. 1B, C). Figure 1B demonstrates the effect of DAMGO in the concentrations of 1 nM ($6 \pm 1\%$, $n=10$, $p < 0.001$), 10 nM ($10 \pm 1\%$, $n=27$, $p < 0.001$) and 100 nM ($12 \pm 1.4\%$, $n=12$, $p < 0.001$). This effect was already well noticeable in concentration of 1 nM and fully saturated at 100 nM. The EC_{50} value obtained from the concentration-response curve was 1.3 ± 0.4 nM and the Hill coefficient was 0.9 ± 0.3 (Fig. 1C). This value is well comparable with K_D of 0.87 nM for binding of DAMGO with μ -opioid receptor [13].

Figure 2 illustrates that DAMGO-induced modulation of P-current is manifested in a wide range of voltages. However, as demonstrated in Fig. 2C, the effect of DAMGO was more prominent at the membrane voltages corresponding to the negative slope of I/V curve. One-way ANOVA ($F(7;80)=3.15$, $P < 0.01$) and *post hoc* analysis using the Tukey–Kramer multiple comparisons test revealed that the effect of DAMGO was significantly larger at -40 mV ($29 \pm 7\%$) than that at membrane potentials of -20 mV ($8 \pm 2\%$), -15 mV ($7 \pm 1.5\%$), -10 mV ($6 \pm 1\%$) and -5 mV ($6 \pm 1\%$).

Facilitation of P-current by DAMGO is not use-dependent: depolarizing pulses were not necessary for the development of this effect. Both deactivation and inactivation kinetics of P-current were not affected by DAMGO (data not shown).

To test possible Ca^{2+} dependence of the described modulation, we performed experiments using Ca^{2+} ions as P-current carriers. The effect of 10 nM DAMGO in these conditions was the same ($9 \pm 0.7\%$, $n=7$, $p<0.001$) as in the experiments with Ba^{2+} ($10 \pm 1\%$, $n=27$, $p<0.001$).

The endogenous selective agonist of μ -opioid receptors, endomorphin-1 in nanomolar concentrations produced facilitation of P-current in 71% of the neurons ($n=10$), whereas in 29% of the neurons ($n=4$) no effect was observed. In the sensitive neurons the effect of 10 nM and 100 nM endomorphin-1 was $8 \pm 1.2\%$ ($n=6$, $p<0.01$) and $10 \pm 1.1\%$ ($n=8$, $p<0.001$) respectively.

To reveal possible involvement of classical opioid receptors in the modulation of P-current by opioid agonists, we used potent and highly selective μ -opioid antagonist, CTOP. As shown in Fig. 3AB, CTOP itself did not affect the P-current ($0.9 \pm 1.2\%$, $n=6$, $p>0.4$) but completely removed the effect of DAMGO: application of 10 nM DAMGO in the presence of 100 nM CTOP did not produce the increase of P-current ($-0.8 \pm 1.4\%$, $n=6$, $p>0.6$) as compared to $9 \pm 1.5\%$ ($n=6$, $p<0.01$) in control. These data indicate that the facilitation of P-current by DAMGO is mediated via the μ -opioid receptor.

Opioid receptors are coupled to various G-proteins. Upon receptor activation, G_α and $G_{\beta\gamma}$ subunits interact with multiple effector systems. Calcium channels have binding sites for direct voltage-independent binding of G_α subunit as well as for voltage-dependent binding of $G_{\beta\gamma}$ complex. Binding to the latter site can be reversed by the application of depolarizing pre-pulse [14]. To check whether DAMGO-induced modulation of P-current is mediated by G-proteins in a voltage-dependent manner, we used a double-pulse protocol [14]. In these experiments the facilitation of P-channels was not abolished by strong depolarizing pre-pulses to +50 mV ($8 \pm 1\%$, $n=5$, $p<0.01$).

To test further possible involvement of G-proteins in the modulation of P-current by DAMGO, GTP in the recording pipette was replaced by GTP γ S so as to reversibly activate G-proteins. It is important to note that intracellular perfusion with 0.5 mM GTP γ S for 15–20 min results in the acceleration of the activation kinetics after strong depolarizing pre-pulses to +50 mV (Fig. 4A). This effect, normally absent in control intracellular solution, indicates at the functional interaction of GTP γ S with G-proteins in our experimental system [14]. However, as shown in Fig. 4B, the presence of GTP γ S (0.5 mM) in the recording patch pipette did not eliminate the effect of 10 nM DAMGO ($9 \pm 1.2\%$, $n=8$, $p<0.001$). Similar results were obtained when GTP was replaced by GDP β S in the patch pipette solution to inhibit G-protein activity. In these experiments the intracellular perfusion of Purkinje neuron with 0.5 mM of GDP β S for 15–20 min failed to prevent facilitation induced by 10 nM of DAMGO ($10 \pm 1.5\%$, $n=9$, $p<0.001$). Taken together, these data indicate that modulation of P-channels induced by DAMGO is not mediated by G-proteins.

