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EFFECT OF MORPHINE ON THE POTASSIUM-INDUCED CHANGE IN THE LEVEL OF REDUCED PYRIDINE NUCLEOTIDES AND CYTOCHROMES IN BRAIN SLICES

JOSEPH T. CUMMINS and HENRY W. ELLIOTT

Addiction Research Laboratory, Veterans Administration Hospital, Sepulveda, California 91343, and Department of Medical Pharmacology and Therapeutics, University of California, Irvine College of Medicine, 101 City Drive South, Orange, California 92668, U.S.A.

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Abstract—Morphine (2×10^{-3} M) inhibits the steady-state changes in rat brain slice NAD(P)H and cytochromes a-a_3 but not b and c, induced by 30 mM K^+ concentrations. The effects of morphine were demonstrated in cerebral cortex and caudate nucleus from naive but not from tolerant rats.

There are some intriguing associations between the actions of morphine on tissue respiration *in vitro* and calcium ion which warrant further investigation. First, although it has been shown that while 10^{-2} M morphine has no effect on the QO_2 of unstimulated rat cerebral cortex slices [1] when rat brain slices are depolarized by potassium, morphine inhibits the potassium-stimulated respiration when the slices are suspended in low or calcium-free Ringer's solution [2, 3]. This may be related to binding or permeability of the cell membrane to morphine since under conditions which favor morphine uptake, morphine inhibits potassium stimulated respiration of brain slices even in conventional Ringer's solution [4]. Second, morphine does not inhibit the oxygen uptake in low or calcium-free Ringer's solution of brain slices taken from rats made tolerant to morphine [2, 3]. Third, when calcium is injected intracisternally in mice, the analgesic effect of morphine is antagonized [5]. Fourth, Shikimi *et al.* [6] have demonstrated that acute morphine lowers brain content of calcium by 25%. Fifth, Ross *et al.* [7] have demonstrated that naloxone can antagonize the effect of morphine on calcium.

Since morphine inhibits O_2 uptake, it should also affect the enzymes (compounds) of the respiratory pathway. Cummins and Bull [8] have established that depolarization of brain tissue leads to rapid changes in the steady-state concentration of respiratory intermediates. Using their procedure, we investigated the effects of morphine on the potassium-induced oxidation of pyridine nucleotides and cytochromes in media containing various amounts of calcium. We felt that this approach might lead to a more definitive interpretation of the interactions between morphine and calcium and the demonstrated effects of morphine on K^+-stimulated respiration of brain slices.

MATERIALS AND METHODS

The respiratory intermediates in isolated brain slices were measured directly as previously described [8]. A Perkin–Elmer 156 dual wavelength spectrophotometer was used to measure continuously the absorbance of NAD(P)H by measuring the difference between the absorption peak at 340 nm and the reference wavelength at 378 nm. Reduced cytochrome a-a_3 was measured at its absorption peak at 445 nm minus 455 nm. Since a 8-nm slit had to be used in the measurements, cytochromes c and b were measured together at 563–575 nm. The absorbance was recorded in arbitrary units on a Varian G-1000 strip-chart recorder at 20 mv setting.

Rats (Sprague–Dawley) were made tolerant by the implantation of a pellet containing 75 mg of morphine formulated as described by Gibson and Tingstad [9]. The pellet was implanted and the extent of the tolerance was measured according to the procedure of Wei, Loh and Way [10] following naloxone-precipitated withdrawal. Ten mg/kg of naloxone caused an 8% diarrhea-induced weight loss in the rats within 3 hr. The animals were sacrificed 3 days after the pellet was implanted.

Rat cerebral cortex slices were cut by hand with a razor blade and guide to a thickness of 0.35 mm. Caudate slices were cut in a Vibratome (Oxford Instrument Co.), under oxygenated Krebs–Ringer at 4°. The caudate nucleus was cut away from surrounding tissue to form a block which was fixed to the slicing stage with instant glue (Devcon 'Zip Grip' 10). The stage with tissue was bolted in the microtome bath under oxygenated Krebs–Ringer cooled to 4°. The top of the tissue block was trimmed with the microtome blade and then successive 0.35-mm slices were made. Two slices of caudate nucleus were needed to cover the window of the tissue holder used in the respiratory measurements.

