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Effect of the endogenous analgesic dipeptide, kyotorphin, on transmitter release in sympathetic ganglia

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1 The effects of kyotorphin and the synthetic analogue, D-kyotorphin, on cholinergic fast excitatory postsynaptic potentials (fast-e.p.s.ps) were studied using intracellular recordings from bullfrog sympathetic ganglion cells.

2 Kyotorphin and D-kyotorphin (1–100 μM) increased the amplitude and the mean quantal content of the fast-e.p.s.p. without changing the mean quantal size in a low Ca^{2+}/high Mg^{2+} medium.

3 Kyotorphin and D-kyotorphin (1–100 μM) did not change the resting membrane potential, input membrane resistance, the amplitude and duration of action potentials and the sensitivity to the transmitter, acetylcholine (ACh), of the ganglion cells.

4 The facilitatory effect of D-kyotorphin on the fast-e.p.s.p. was reversibly inhibited by naloxone (10 μM).

5 These results indicate that kyotorphin may increase transmitter release from preganglionic nerve terminals. The possible mechanisms underlying this action of kyotorphin are discussed.

Introduction

Kyotorphin (L-Tyr-L-Arg) is an endogenous analgesic substance which has been isolated from bovine brain extracts (Takagi *et al.*, 1979a). The analgesic action of kyotorphin in the mouse is antagonized by naloxone (Takagi *et al.*, 1979a; Rackham *et al.*, 1982) and the kyotorphin-induced inhibition of the nociceptive response of rabbit spinal neurones is also antagonized by naloxone (Satoh *et al.*, 1980). Furthermore, the regional distribution of kyotorphin in the rat brain shows good correlation with opiate binding sites (Ueda *et al.*, 1980). These results indicate the participation of opiate receptors in the analgesic action of kyotorphin. However, kyotorphin does not bind to any type of opiate receptor in rat brain (Takagi *et al.*, 1979a; Rackham *et al.*, 1982), suggesting that kyotorphin does not act on opiate receptors but may induce the release of endogenous opioids.

On the other hand, increased activity in rat cerebral cortex induced by kyotorphin (applied microelectrophoretically) and inhibition of extinction of the pole-jump avoidance response in the rat effected by the intraventricular administration of kyotorphin are not antagonized by naloxone (Yamamoto *et al.*, 1981; 1982; Stone, 1983). About half of the total kyotorphin in the rat brain is found in the cerebral cortex (Ueda *et al.*, 1980). These findings suggest that endogenous opioids are not involved in those effects of kyotorphin unrelated to analgesia. There is still the possibility that kyotorphin affects the release of neurotransmitters other than enkephalins.

In this study we examined, using an intracellular recording technique, whether or not kyotorphin could influence cholinergic neurotransmission. Bullfrog sympathetic ganglia were used since their electrophysiological properties of synaptic events have already been well-defined (Kuba & Koketsu, 1978) and recently quantitative analyses of neurotransmitter release have been carried out (Hirai & Koketsu, 1980; Kuba *et al.*, 1981; Koketsu *et al.*, 1982).

D-Kyotorphin (L-Tyr-D-Arg), the synthetic analogue of kyotorphin, was mainly used in this study because it is thought to be resistant to degradative enzymes and has a greater analgesic effect than natural kyotorphin (Takagi *et al.*, 1979b).

A preliminary account of this work has been published previously (Hirai & Katayama, 1983).

Methods

The ninth or tenth lumbar sympathetic ganglia were isolated from bullfrogs of either sex (Rana catesbiana), together with preganglionic nerves. The preparations were continuously superfused with a Ringer solution of the following composition (mM): NaCl 112, KCl 2, $CaCl_2$ 1.8, and $NaHCO_3$ 2.4.

