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SHORT COMMUNICATION

CYCLIC-AMP-MEDIATED EXCITATORY RESPONSES TO LEUCINE ENKEPHALIN IN *APLYSIA* NEURONES

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Although there are several different types of opiate receptors in mammals (Goldstein and James, 1984) and a variety of different ionic responses can be elicited by each of the receptor types on different neurones (Crain and Shen, 1990), relatively little attention has been given to the role of opiate receptors in invertebrates. We have previously demonstrated that the nervous system of the marine mollusc *Aplysia californica* contains both leucine-enkephalin-like and methionine-enkephalin-like opiate peptides (Leung *et al.* 1986) and that receptors for these substances exist on many neurones (Carpenter and Hall, 1986; S.-Rózsa *et al.* 1991; Kemenes *et al.* 1992). Although these responses are not identical to those observed in mammalian neurones, there are several common features.

A common response to leucine enkephalin and the stable analogue D-Ala^2-leucine enkephalin (DALEU) is a slow depolarization which is not associated with a significant conductance increase. This response has been characterized using an identified giant neurone, the metacerebral giant cell (MGC) or neurone C-1 (Kemenes *et al.* 1992), but similar responses are found in many neurones in the B cluster of the cerebral ganglion (S.-Rózsa *et al.* 1991). We have explored the effects of cyclic AMP derivatives and phosphodiesterase inhibitors on these responses in order to test the possibility that the receptors activated by DALEU and leucine enkephalin act through stimulation of cyclic AMP synthesis. Such second-messenger-mediated slow responses to endogenous opiates may contribute to the modulatory action of these peptides on a variety of physiological and behavioural processes (for a review, see Crain and Shen, 1990).

Most of the methods used here have previously been described in detail (Kemenes *et al.* 1992). Experiments were performed on the cerebral ganglia of adult *Aplysia californica*. The connective tissue sheath was removed by dissection above the MGCs or the B cluster cells and the neurones were penetrated with two independent glass microelectrodes filled with 3moll^{-1} potassium acetate and connected to a Dagan model

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8500 voltage-clamp amplifier. Neurones in the B cluster were identified according to the criteria of S.-Rózsa *et al.* (1991). The responses of two types of identified neurones were tested: the paired MGCs and the largest cell in the B cluster of the right cerebral ganglion, the CBR1 neurone. Both types of cells have previously been used in studies of the effects of FMRFamide and opiates in *Aplysia* (S.-Rózsa *et al.* 1991; Kemenes *et al.* 1992). Five of each of the two different cells were investigated in ten preparations for responses to 5×10^{-6} mol l^{-1} or 10^{-5} mol l^{-1} DALEU before and after the application of 10^{-4} mol l^{-1} isobutyl-l-methylxanthine (IBMX, Sigma), a potent inhibitor of phosphodiesterase. In preliminary experiments with other B cluster cells, another phosphodiesterase inhibitor (RO20-1724, Calbiochem) and the adenylate cyclase activator forskolin (Calbiochem) were also applied. They had the same effect as IBMX on the enkephalin- or FMRFamide-induced responses of all the neurones tested (S.-Rózsa *et al.* 1991). In the present paper, we only analyzed the effect of IBMX in detail, making the assumption that its application leads to increased intracellular cyclic AMP levels, as do any other phosphodiesterase inhibitors or activators of adenylate cyclase.

In each test, DALEU was microperfused five times prior to loading the cell with IBMX. Individual applications of DALEU lasted 30–45s and were separated by 2min intervals, during which only artificial sea water (ASW) was perfused over the ganglion. This protocol was repeated after treatment of the cells with IBMX. IBMX was microperfused over the MGC or CBR1 for 10min and the effect of DALEU was first tested after a 10min wash with ASW (i.e. when there was no IBMX left in the external medium). The peak depolarization of the MGC and the spike frequency of the CBR1 neurone in response to the DALEU stimulus were measured and the means of the five pre-IBMX and five post-IBMX results for each cell were calculated. From these, the means (±S.E.M.) of the pre-IBMX and post-IBMX results for all five CBR1 and MGC neurones were calculated and compared using two-tailed *t*-tests.