The rat brain slice was mounted in the special chamber previously described [8], and perfused with Krebs–Ringer solution which had been heated to 37° and gassed with 95% O_2–5% CO_2 in an insulated reservoir. Uniform perfusion of the tissue chamber was accomplished with a 4-channel Gilson Minipulse II peristaltic pump. The temperature was monitored on a digital thermometer (Technical Hardware, Model 401) by means of a thermistor probe placed within the tissue chamber. Potassium or drugs were added to the reservoir and pumped to the tissue. In a typical experiment morphine at a concentration of 2×10^{-3} M was added to the medium and 15 min

were allowed for the chamber to warm to 37 . After a steady state baseline was recorded, 30 mM potassium ion was added and changes in absorbance were determined for at least 150 sec.

RESULTS

The effects of 2×10^{-3} M morphine on the potassium-induced steady-state changes in NAD(P)H levels in brain cortex slices at various calcium concentrations are shown in Fig. 1. Morphine strikingly inhibits the potassium induced decrease in NAD(P)H in the 0.19 mM calcium medium but not in 0.75 mM calcium medium. The effect of morphine at 0.19 mM calcium is significant at the 0.01% level. On the other hand, in slices from rats made tolerant to morphine by pellet implantation, additional morphine has no effect in 0.19 mM and 0.75 mM calcium media and a delayed effect in 2.7 mM calcium medium. An attempt was made to remove all calcium from the system by using a calcium-free medium containing 2×10^{-4} M EGTA as a chelator to remove tissue calcium. In this experiment 2×10^{-3} M morphine added to the media had little effect on brain slices from either control or morphine-tolerant rats. The minimal effect of morphine on the medium containing EGTA in Figure 1d suggests inhibition of membrane function due to the complete removal of Ca^{2+}. Inhibition by morphine of the potassium-induced decrease in NAD(P)H in slices from control (naive) rats is strikingly shown in Figure 1c as is the lack of effect

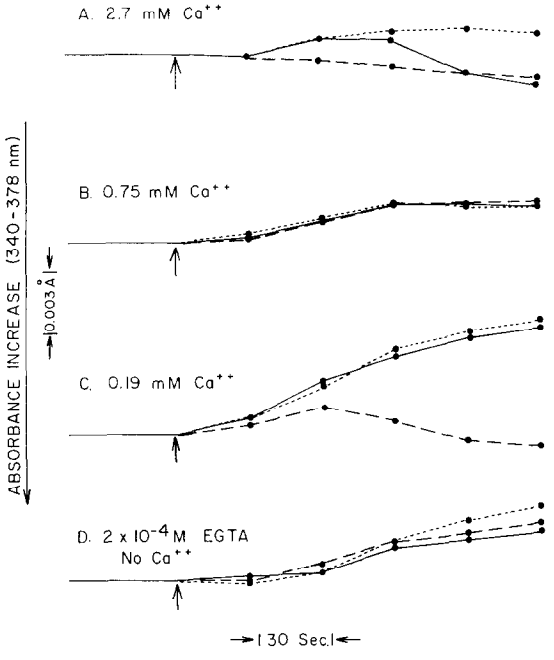

Fig. 1. The effect of 2×10^{-3} M morphine on K^+-induced steady-state changes in NAD(P)H levels in cortical slices at various Ca^{2+} concentrations. NAD(P)H changes measured at absorption peak 340 nm minus reference wavelength 378 nm. Four concentrations of Ca^{2+} were used: A is at 2.7 nM Ca^{2+}; B is at 0.75 mM Ca^{2+}; C is at 0.19 mM Ca^{2+}; and D has no Ca^{2+} plus 2×10^{-4} M EGTA. 30 mM K^+ added at arrow. Control (----●----●---); Naive + 2×10^{-3} morphine (--●--); Tolerant + 2×10^{-3} morphine (— ●—).