A conventional intracellular microelectrode technique was employed; microelectrodes filled with 3 M KCl had tip resistances of 30 to 60 MΩ. Action potentials and electrotonic hyperpolarizing potentials were evoked by passing transmembrane current through the recording electrode via a bridge circuit of a preamplifier. Fast excitatory postsynaptic potentials (fast-e.p.s.ps) were evoked by supramaximal preganglionic stimulation at 0.2 Hz in a low Ca^{2+}/high Mg^{2+} medium of the following composition (mM): NaCl 102.8, KCl 2, $CaCl_2$ 0.54, $MgCl_2$ 7.56 and $NaHCO_3$ 2.4. The average quantal content of the fast-e.p.s.p. in the low Ca^{2+}/high Mg^{2+} medium, was calculated for sixty consecutive fast-e.p.s.ps by both variance and failure methods (del Castillo & Katz, 1954; Martin, 1955). Acetylcholine (ACh) potentials were induced by iontophoretic application of ACh from micropipettes filled with 2 M ACh. Recorded signals were immediately stored in the digital memory of an oscilloscope (VC-10; Nihon Kohden) and subsequently displayed on a pen recorder chart and fed to a computer for on-line calculation of the mean quantal content. All experiments were carried out at room temperature (20–24°C).

Drugs used in this study were acetylcholine chloride (Sigma), kyotorphin (gift from Professor Takagi), D-kyotorphin (gift from Daiichiseiyaku Co.) and naloxone chloride (gift from Du Pont). Results are presented as mean ± standard error of the mean (s.e.mean).

Results

The present results were obtained from sixty-seven B neurones whose electrophysiological properties were essentially the same as those described previously (Nishi & Koketsu, 1960).

Kyotorphin and D-kyotorphin (1 to 100 μM; $n = 3$ cells for each drug) had no significant effects on the resting membrane potential and the anelectrotonic potentials of the ganglion cells (Figure 1a). Also the peak amplitude, the duration and after hyperpolariza-

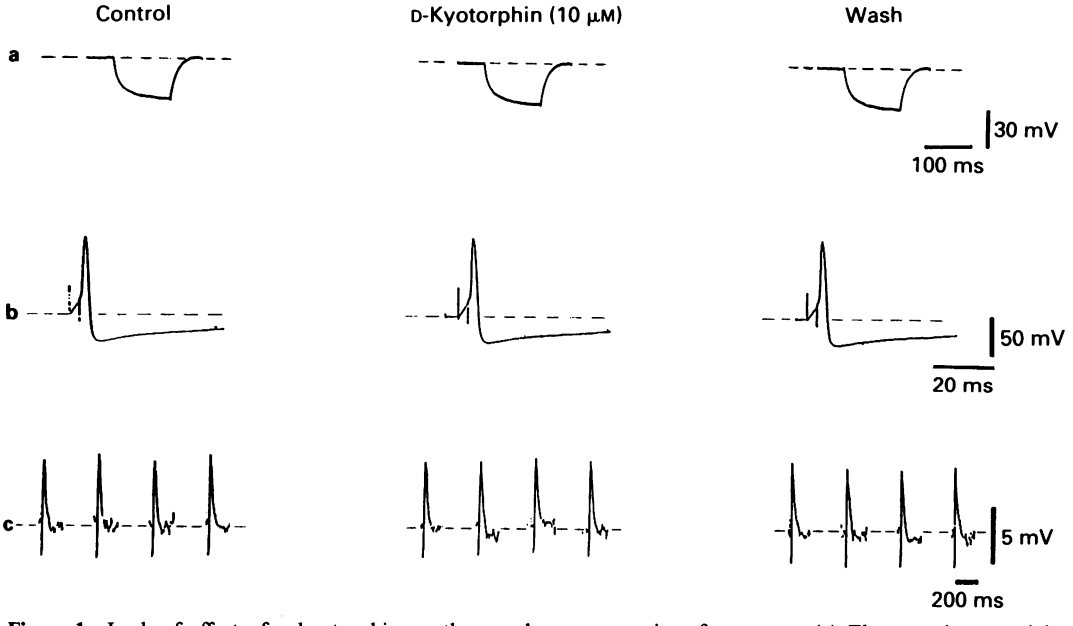

Figure 1 Lack of effect of D-kyotorphin on the membrane properties of neurones. (a) Electrotonic potentials generated by constant hyperpolarizing current pulses through the recording electrode (duration, 130 ms; intensity, 0.5 nA). (b) Action potentials elicited by short depolarizing current pulses through the recording electrode (duration, 3 ms). (c) Acetylcholine (ACh)-induced potentials elicited by iontophoresis of ACh (duration, 5 ms; intensity, 100 nA; interval 5 s).

