Biochemical Pharmacology, Vol. 30, pp. 674–676.

0006-2952/81/060674-03 $02.00/0

Interactions of narcotics with synaptosomal calcium transport

(*Received* 13 *August* 1979; *accepted* 12 *September* 1980)

Several lines of evidence have implicated a role for Ca^{2+} in the pharmacodynamics of narcotic analgesics. As early as 40 years ago, it was demonstrated that high Ca^{2+} diets attenuated the development of tolerance and physical dependence produced by chronic morphine administration [1, 2]. Parenteral [3] and intraventricular [4] administration of Ca^{2+} has since been shown to antagonize the acute, antinociceptive effects of morphine. Coadministration of the divalent cation ionophore X537A and Ca^{2+} enhanced the antagonistic actions of the ion, suggesting that an elevation of intracellular Ca^{2+} reversed the antinociceptive effects of morphine [4]. With the demonstration that intraventricular administration of lanthanum (La^{3+}), a trivalent cation that inhibits the binding and transport of Ca^{2+} in biological membranes [5], produced naloxone-reversible antinociception that showed cross tolerance to morphine [6], an inhibition of neuronal Ca^{2+} transport was postulated as the molecular event that contributes to the production of antinociception. Consistent with this hypothesis were the reports of Ca^{2+} depletions from various brain regions [7] and from subcellular synaptosomal fractions following acute administration of narcotics [6, 8–11]. Additionally, *in vitro*, the narcotic agonist levorphanal has been reported to inhibit the high affinity binding of $^{45}Ca^{2+}$ to synaptosomal membranes [12], while morphine has been shown to inhibit a synaptosomal Ca^{2+} transport process that is supported by exogenously supplied ATP [13].

Given the obligatory role of Ca^{2+} in excitation–neurosecretion coupling [14] and the well-recognized anti-secretory activity of the narcotics toward acetylcholine [15–19] and norepinephrine release [20–21], investigators have speculated that these narcotic Ca^{2+} interactions underlie narcotic modifications of central neutrotransmission. This concept was borne out in the studies of Sanfacon *et al.* [22], wherein parenteral administrations of Ca^{2+} reversed the morphine-induced inhibition of acetylcholine release into cortical cups.

The extensive investigations of Blaustein and co-workers [23–26] into molecular events of excitation–neurosecretion coupling in rat brain synaptosomes have developed an invaluable *in vitro* model for investigating the effects of drugs on the neuronal Ca^{2+} transport that regulates neurotransmitter release. The techniques of Blaustein were used in the present investigations to characterize more fully the effects of morphine on a process of nerve terminal Ca^{2+} transport which is relevant to excitation-secretion coupling.

Synaptosomes were prepared from whole brains (minus cerebella) of male Sprague–Dawley rats (175–200 g) on the ficoll flotation gradient described by McGovern *et al.* [27]. Electron microscopic examination of this subcellular preparation revealed a highly enriched, intact synaptosomal fraction carrying slight contamination with vesicularized myelin but containing no free mitochondria. Synaptosomal $^{45}Ca^{2+}$ uptake under basal (5 mM KCl) and depolarized (60 mM KCl) conditions at 37° was studied by the method of Blaustein and Weisman [23]. Synaptosomal pellets obtained from the rapid centrifugations (18,000 $g \times 4$ min), which were used to reisolate the tissue from the radioactive media, were digested in 0.5 N NaOH. Following acid neutralization, samples were transferred to 10 ml of aqueous counting scintillant (ACS™, Amersham–Searle, Arlington Heights, IL) for the determination of synaptosomal $^{45}Ca^{2+}$ content by liquid scintillation spectrometry. Protein determinations were performed in synaptosomal pellets by the method described by Sutherland *et al.* [28].