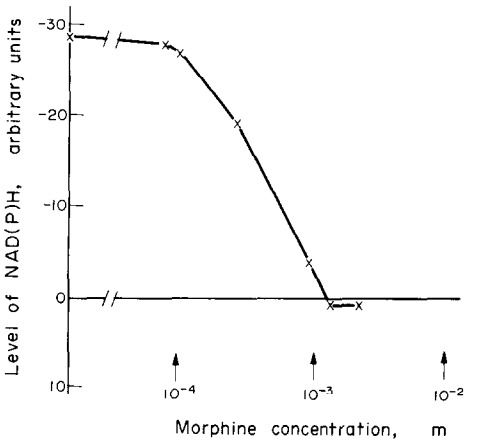

Fig. 2. Dose-response curve for morphine on the K^+-induced change in the steady-state level of NAD(P)H.

in slices from morphine-tolerant rats. The reversal of potassium effect in both naive and tolerant rats at 2.7 mM of calcium (Figure 1a) while only barely significant at the 0.1% level suggests antagonism of morphine by the calcium ion.

The inhibiting effect of various concentrations of morphine on the K^+-induced decrease in NAD(P)H levels in the medium containing 0.19 mM calcium is shown in Fig. 2. Morphine had no effect at a concentration of 10^{-4} M, minimal effect at 5×10^{-4} M and maximal effect at 2×10^{-3} M.

As shown in Fig. 3b the effects of morphine on the potassium induced changes in reduced cytochrome a-a_3 are similar to the changes in reduced NAD(P)H. In slices fron tolerant rats the morphine appears to delay the decrease in reduced cytochrome a-a_3, but in slices from naive rats it inhibits the changes at least for the period of 150 sec studied. As shown in Fig. 3-a, morphine has little if any effect on potassium induced steady-state changes in the

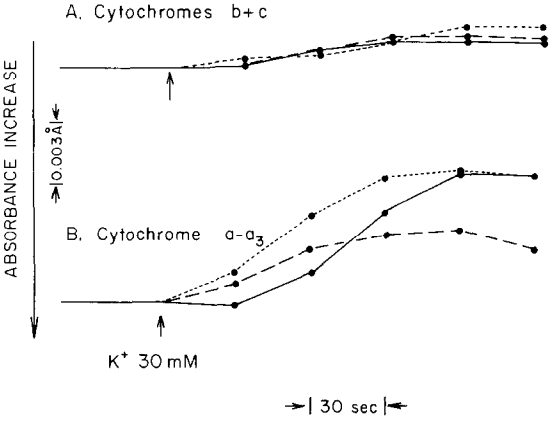

Fig. 3. The effect of 2×10^{-3} M morphine on K^+-induced steady-state changes in reduced cytochromes b and c (A) and reduced cytochrome a-a_3 (B) in 0.19 mM Ca^{2+}. Reduced cytochrome b and c were measured by the difference between 562 and 575 nm, and reduced cytochrome a-a_3 was measured at the wavelength settings of 445–455 nm. Control (----●----●---); naive + 2×10^{-3} M morphine (--●--); tolerant + 2×10^{-3} M morphine (—●—●—).

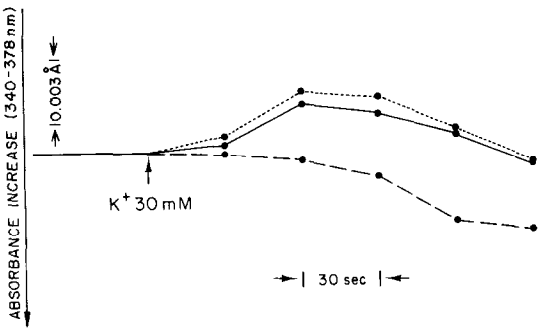

Fig. 4. The effect of 2×10^{-3} M morphine on K^+-induced steady state changes in NAD(P)H levels of caudate slices at 0.19 mM Ca^{2+} NAD(P)H changes measured at absorption peak of 340 nm minus reference wavelength 378 nm. Control (----●----●---); naive 2×10^{-3} M morphine (--●--); tolerant + 2×10^{-3} M morphine (—●—●—).

levels of reduced cytochromes *b* and *c* in slices from either control or morphine-tolerant rats.

As shown in Fig. 4, in low calcium-containing media morphine has the same effect on potassium-induced steady-state changes in NAD(P)H levels of slices from the caudate nucleus as seen in the slices of cerebral cortex. Decrease in NAD(P)H levels was inhibited in slices from naive animals and not inhibited in slices from morphine-tolerant animals. The morphine inhibition was significant at the 0.01% level. It should be noted that potassium induces a higher steady-state level of NAD(P)H in caudate nucleus than it does in cortical tissue.