Following a wash of at least 30min with ASW, during which the DALEU-induced responses returned to control level, a membrane-permeable cyclic AMP derivative, 8-bromo-cyclic AMP (10^{-5} mol l^{-1}) was microperfused onto the neurones and their responses to this were compared with their responses to 10^{-5} mol l^{-1} DALEU.

At resting membrane potential, 10^{-6}–10^{-4} mol l^{-1} DALEU caused each of the MGC and CBR1 cells tested to give a consistent slow depolarizing response which, under voltage-clamp, was shown to be caused by an inward current.

Fig. 1A illustrates depolarizing responses of neurone CBR1 to microperfusion of DALEU at various concentrations. The threshold for a response was usually about 10^{-6} mol l^{-1}, and maximal responses were obtained with 10^{-5} mol l^{-1} DALEU. The response in each CBR1 neurone was very similar to that previously described for the MGC: the response did not desensitize during individual or consecutive applications (Fig. 2A,B), was not associated with any marked conductance change (Fig. 2D), but was quite voltage-dependent (Fig. 2C) and decreased with depolarization (Kemenes *et al.* 1992).

Fig. 1B illustrates the effect of IBMX perfusion on the CBR1 neurone's response to DALEU. After treatment of the cell with 10^{-4} mol l^{-1} IBMX for 10min, the DALEU response was considerably potentiated, and this effect was reversed after washing.