Depolarization of synaptosomes with 60 mM KCl enhanced the uptake of $^{45}Ca^{2+}$ 2 to 3-fold over the uptake with 5 mM KCl (uptake over a 10-min time course, Figs. 1 and 2). The effects of depolarization were observed as a rapid, initial (1–2 min) increase in $^{45}Ca^{2+}$ uptake which plateaued to rates paralleling the basal uptake of Ca^{2+}, in agreement with the original observations of Blaustein and Weisman [23].

Agents that have previously been shown to block the voltage-sensitive, late Ca^{2+} channel in squid giant axon (D600, La^{3+}, and tetracaine) [29, 30] selectively blocked the depolarization-induced uptake of $^{45}Ca^{2+}$ in synaptosomes, without altering basal uptake. In Fig. 1, data representative of the effects of a standard Ca^{2+} antagonist are reported for 10^{-5} M La^{3+}. This selective action of the Ca^{2+} antagonists toward the potassium-depolarized state has extensive precedence in the literature [23, 25, 31, 32] and suggested that depolarization with 60 mM KCl activated a synaptosomal Ca^{2+} channel that was analogous to the voltage-dependent Ca^{2+} channel in squid giant axon.

Morphine also selectively inhibited only the depolarization-induced influx of $^{45}Ca^{2+}$ (Fig. 2). No concentration-effect relationships were observed from 10^{-7} M to 10^{-5} M morphine, but effects did disappear at 10^{-8} M morphine (data not shown). The failure to detect a graded inhibition by morphine probably was due to the lack of sensitivity in our test system within the small (approximately 30 per cent) range of the maximal inhibition. The narcotic antagonist naloxone (10^{-8} M) reversed the inhibition observed

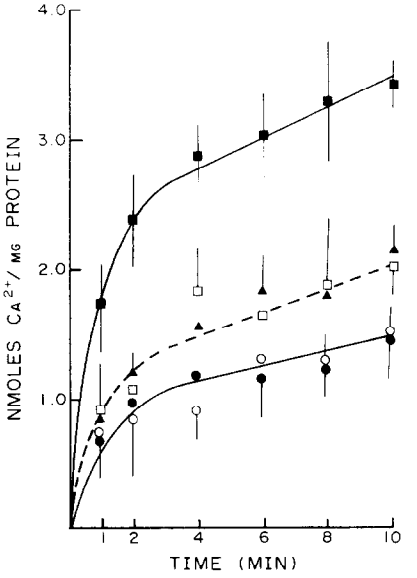

Fig. 1. Selective inhibition by 10^{-5} M La^{3+} of synaptosomal $^{45}Ca^{2+}$ uptake (nmoles Ca^{2+}/mg protein). Each value is the mean ± S.D. (N = 3); the experiment was repeated three times with similar results. Two-way analysis of variance revealed significant effects of La^{3+} on 60 mM KCl depolarization-induced Ca^{2+} uptake ($P < 0.001$). Key: (●) 5 mM KCl, basal uptake; (■) 60 mM KCl, stimulated uptake; (○) 5 mM KCl plus 10^{-5} M La^{3+} (□) 60 mM KCl plus 10^{-5} M La^{3+} and (▲) 60 mM KCl plus 10^{-5} M La^{3+} plus 10^{-6} M naloxone.

for 10^{-7} M morphine, and dextrorphan, the nonanalgesic (+) isomer of the narcotic analgesic levorphanol, produced no inhibition of synaptosomal $^{45}Ca^{2+}$ uptake (Fig. 3). Therefore, narcotic stereospecific binding sites (i.e. receptors) [33] appeared to be mediating the inhibitory action of morphine. Morphine in concentrations from 10^{-7} M to 10^{-5} M did not alter the efflux of $^{45}Ca^{2+}$ from synaptosomes preloaded with radiotracer (unpublished observation), suggesting that morphine inhibited the uptake process itself rather than modified synaptosomal Ca^{2+} retention.