DISCUSSION

Our data indicate that calcium is important in the action of morphine. Morphine inhibits the K^+-induced respiratory response in NAD(P)H levels in media low in Ca^{2+} (0.19 mM) but does not affect the response at zero or near-physiological levels of Ca^{2+}. Previous studies on oxygen uptake have given somewhat different results. Elliott, Kokka and Way [2] used a Ca^{2+}-free Ringer's but no chelating agent to complex the tissue Ca^{2+} so leaching of Ca^{2+} from brain tissue could have produced a low but not zero Ca^{2+} medium as was provided by the EGTA used in the present study.

The earlier report of Takemori [11] that morphine inhibited potassium-stimulated respiration of brain slices suspended in a medium containing 1.3 mM Ca^{2+} from control but not morphine-tolerant rats is harder to explain because in our laboratories numerous experiments using normal Ca^{2+} concentrations have shown that 2×10^{-3} M morphine has no effect on either QO_2 or short term responses in NAD(P)H levels evoked by high K^+ concentrations in suspending media for brain slices.

Unpublished studies from our laboratory demonstrate that 2×10^{-3} M theophylline enhances the K^+-induced decrease in NAD(P)H levels and that the decrease is more strongly inhibited by a lower concentration (10^{-4} M) of morphine than used in the present study. This observation fits nicely with the observed maximum response at low Ca^{2+} concentrations which closely resembles the Ca^{2+}-activation of adenylcyclase systems such as those prepared from homogenates of calf brain [12] and parotid gland [13]. These and similar preparations require Ca^{2+} but are inhibited by 1 mM Ca^{2+} or higher concentrations. It has been suggested that calcium may regulate phosphodiesterase activity [14]. Also, morphine has been shown directly to affect adenylcyclase activity in cultures of neuroblastoma glioma hybrid cells [15].

One explanation of our results is that part of the K^+-activated response may require low Ca^{2+} but is inhibited at normal calcium concentrations. The respiratory activity is higher at low Ca^{2+} concentrations but morphine inhibition of the higher respiratory rate is only marginal. High concentrations of morphine are needed to affect the K^+-responsive system when phosphodiesterase is not inhibited because of the high rate of cAMP breakdown. In the tolerant animal an adaptive change takes place possibly in cellular regulation of the adenylcyclase system resulting in higher cAMP levels so that even high concentrations of morphine are not inhibitory.

The increase in metabolic rate caused by depolarization of the cell membrane by K^+ results in a shift in the levels of the various oxidized and reduced respiratory intermediates [7]. The inhibition by morphine of the K^+ response shifts this equilibrium in a predictable manner. Bilodeau and Elliott [16] suggested that the energy demands of K^+ depolarization may be met by ATP and the resulting ADP and Pi establish a new steady-state equilibrium. It is likely that the respiratory enzymes of the mitochondria within the cells of brain are in a state of near equilibrium similar to that demonstrated by Wilson *et al.* [17] for isolated liver cells. Increased cellular energy demand brought about by K^+ depolarization and lessened by morphine inhibition of K^+ depolarization could alter the concentration of extramitochondrial nucleotides which in turn controls electron transfer at the three coupling sites of the mitochondrial chain. However, one should keep in mind that K^+ induces complex membrane changes that involve the release of neurochemical agents such as adenosine [18] and that the cell regulatory processes controlling the flow of reducing equivalents are poorly understood.

Caudate nucleus responds to K^+ in a manner similar to cortical tissue. The response to K^+ of both caudate nucleus and cortex slices is inhibited by morphine in the naive but not in the tolerant rat. The extent of the morphine inhibition of the K^+ response in the caudate nucleus approximates that of the cortex. Although caudate nucleus has a higher stereospecific binding activity for narcotics [19], our results suggest that the amount of binding may not be the sole determinant of effect of narcotics on brain metabolism. If so, it is possible that the metabolic actions of morphine may be common to all brain tissue but mediate its pharmacologic actions only in certain key centers. This is supported by the observation that acute morphine injection induces a similar degree of Ca^{2+} depletion in eight discrete brain areas [20].

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