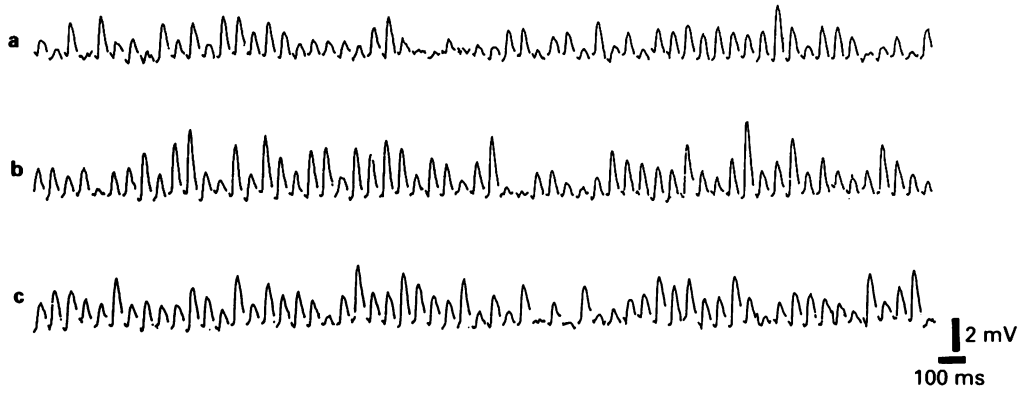

Figure 2 Effect of D-kyotorphin on the fast-excitatory postsynaptic potentials evoked by supramaximal preganglionic nerve stimulation at 0.2 Hz in a low Ca^{2+}/high Mg^{2+} medium. Before (a), during the application of D-kyotorphin (10 μM) (b) and after washout (c).

tion of the action potentials elicited by cathodal current were unaffected by the drugs (Figure 1b). Since synaptic transmission in this preparation is known to be cholinergic in nature, the effect of D-kyotorphin on the sensitivity of postsynaptic neurones to exogenously-applied ACh was examined. The amplitudes of ACh potentials induced by iontophoretic application of ACh were not significantly changed by D-kyotorphin (1 to 100 μM; $n = 4$ cells for each concentration) (Figure 1c). The passive and active electrophysiological properties of postsynaptic neurones were not appreciably affected by either kyotorphin or D-kyotorphin (1 to 100 μM).

The amplitude of the fast-e.p.s.p. evoked by preganglionic stimulation was reversibly augmented by superfusion of D-kyotorphin (1 to 100 μM) (Figures 2 and 3a); a facilitatory effect which was investigated further. The amount of neurotransmitter released from presynaptic nerve terminals was subjected to quantal analysis. The control values of the mean quantal content of the fast-e.p.s.ps obtained using the variance and the failure methods, were 2.6 ± 0.6 ($n = 8$ cells) and 2.2 ± 0.8 ($n = 8$ cells), respectively. The mean quantal content was significantly increased by D-kyotorphin (1 to 100 μM), while the mean quantal size (i.e. ratio of the mean amplitude to mean quantal content of the e.p.s.ps) was not changed; results obtained by the variance method are shown in Figure 3. The same results were also obtained by the failure method (not shown). The facilitatory effect of D-kyotorphin on transmitter (ACh) release was concentration-dependent within the concentration range examined (Figure 4). The natural dipeptide, kyotorphin, also increased the amplitude and the mean quantal content of the fast-e.p.s.p. ($n = 6$ cells) and the potency of kyotorphin was almost equal to that of D-kyotorphin (data not shown).

The participation of endogenous opioids in the action of D-kyotorphin was investigated by using the opiate antagonist, naloxone. Since the concentrations of D-kyotorphin tested were 1 to 100 μM, 1 to 10 μM of naloxone were tried for this purpose. Figure 5 shows that pretreatment with naloxone (10 μM) prevented the D-kyotorphin-induced increase in the amplitude and mean quantal content of the fast-e.p.s.ps, but these effects of D-kyotorphin again became apparent immediately on discontinuing exposure to naloxone. When given alone at concentrations of 1 to 10 μM, naloxone had no detectable effect on the electrophysiological properties of the postsynaptic membrane and on the amplitude and mean quantal content of fast-e.p.s.ps. However, in the low concentration range, 0.1 to 1 nM, naloxone sometimes increased the quantal content of fast-e.p.s.ps.