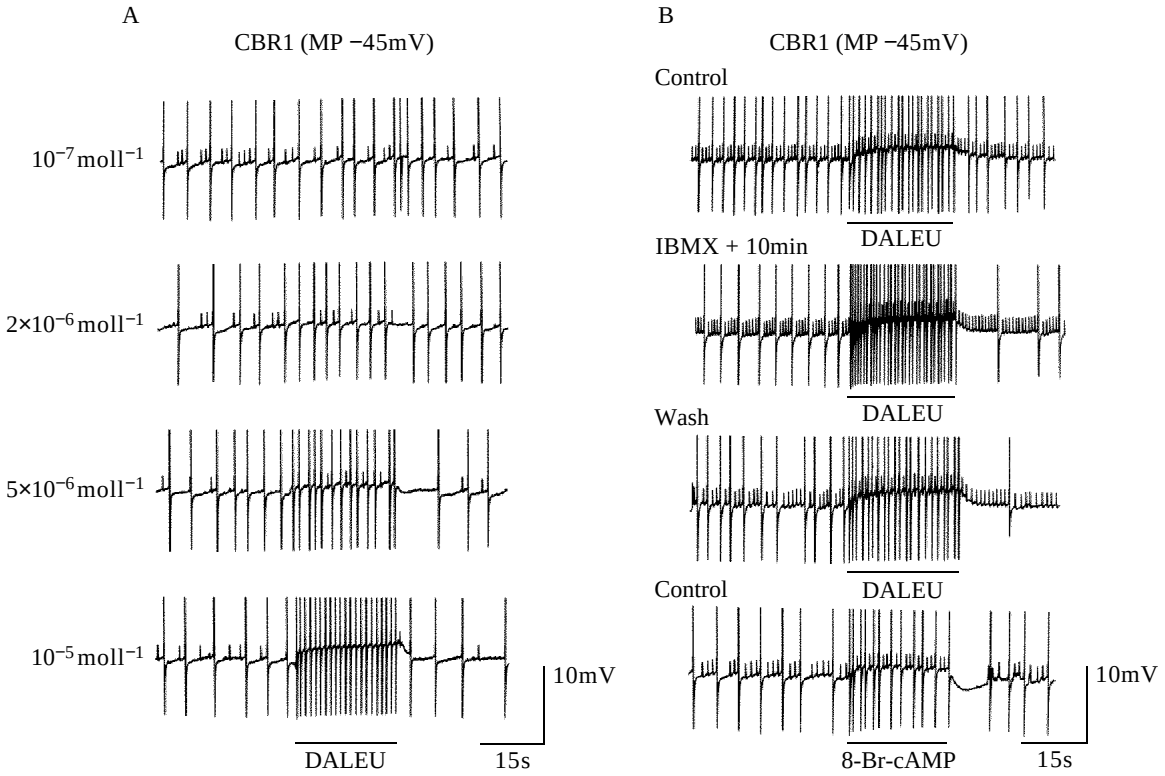

Fig. 1. Characterization of the response of neurone CBR1 in *Aplysia californica* to D-ala^2-leucine-enkephalin (DALEU). (A) Responses of neurone CBR1 to microperfusion of various concentrations of DALEU (current-clamp recording at −45mV resting membrane potential, MP). This neurone was spontaneously active, but there was an increase in spike discharge frequency and a membrane depolarization in response to DALEU application. At the gain used, the peak of the spikes is not shown. The small deflections are excitatory postsynaptic potentials (EPSPs). (B) Recordings from the same CBR1 neurone as in A. The first trace shows the control response to 10^{-5} mol l^{-1} DALEU. The second trace shows the effect of a 10min perfusion with 10^{-4} mol l^{-1} isobutyl-l-methylxanthine (IBMX), and the third trace shows that the IBMX-induced potentiation of the response was reversed by washing. In the experiment shown in the bottom trace, microperfusion of 10^{-5} mol l^{-1} 8-bromo-cAMP (8-Br-cAMP) caused a depolarizing response.

Similar results were obtained with the non-methylxanthine inhibitor of phosphodiesterase, RO20-1724 (see S.-Rózsa *et al.* 1991).

These observations suggest that DALEU acts by stimulating adenylate cyclase through the production of cyclic AMP. If this is the case, one would expect that a membrane-permeable derivative of cyclic AMP would induce a similar depolarizing response. The record in Fig. 1B shows that 8-bromo-cyclic-AMP mimics the response of this cell to DALEU.

Similar observations were obtained in studies on the MGC, a neurone that does not normally discharge spontaneously. Fig. 3A shows one such recording made in current-

