*Neuroscience Letters*, 137 (1992) 137–140



NSL 08478

# 8-Bromo-cAMP blocks opioid activation of a voltage-gated potassium current in isolated hippocampal neurons

Terry L. Wimpey and Charles Chavkin

*Department of Pharmacology, University of Washington School of Medicine, Seattle, WA 98195 (USA)*

(Received 3 July 1991; Revised version received 3 December 1991; Accepted 9 December 1991)

*Key words:* Opioid receptor; Delayed rectifier; Potassium conductance; Hippocampus; Subiculum; cAMP; cGMP; Adenylate cyclase

We have previously shown that $\mu$-selective opioid agonists activate both an inward rectifying and a voltage-gated potassium conductance in acutely dissociated non-pyramidal neurons from rat hippocampus. We now report that the opioid-activated voltage-gated potassium conductance was blocked by the membrane permeable cAMP analogue 8-bromo-cAMP. In contrast, 8-bromo-cGMP failed to inhibit opioid activation of the voltage-gated potassium current. These results suggest that the opioid-activated potassium channel is regulated by cAMP-dependent phosphorylation.

Opioid agonists produce excitatory actions in the hippocampus by reducing inhibitory input to pyramidal and granule neurons [9, 11, 19, 22]. Specifically, opioids reduce the release of acetylcholine and norepinephrine from fibers that project to the hippocampus and act directly on hippocampal GABAergic interneurons to decrease inhibitory postsynaptic potentials in hippocampal projection neurons [5, 9, 15, 19]. We have previously shown that $\mu$-selective opioid agonists activate both an inward rectifying and a voltage-gated potassium conductance in acutely dissociated neurons of the hippocampal formation [18]. The inward rectifying potassium conductance had properties similar to those described for opioid regulated currents in other parts of the brain [8, 17, 20]. Opioid activation of the inward rectifier resulted in small outward currents at resting membrane potentials and increased inward currents at hyperpolarized potentials. In a second population of neurons, $\mu$-selective opioid agonists increased a novel voltage-gated potassium conductance. In contrast to the inward rectifying potassium conductance, the voltage-gated conductance was not activated at resting membrane potentials or hyperpolarized potentials. Opioid agonists activated this potassium conductance only at depolarized membrane potentials. In the present study, we show that opioid activation of the voltage-gated potassium conductance was inhibited by the membrane permeable cAMP analogue 8-bromo-cAMP, but not by 8-bromo-cGMP.

Male Sprague–Dawley rats (125–150 g) were decapitated, the brains were rapidly removed and the hippocampal region dissected. Hippocampal slices (650 $\mu$m) were cut transverse to the long hippocampal axis using a McIlwain tissue chopper. The portion of the CA1 closest to the subiculum and the subicular formation (including the subiculum proper, presubiculum and parasubiculum) were sub-dissected as a single piece; transverse hippocampal slices were cut using a razor blade through the hippocampal fissure excluding the dentate gyrus. Both CA1 and subicular formation contain high densities of opioid receptors as revealed by radioligand binding and autoradiography [10, 16]. Neurons were isolated using a modified method of trypsin digestion described by Kay and Wong [6]. The tissue was stirred in a bicarbonate-buffered salt solution (consisting of (mM): NaCl 124, KCl 4.9, $KH_2PO_4$ 1.2, $MgCl_2$ 2.4, $CaCl_2$ 2.5, glucose 10 and $NaHCO_3$ 25.6, equilibrated with 95% $O_2$ 5% $CO_2$, pH 7.4) containing 1 mg/ml trypsin type XI (7000–7500 BAEE units/mg) (Sigma) at 34°C. After 1–1.5 h, the tissue was placed in fresh buffer containing trypsin inhibitor type II-O (1 mg/ml) (Sigma). Cells were then dissociated by trituration of a trypsin-treated slice through a series of fire-polished pasteur pipettes. Dissociated cells were allowed to adhere to the bottom of recording dishes.