Opioids are known to regulate the activity of different types of protein kinases, such as protein kinase C (PKC), cyclic AMP dependent protein kinase A (PKA), calcium/calmodulin-dependent protein kinase II (CaMKII) and protein kinase G (PKG). Activation of PKA [17] or PKC [4] facilitates the activity of P-type calcium channels. To investigate whether PKA or PKC were involved in the modulation of P-current by DAMGO, non-specific inhibitor of both kinases, H7 was used. As shown in Fig. 4C the intracellular perfusion of cell with H7 (10 μ M) for 20 min did not eliminate the effect of DAMGO on P-current ($7.3 \pm 0.5\%$, $n=7$, $p<0.001$). These results indicate that DAMGO-induced facilitation of P-current is not mediated by PKA or PKC. This conclusion is in concert with rapid onset and offset of DAMGO-induced facilitation.

Discussion

Voltage-dependent P/Q-type calcium channels ($\text{Ca}_v2.1$) are among the most critical components of synaptic transmission in mammalian brain [20]. Even subtle changes in the properties of these channels may substantially influence the efficacy of synaptic transmission. In the present study we demonstrate that μ -opioids in nanomolar concentrations produce facilitation of the P-type calcium channels acting via G-protein-independent mechanism. The speed and reversibility of the effect of DAMGO is more rapid (less than 10 sec) than the effect of κ -opioid agonist U50488 which needs about minute to develop [8]. In contrast to the frequency-dependent inhibition of P-current by U50488, DAMGO-induced facilitation of this current is not use-dependent and cannot be prevented by strong depolarizing pre-pulses to +50 mV. Furthermore, intracellular perfusion of Purkinje neuron with GTP γ S or GDP β S did not abolish the effect of DAMGO, whereas high affinity U50488-induced modulation of P-current was completely eliminated in the presence of GDP β S [8]. Finally, the effect of DAMGO was completely antagonized by selective μ -opioid antagonist CTOP, indicating the involvement of μ -opioid receptors, while the high affinity inhibition of U50488 was attenuated by norbinartorphimine (nor-BNI), suggesting that κ -opioid receptors were involved [8]. Comparing these two sets of results it can be suggested that there are different pathways by which various opioids can modulate P-current in Purkinje neurons depending on which receptor type and transduction mechanism is activated.

Opioid receptors belong to a large superfamily of GPCRs. As a class, GPCRs are of fundamental physiological importance mediating the effects of the majority of known neurotransmitters and hormones. According to the accepted paradigm, the first step in GPCRs transduction process requires activation of heterotrimeric G-proteins. However, increasing number of observations prompt a novel G-protein-independent mechanism through which GPCRs can initiate biochemical signals and modulate neuronal excitability [2,5]. According to these data, GPCRs are capable to exert certain effects by activating transduction systems that involve scaffold proteins. These GPCR-associated proteins may directly mediate receptor signaling, as in the case of G-proteins, or physically link the receptor to various effectors via different scaffold proteins [5].

Still much earlier it became known that opioid receptor agonists produce G-protein-independent ("non-classical") modulation of ion channels by interacting with different types of opioid receptors [1,9,10,19]. Abdulla and Smith [1] demonstrated that nociceptin (with high affinity, 100 nM) inhibited T-type calcium channels through G-protein-independent mechanism by activating opioid receptor-like (ORL1) receptors. This inhibition did not alter kinetics of the current and did not exhibit "use-dependence". Similar to these observations, in our experiments modulation of P-type calcium channels by DAMGO did not require G-proteins, there were no changes in the kinetics of this current and no "use-dependence". Twitchell and Rane [19] found that DAMGO increases the activity of Ca^{2+} -sensitive K^+ current via mechanism which is independent of G-proteins, protein kinases or protein phosphatases. In our experiments we were also unable to implicate PKA or PKC in the facilitation of P-current by DAMGO. Altogether this bulk of data clearly indicates that there is an alternative mechanism by which opioids can regulate ion channel activity independently of either G-proteins or phosphorylation-dependent processes. Still very little can be said about this molecular mechanism and further investigation is required. It is possible that μ -opioid receptors can modulate P-current through association with scaffold protein alike κ -opioid receptors stimulate Na^+/H^+ exchange [7] or through direct protein-protein interactions in the same way as metabotropic glutamate receptor subtype 1 (mGluR1) modulates P-type calcium channels [12]. We also do not exclude the possibility that opioid receptor agonists interact with modulatory site within P-channel itself.