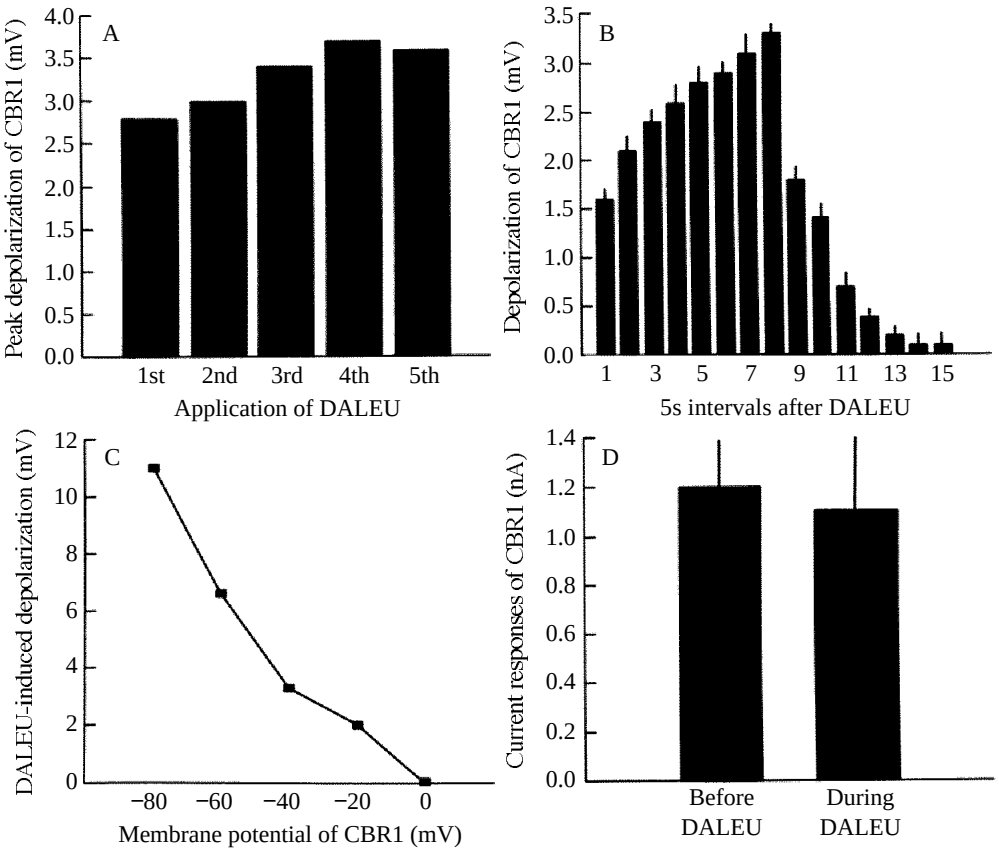

Fig. 2. Characterization of the response of neurone CBR1 in *Aplysia californica* to D-ala^2-leucine-enkephalin (DALEU). (A) The peak depolarization during five consecutive 40 s applications of 10^{-5} mol l^{-1} DALEU onto a CBR1 neurone. The applications by microperfusion of the peptide were separated by 2min intervals when only artificial sea water was perfused over the brain. Note that there was no desensitization after the first application; on the contrary, the responses to subsequent applications were slightly stronger. (B) Illustration of the lack of desensitization during 40s applications of 10^{-5} mol l^{-1} DALEU to the same CBR1 cell. The bars represent the means (+S.E.M.) of the depolarization from the resting potential level measured at 5s intervals after the onset of the DALEU-induced stimulus in five consecutive applications of the peptide. Note that the response built up slowly and outlasted the application of the DALEU, despite the fact that with microperfusion the final concentration is obtained within 100 μs of the onset of the stimulus and the drug is washed out within 100 μs of the offset (Slater *et al.* 1984). (C) Illustration that the DALEU-induced depolarization of a CBR1 cell decreased as the membrane potential was shifted towards less negative values. (D) The inward current responses (means + S.E.M.) of a voltage-clamped CBR1 cell to five voltage shifts (−2 mV, 0.5s) before and during the microperfusion of DALEU to the soma membrane. In this cell, DALEU caused no significant change in the inward current responses (P>0.5, N=5), indicating that the membrane resistance was also unchanged.