Membrane current was measured under whole cell

*Correspondence:* C. Chavkin, Department of Pharmacology, SJ-30 University of Washington, Seattle, WA 98195, USA. Fax: (1) (206) 685-3822.

voltage clamp using an Axoclamp 2-A (Axon Instruments) in the single electrode continuous mode and records were saved by digitizing and storing on computer using the Fastlab software (Indec Systems). All traces are averages of two or three sweeps. Fire-polished microelectrodes had resistances of 2–4 MΩ and were filled with the following solution (in mM): EGTA 11, KCl 140, $CaCl_2$ 1, $MgCl_2$ 2, HEPES 10, pH 7.4 with KOH. No differences were observed in neurons voltage-clamped with electrodes also containing 1 mM ATP-Mg (Sigma). The cells were continuously superfused at room temperature with an oxygenated solution containing (in mM): TTX 0.001, HEPES 10, NaCl 149, KCl 6.1, $MgCl_2$ 2.4, $CaCl_2$ 2.0, $CdCl_2$0.5 and glucose 10, pH 7.4. Drugs were applied by pressure ejection through low resistance drug pipettes positioned close to neurons using a Picospritzer II (General Valve Corporation). Drugs used in this study included PL017 and DAMGO (Peninsula Laboratories, San Carlos, CA), and 8-bromo-cAMP, 8-bromo-cGMP and naloxone-HCl (Sigma Chemical Co., St. Louis, MO). Drugs were diluted to final concentration in the same buffer as used for perfusion.

For this study, successful recordings from a total of 61 neurons were made. Only dissociated neurons that were not pyramidal in appearance were selected for recordings. As previously reported, the opioid-responsive neurons make up only a small fraction of the total neuronal population in this brain region [18]; the results presented were from the 11 non-pyramidal neurons that responded to opioid application with an increase in outward current at depolarized membrane potentials (none of the remaining neurons was significantly affected by opioid). The average resting membrane potential of the opioid-responsive cells was −57.7±2.5 mV ($n$=11) which was not significantly different from the resting membrane potential of the unresponsive neurons, −57.9±2.1 mV ($n$=50).

Outward currents were elicited in voltage-clamped neurons by depolarizing voltage steps (200 ms) from a holding potential of −60 mV. Hyperpolarizing prepulses (200 ms) were delivered immediately prior to the depolarizing steps to reduce potassium channel inactivation. The opioid activated voltage-gated potassium conductance was previously found to be inactivated by sustained depolarization and maximally activated during depolarizing voltage steps that followed prepulses to −120 mV [18]. Fig. 1A shows outward current elicited in a typical opioid-responsive neuron depolarized in steps from resting membrane potential (−60 mV) to +20 mV. Note that sodium currents were blocked by TTX (1 μM) and calcium currents were blocked by cadmium (500 μM) in the perfusion buffer. Application of the μ-selective opioid agonist [$N$-MePhe$^3$,D-Pro$^4$]morphiceptin