In summary, opioids exert bi-directional effects on P-type calcium channels: inhibition through G-protein dependent pathway activated by κ -opioid receptors or via direct action on the P-channels [8] and facilitation via G-protein-independent pathway which involves interaction with μ -opioid receptors. Our data also support a possibility that opioids can act on distinct μ - or κ -opioid receptors in the same cell producing competing effects on P-type calcium channels.

Acknowledgments

This work was supported in parts by INTAS grant 05-100008-7843 and NIH grant 1R03TW008228-01A1.

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Figure 1. DAMGO produces small, but consistent facilitation of P-type calcium current

(A) Typical time course of the effect of two subsequent application of 10 nM DAMGO on the normalized P-current peak amplitude. The current was evoked every 20 sec by 50 msec voltage step from the holding voltage -70 mV to -25 mV. The traces of P-currents recorded at the moments indicated by the corresponding symbols are demonstrated in the inset.

(B) Concentration dependence of the facilitatory effect of DAMGO. Current traces are in the inset. The neuron was kept at -70 mV and the current was elicited by step depolarization to -20 mV every 20 sec.

(C) The dose-dependence relationship for DAMGO-induced facilitation of P-current. The

smooth curve was drawn according to the Hill equation
$$\frac{I - I_{con}}{I_{con}} = \frac{a \cdot [A]^n}{[A]^n + EC_{50}^n}$$
. The best fit was achieved for $EC_{50} = 1.3 \pm 0.4$ nM, $n = 0.9 \pm 0.3$ and $a = 12 \pm 1\%$. Points in the plot represent the mean $\pm$ SE of the values obtained from the number of cells given in the parentheses. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ vs control; Student's t-test.

Figure 2. P-current is facilitated by DAMGO in the whole range of membrane voltages

(A) Typical families of P-currents measured in control solution (○), in the presence of 10 nM DAMGO (●) and after wash-out (□). The voltages of the test pulses are indicated near the current traces. The neuron was held at -70 mV and stimulated every 5 sec by 50 ms long voltage steps in 5 mV increment.

(B) Current-voltage (I/V) relationships for the recordings demonstrated in (A).

(C) Summary data showing the voltage-dependence of the effect of DAMGO. One-way ANOVA ($F(7;80)=3.15$, $P<0.01$) and *post hoc* Tukey-Kramer test revealed significant differences between the measurements at the following voltages: -40 mV and -20 mV, -40 mV and -15 mV, -40 mV and -10 mV, -40 mV and -5 mV ($*p<0.05$). $*p<0.05$, $**p<0.01$, $***p<0.001$ vs control; ANOVA. Here and below, vertical bar: mean $\pm$ S.E.

Figure 3. DAMGO-induced facilitation of P-current was almost completely abolished in the presence of selective μ -opioid antagonist CTOP

(A) Typical effect of CTOP (100 nM) on facilitation of P-currents induced by DAMGO (10 nM). Holding voltage -70 mV. Test pulses were given every 20 sec by 50 msec voltage step to -25 mV. Original traces of P-currents recorded at the moments indicated by the corresponding symbols are in the inset.

(B) Cumulative data for the experiments demonstrated in A. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ vs control; Student's t-test.

Figure 4. The effect of DAMGO is not mediated either via G-proteins nor PKA and PKC activation

(A) Superposition of the original traces were recorded using standard stimulation protocol (-□-) and after a 50-ms conditioning pre-pulse to +50 mV (-■-). The neuron loaded with 0.5 mM of GTPγS for 15–20 min displayed facilitation of P-current by a 50 ms conditioning pre-pulse to +50 mV, indicating the functional interaction of GTPγS with G-proteins.

(B) DAMGO induces facilitation of P-current in the neuron demonstrated in (A). Holding voltage -70 mV. Test pulses were given every 20 sec by 50 msec voltage step to -30 mV. Current traces are in the inset.

(C) The intracellular perfusion of cell with 10 μM of H7 for 20 min failed to prevent facilitation induced by 10 nM of DAMGO.

(D) Cumulative data demonstrating the relative facilitation of P-current after depolarizing pre-pulses, with GTPγS (0.5 mM), GDPβS (0.5 mM) or H7 (10 μM) inside the cell.

* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ vs control; Student's t-test.