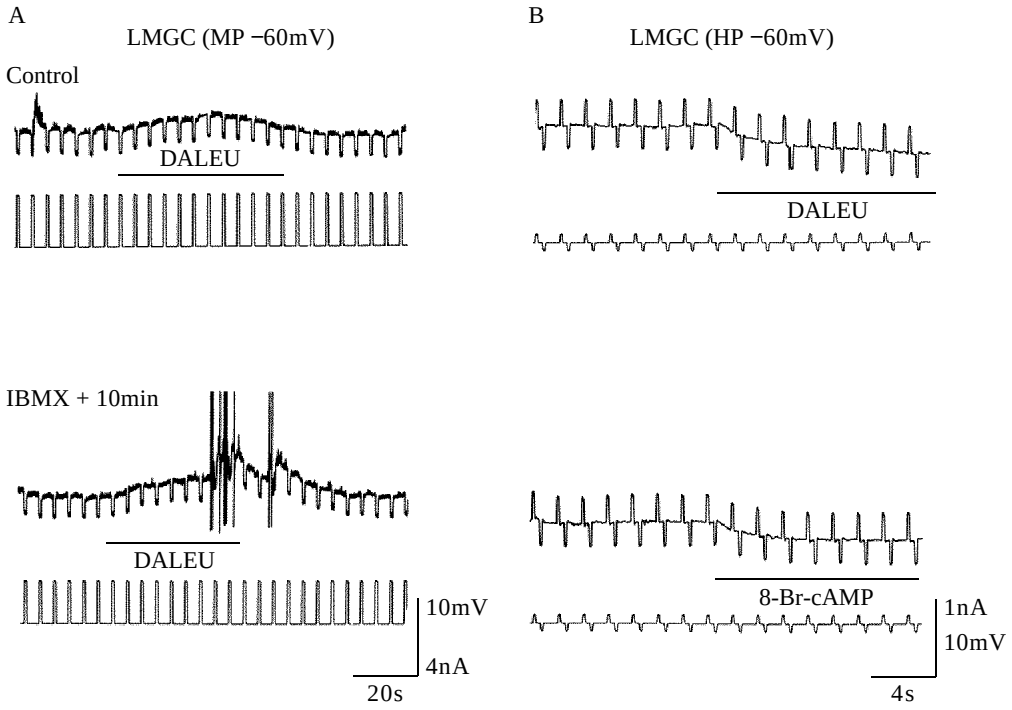

Fig. 3. Voltage and current responses of the metacerebral giant cell (MGC) in *Aplysia californica* to D-ala^2-leucine-enkephalin (DALEU) and 8-bromo-cyclic AMP (8-Br-cAMP). (A) Current-clamp recordings from the left metacerebral giant cell (LMGC). The upper trace is the voltage recording of the neurone, which had a resting membrane potential (MP) of approximately −60mV. Superimposed on the resting potential are deflections resulting from constant-current pulses passed through the second intracellular electrode. The pulses were 4nA in amplitude and are indicated in the lower trace. 5×10^{-6} mol l^{-1} DALEU was perfused for the duration indicated by the line drawn between the traces. The control records were obtained in normal artificial sea water (ASW), whereas the experimental records were obtained after a 10min perfusion with 10^{-4} mol l^{-1} IBMX followed by a 10min wash in ASW. Note the lack of a clear conductance change during the DALEU response and the potentiation of the response in the presence of intracellular IBMX. (B) Voltage-clamp recordings from the LMGC made during application of DALEU as in A. The upper record is the current trace obtained at a holding potential (HP) of −60mV on which are superimposed the current deflections resulting from brief voltage commands (±2mV). DALEU (10^{-5} mol l^{-1}) induced an inward current without any evidence of a change in input resistance. Perfusion of 8-bromo-cyclic AMP (8-Br-cAMP) at 10^{-5} mol l^{-1} induced a similar inward current in this neurone and this response was also not associated with a clear conductance change.

clamp mode. The constant-current pulses superimposed on the recording demonstrate the lack of a clear change in membrane conductance during the response. After 10min of 10^{-4} mol l^{-1} IBMX, the response to the same concentration of DALEU was increased and the neurone fired several action potentials.

Fig. 3B is a voltage-clamp recording of an MGC, and again shows the lack of a

conductance change. The current induced by DALEU application was inward but the currents induced by ±2 mV voltage shifts were not altered during the DALEU response. Perfusion of 10^{-5} mol l^{-1} 8-bromo-cyclic AMP generated an inward current which had similar characteristics to that produced by DALEU.

When 10^{-5} mol l^{-1} DALEU was applied to CBR1 cells in control medium, the average spike frequency was 45±7 action potentials per minute. This increased to 85±10 min^{-1} after microperfusion of IBMX at 10^{-4} mol l^{-1} for 10min (N=5). Similarly, the average depolarization in five MGCs induced by 10^{-5} mol l^{-1} DALEU was 4.2±0.7mV in control conditions and 6.7±0.8mV after 10min in 10^{-4} mol l^{-1} IBMX. Both increases were significant at the P<0.001 level (two-tailed t-tests, N=5). These observations indicate that both the depolarization and the resulting effect on spontaneous discharge are increased by inhibition of cyclic AMP breakdown caused by IBMX.