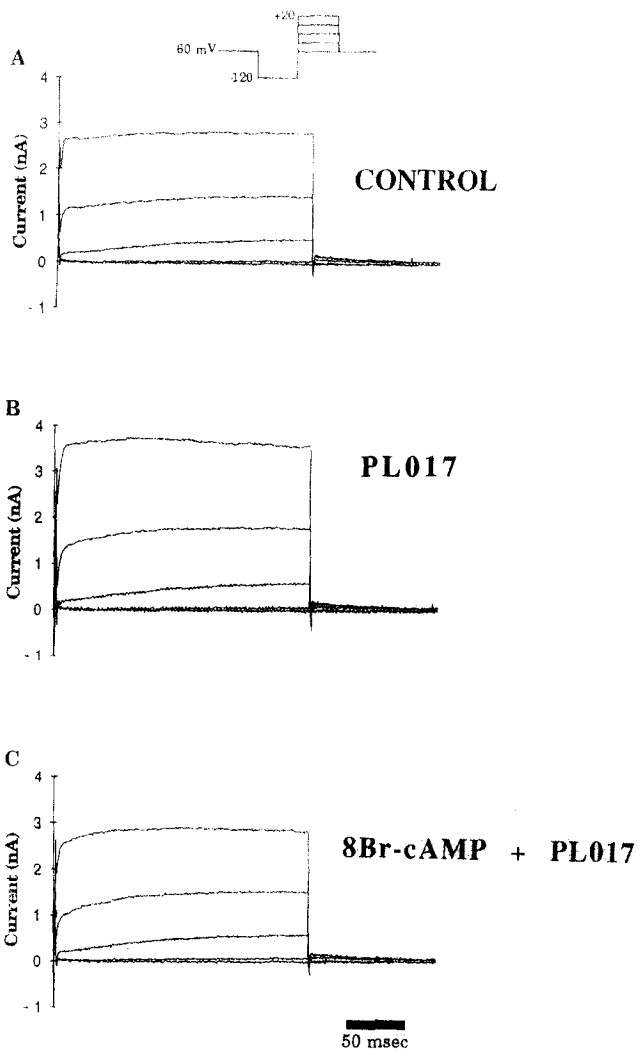


Fig. 1. The opioid-activated voltage-gated potassium current is blocked by 8-bromo-cAMP. A: current traces in a representative cell were elicited from a holding potential of −60 mV by steps to −60, −40, −20, 0 and +20 mV (200 ms) following hyperpolarizing conditioning prepulses to −120 mV (200 ms) (see inset). B: application of PL017 caused a reversible increase in the outward potassium currents at the depolarized potentials without producing a current at the resting potential (−60 mV). The opioid effect peaked between 1–2 min following opioid application and persisted for 3–4 min. The increase in potassium current caused by opioids was completely blocked by naloxone (data not shown). C: Application of 8-bromo-cAMP from a second pipette during the peak of the opioid action reversed the opioid-induced increase in the potassium current. Subsequent applications of PL017 had no effect on the currents. Capacitance transients and leak currents were not subtracted. PL017 was applied by pressure ejection (1 μM, pressure pulse 10 psi for 200 ms) from a nearby (0.1–0.2 mm) drug pipette 1 min before the beginning of the series of sweeps. 8-Bromo-cAMP was applied by pressure ejection (5 mM, pressure pulse 10 psi for 200 ms) from a second pipette.

(PL017) [1] (1 μM, pressure pulse 10 psi for 200 ms) caused an increase in the outward current in subsequent voltage steps to 0 or +20 mV (Fig. 1B). PL017 increased the sustained component of the outward potassium current without affecting the transient component as de-

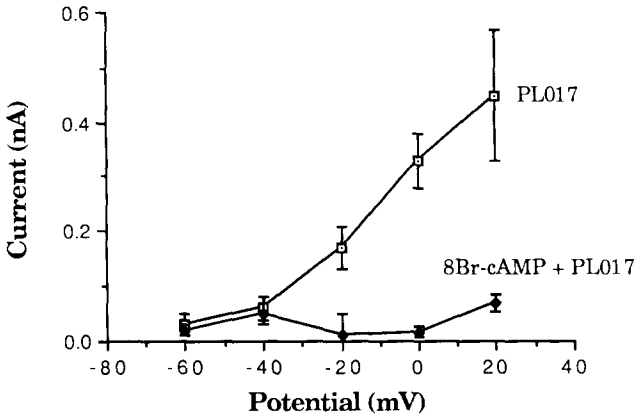


Fig. 2. The average current voltage relationship plotted for the voltage-gated current activated by the $\mu$ agonist PL017 and the inhibition of the current by 8-bromo-cAMP. Opioids consistently elicited an increase in the voltage-activated potassium current in these neurons without altering the resting membrane conductance (□). Application of 8-bromo-cAMP blocked the opioid-activated current (◆). Data points for the opioid-activated current were determined by subtracting currents elicited in the absence of opioid from those in the presence of opioid. Currents were measured 150 ms after the beginning of the step to the indicated potential. Values are means±S.E.M., $n$=6 different neurons.

scribed previously [18]. The $\mu$-selective opioid agonist [D-Ala$^2$,MePhe$^4$,Gly-(ol)$^5$]enkephalin (DAMGO) [7] was found to be equally potent at activating the potassium current (data not shown). Opioid application had no effect on resting membrane conductance (Fig. 1B). The opioid effect on the outward potassium current persisted for 1–10 min following a single application of the agonist, and could be blocked by coapplication of the opioid antagonist naloxone (100 nM, pressure pulse 10 psi for 200 ms) from a second drug pipette. Subsequent application of the opioid agonist produced a similar increase in the potassium current. Results are shown for a representative cell; however, the average outward current evoked by a step to +20 mV was 2.6±0.4 nA and the increase by PL017 application was 0.45±0.12 nA ($n$=11) or approximately 17%.

Application of the membrane permeable cAMP analogue 8-bromo-cAMP from a drug pipette (5 mM, pressure pulse 10 psi for 200 ms) blocked the opioid-induced increase of the voltage-gated potassium current on cells previously shown to respond to opioid application (Fig. 1C). In addition, application of 8-bromo-cAMP rapidly reversed the opioid action when applied during the peak of the PL017 effect and then blocked the effects of subsequent opioid agonist application.

Fig. 2 shows the mean current–voltage relationship for the opioid-activated voltage-gated current. Values for the opioid-activated current were determined by subtracting whole currents observed in the absence of opioid from those currents observed in the presence of opioids. The opioid-activated voltage-gated potassium current was activated only at depolarized membrane potentials. The opioid-activated current had a threshold of about −50 mV and peaked between 0 and +20 mV. 8-Bromo-cAMP blocked the opioid-activated current at all potentials. Application of 8-bromo-cAMP in the absence of opioid agonist had no significant effects on the resting membrane properties or the step depolarization elicited outward currents (data not shown). The inhibitory effect of 8-bromo-cAMP on opioid action was not reversed by washing during the time neurons could typically be voltage clamped (20–40 min). These results are consistent with the antagonist effects of forskolin and dibutyryl cAMP on the inhibitory responses caused by opioids in spinal cord explants reported by Crain et al. [3, 12]. However, opioid regulation of the inward rectifying potassium conductance is not affected by changes in cAMP concentration [17].