Discussion

These results indicate that kyotorphin and D-kyotorphin are capable of affecting cholinergic neurotransmission in bullfrog sympathetic ganglia. The dipeptides substantially increased the amplitude and mean quantal content of the fast e.p.s.p., while mean quantal size was unchanged. The mean quantal size is an indication of the amplitude of a response to a quantum of transmitter assuming a constant amount of ACh in each quantum. No change in mean quantal size means that the postsynaptic sensitivity to the neurotransmitter was not affected by kyotorphin. In support of this conclusion, the sensitivity of postsynaptic neurones to ACh was found to be unaltered by kyotorphin, as judged from the amplitude of the ACh-induced potential. Thus it may be concluded that kyotorphin and D-kyotorphin facilitate release of ACh from presynaptic

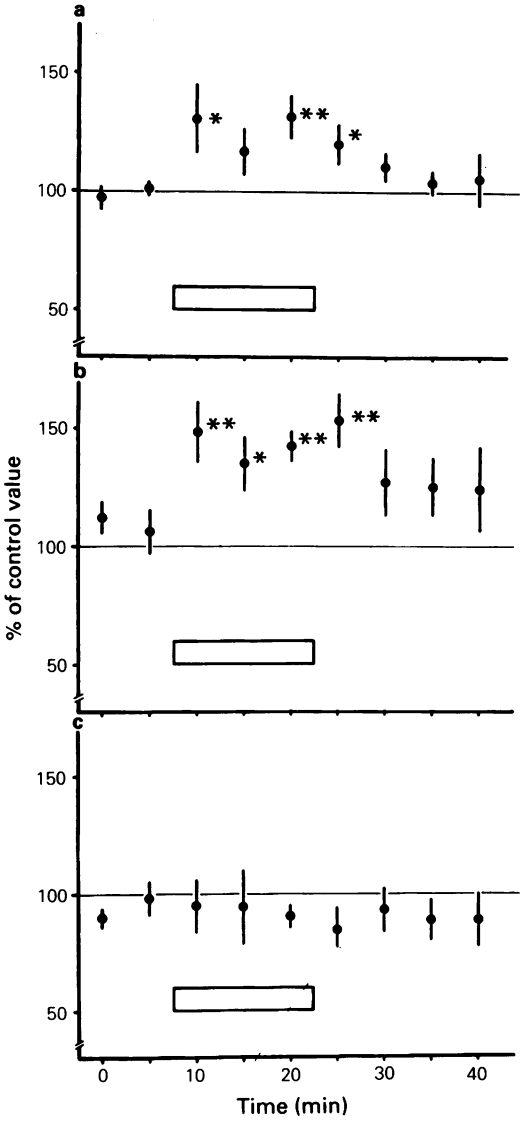

Figure 3 Effect of D-kyotorphin on the relative amplitude (a), quantal content (b) and quantal size (c) of the fast-excitatory postsynaptic potential. D-Kyotorphin (10 μM) was superfused during the period indicated by the open bar. The mean quantal content was calculated using the variance method. Each point indicates a mean value from eight experiments and vertical lines represent s.e.mean. * $P < 0.05$, ** $P < 0.01$, compared to the control value immediately before the application of the drug.

nerve terminals in bullfrog sympathetic ganglia, without affecting postsynaptic sensitivity to ACh.

Kyotorphin has been shown to provoke a naloxone-sensitive analgesia via its enkephalin-releasing action (Takagi *et al.*, 1979a; Satoh *et al.*, 1980; Shiomi *et al.*, 1981). In this study it was found that the D-kyotorphin-induced facilitation of ACh release was reversibly inhibited by naloxone. Antagonism by naloxone suggests the involvement of some kind of endogenous opioid in the facilitatory action of kyotorphin. Kondo & Yui (1981) demonstrated the localization of enkephalin-like immunoreactivities in the bullfrog sympathetic ganglion. Beani *et al.* (1982) found that the electrically-evoked release of ACh from brain slices was enhanced by morphine and [Met^5] enkephalin at low concentrations but inhibited at higher concentrations, although there is an abundance of evidence that enkephalin decreases transmitter release in many types of nervous system (see Beaumont & Hughes, 1979). It was speculated that, in the bullfrog sympathetic ganglion, kyotorphin may induce the release of enkephalin, as in the central nervous system (Takagi *et al.*, 1979a; Satoh *et al.*, 1980; Shiomi *et al.*, 1981). The effect of this enkephalin, released in low concentrations, might explain the facilitatory action of kyotorphin on transmitter (ACh) release. On the other hand, the possibility that kyotorphin acts directly on naloxone-sensitive receptor sites on the presynaptic nerve terminals cannot be excluded.