Although the present experiments were performed in normal saline (inhibitors of synaptic transmission, particularly low-Ca^{2+} solutions, have been shown to alter intracellular cyclic nucleotide levels; Kehoe, 1990), we are confident that both the basic DALEU-evoked response and its potentiation by IBMX were caused by direct effects on the CBR1 and MGC neurones and were not mediated indirectly by synaptic inputs. The following reasons justify this statement: (1) the microperfusion pipette was positioned so that no other cells downstream of CBR1 or MGC were affected by the drugs; (2) the responses had a short latency; (3) the overall number of EPSPs plus action potentials in CBR1 was not significantly changed by DALEU application (data not shown); (4) the overall number of EPSPs plus action potentials in CBR1 was not significantly changed by IBMX treatment (data not shown); (5) short-latency inward current responses were present in voltage-clamped cells, indicating that the basic depolarization and the increase in the firing rate seen in control cells after DALEU application and the stronger depolarization and firing rate increase seen in IBMX-treated cells after DALEU application were not caused by an increase in firing of presynaptic cells. However, we cannot entirely rule out the possibility that activation by DALEU or IBMX of neurones presynaptic to CBR1 and MGC may also have contributed to the effects observed in our intact central nervous system preparations. Only further experiments on isolated cells will provide evidence for or against the role of such presynaptic effects.

Although several actions of mu, delta and kappa receptors in mammals are known to be associated with an inhibition of adenylate cyclase (Collier, 1984), there is strong evidence that some of the excitatory effects of opiate peptides are mediated by stimulation of adenylate cyclase (Crain and Shen, 1990; Terwilliger *et al.* 1991). Such actions in the mammalian central nervous system are specific to particular brain areas and cell types and may be mediated by mu, delta or kappa receptors. Crain and Shen (1990) have proposed that increases in levels of cyclic AMP are associated with essentially all excitatory actions of opiates, whether the major ionic mechanism is a decrease in K^+ conductance or an increase in Ca^{2+} conductance (these being the major ionic bases of excitatory responses to opiate peptides in mammals). However, they propose that decreases in cyclic AMP levels are associated with almost all inhibitory actions of opiates, whether the inhibitory effects are mediated through increases in K^+ conductance or decreases in Ca^{2+} conductance.

The results of our studies of excitatory responses of *Aplysia* neurones to DALEU are

consistent with the conclusion that cyclic AMP is a second messenger responsible for mediation of the response. A further indication that the DALEU responses of both the MGC and CBR1 are mediated by a second-messenger system is the lack of desensitization and the slow build-up of the responses, together with the observation that the effect outlasts the application of DALEU. Our investigations have shown that this response is reduced by depolarization and is essentially abolished at about −30 mV in the MGCs (Kemenes *et al.* 1992) and at about 0mV in CBR1 (present study). Cyclic-AMP-induced currents in *Aplysia* neurones have been characterized by Kehoe (1990), who showed that this response may be the sum of the opening of an ion channel permeable to both Na^+ and K^+ (with a reversal potential of about +25mV) and the diminution of a Ca^{2+}-activated K^+ conductance. Very similar conclusions were drawn by Swandulla and Lux (1984), who showed that injection of cyclic AMP into *Helix pomatia* neurones resulted in induction of an inward current carried principally by Na^+, but also by K^+ and Ca^{2+}, as well as a reduction in the resting permeability to K^+. They noted that the nearly compensatory increase and decrease of two membrane conductances explained the lack of a significant change in cell input resistance even though the injection of cyclic AMP caused a considerable depolarization. It is likely that the responses activated by DALEU include both of these effects. This would explain why there was no consistent conductance change and why the response disappeared at a relatively hyperpolarized level.

S.-Rózsa *et al.* (1991) have previously reported that forskolin potentiates an inward current induced by the molluscan peptide FMRFamide and that IBMX potentiates the inward currents induced by both FMRFamide and methionine enkephalin in cerebral B neurones. Kemenes *et al.* (1992) have shown that leucine enkephalin and methionine enkephalin activate distinct receptors on one single neurone, so these observations, together with the present results, suggest that cyclic AMP is a common second messenger for a class of responses that may be elicited by activation of a variety of distinct receptors, each causing a slow depolarizing response. The concept that distinct receptors may activate a common adenylate cyclase is not new, having been first shown clearly by Tolkovsky and Levitzki (1978). However, the demonstration that a variety of small peptides, including the opiate peptides, all increase cyclic AMP levels through actions at distinct receptors provides yet another example of the ways in which substances regulate neuronal activity in complex ways.

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