In contrast to the effects of 8-bromo-cAMP, application of the membrane permeable cGMP analogue 8-bromo-cGMP failed to inhibit opioid activation of the voltage-gated current (Fig. 3). 8-Bromo-cGMP had no effect on the resting membrane properties and did not block opioid induced increases in the voltage-gated potassium current. In some neurons, 8-bromo-cGMP by itself increased the amplitude of the outward current during depolarizing voltage steps; however, the effect of opioids was unchanged. The lack of effect of 8-bromo-cGMP on opioid activation of the voltage- gated current indicates that the inhibition caused by 8- bromo-cAMP was likely to be a specific effect.

The opioid-activated voltage-gated potassium current was previously shown to be irreversibly activated by opioids when the non-hydrolyzable GTP analogue GTP$\gamma$S was included in the recording pipette [18]. These experiments suggest that the opioid receptor couples to the voltage-gated potassium channel through a GTP-binding protein. In addition, the long latency and duration of the opioid effect may have been due to activation of a second messenger system. We previously found rapid onset and duration of opioid action on an inward rectifying potassium current present in another population of hippocampal neurons under the same recording conditions. Although the drug application method was identical, the opioid-induced response in neurons with the inward rectifying current was completely reversed several seconds after drug application. In contrast, opioid responses in neurons with the voltage-gated type of opioid-regulated current lasted several minutes following a single drug application.

Although the underlying mechanism responsible for the opioid-induced increase in potassium conductance is unknown, our hypothesis is that $\mu$ receptor activation

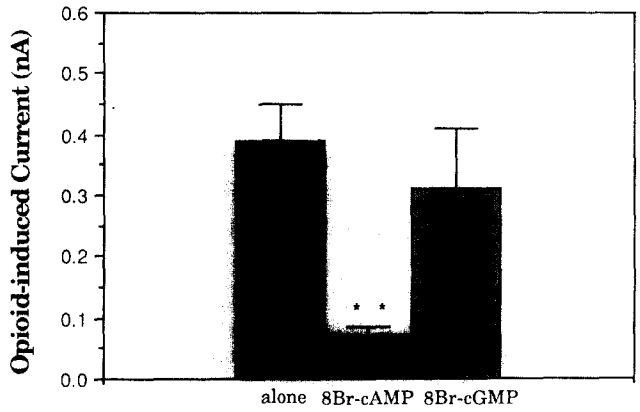


Fig. 3. cAMP but not cGMP analogues block the voltage-gated current activated by the $\mu$ agonist PL017. The graph summarizes the results from 11 independent experiments in which opioid application was followed by either 8-bromo-cAMP or 8-bromo-cGMP (data bars show the effects of PL017 application alone or following application of either 8Br-cAMP or 8Br-cGMP). Neurons were held at −60 mV then depolarized by a step to +20 mV. In the absence of opioid, the step resulted in an outward current of 2.6±0.4 nA. PL017 alone increased the current by 10–50% (mean=17%). 8-Bromo-cAMP significantly reduced the magnitude of the response to subsequent opioid application ($^{**}P<0.01$, Student's $t$-test) without directly affecting conductance. In contrast, 8-bromo-cGMP had no significant effect on conductance either in the presence or absence of opioid: for these cells, PL017 alone increased the outward current by 0.39±0.06 nA ($n=11$), and in the presence of 8-bromo-cGMP, the opioid increase was 0.31±0.10 nA ($n=5$).

results in a reduction in cAMP concentration leading to a reduction in the phosphorylation of the ion channel. This mechanism is consistent with actions seen in other neuronal cell types where $\mu$ opioid receptor activation inhibits adenylate cyclase activity [4] and activates a cAMP-dependent phosphodiesterase [21]. In other preparations, $\mu$ receptor activation has been suggested to activate PKC [2] or stimulate adenylate cyclase [13]; however, neither of those signal transduction mechanisms are likely to underlie the effects observed with hippocampal neurons. Thus opioids may increase potassium conductance by two mechanisms: a membrane-delimited activation of the inward rectifier and an activation of the delayed rectifier by dephosphorylation.

Supported by Grant DA-04123 from the US Public Health Service.

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