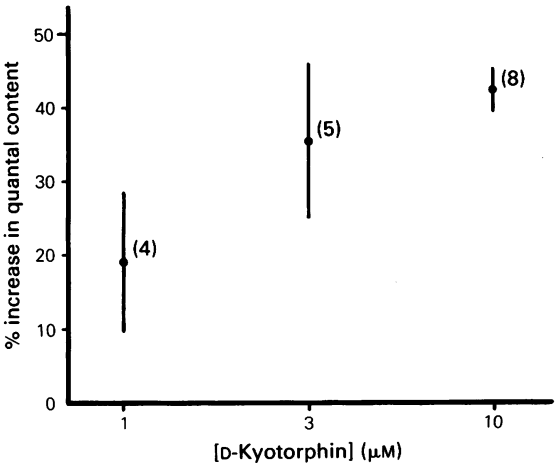

Figure 4 Concentration-response relationship for the facilitatory effect of D-kyotorphin on the quantal content relative to control of the fast-excitatory postsynaptic potentials, calculated by the variance method. Each point indicates a mean value and vertical lines represent s.e.mean. Figures in parentheses show the number of observations for each point.

Takagi *et al.* (1979b) showed that the analgesic potency of D-kyotorphin, measured *in vivo*, is about 5.6 times higher than that of kyotorphin. However, in this *in vitro* study the potencies were almost equal in facilitating ACh release, suggesting that the degradative enzyme system might not be active or present in isolated sympathetic ganglia.

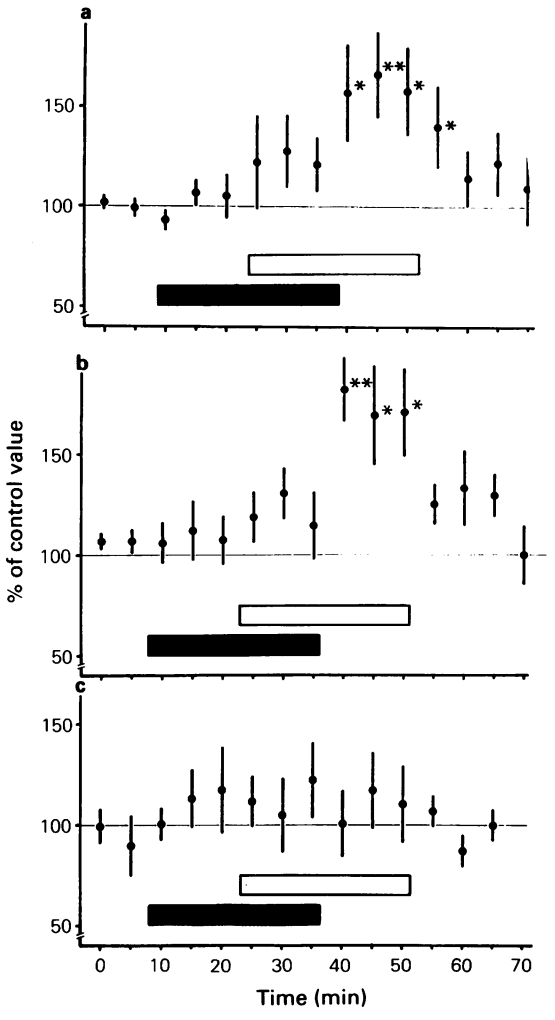

Figure 5 Prevention by naloxone of the effect of D-kyotorphin on relative amplitude (a), quantal content (b) and quantal size (c) of the fast-excitatory postsynaptic potentials. Naloxone (10 μM) and D-kyotorphin (10 μM) were added to the perfusate during the periods indicated by the solid and open bars, respectively. Each point indicates a mean value of eight experiments and vertical lines represent s.e.mean. $*P<0.05$, $**P<0.01$, compared to the control value immediately before the application of D-kyotorphin.

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