Naloxone did not reverse the inhibition of 60 mM KCl-induced synaptosomal $^{45}Ca^{2+}$ uptake observed with the standard Ca^{2+} antagonists D600, tetracaine, or La^{3+} (data for La^{3+} in Fig. 1). These findings were unexpected in light of the reports of the narcotic-like properties of intraventricularly administered La^{3+}, an effect which may involve more than a simple competitive displacement of Ca^{2+} from neuronal membrane binding and transport sites. These data further attest to the specificity of narcotic interactions with synaptosomal Ca^{2+} transport and also suggest that the narcotic receptor may not be the Ca^{2+} channel *per se* (or at least not the site of calcium binding) but rather a modulator associated with the Ca^{2+} channel.

A further point of divergence of the effects of morphine from the actions of standard Ca^{2+} antagonists was the small maximal inhibition of synaptosomal Ca^{2+} uptake obtained with morphine. This was consistent with the hypothesis that morphine acts at receptors associated with a specific population of calcium channels or nerve endings, a postulate that is currently being investigated by testing morphine against the Ca^{2+}-dependent release of neurotransmitters from synaptosomes.

In conclusion, morphine selectively blocked the synaptosomal $^{45}Ca^{2+}$ uptake induced by depolarization with 60 mM KCl. This essentially agrees with previous reports on a morphine-induced disruption of neuronal Ca^{2+} transport under a variety of conditions [9, 10, 13]. Since the potassium-induced influx of Ca^{2+} has been shown previously to be obligatory to synaptosomal secretion coupling [23, 26], the present findings may represent a molecular mechanism contributing to the anti-secretory activity of the narcotics, as was suggested previously in the work of Sanfacon *et al.* [22]. Further studies on the release of neurotransmitters from synaptosomes are required to confirm this. Although it is tempting to speculate that these interactions of morphine with the nerve terminal Ca^{2+} channels subserve the analgesic activity of morphine in light of previous reports on antagonism by Ca^{2+} of narcotic antinociception [3, 4], similar selective inhibitions of potassium-induced uptake have been associated with the central depressant actions of chlorpromazine [34] and the barbiturates [25, 35]. Thus, the relevance of these *in vitro* neurochemical alterations to a specific aspect of the pharmacological activity of morphine (e.g. antinociception vs central nervous system depression) requires much further investigation.

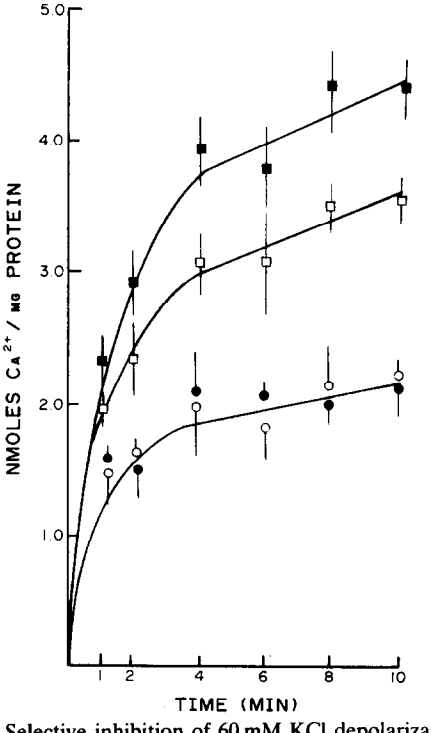

Fig. 2. Selective inhibition of 60 mM KCl depolarization-induced synaptosomal $^{45}Ca^{2+}$ uptake by 10^{-7} M morphine. Values are means ± S.D. (N = 3) at each time point; the experiment was repeated four times with similar results. Two-way analysis of variance revealed significant effects of morphine inhibition of 60 mM KCl-induced $^{45}Ca^{2+}$ uptake ($P < 0.001$). Key: (●) 5 mM KCl, basal uptake; (■) 60 mM KCl, stimulated uptake; (○) 5 mM KCl plus 10^{-7} M morphine; and (□) 60 mM KCl plus 10^{-7} M morphine.

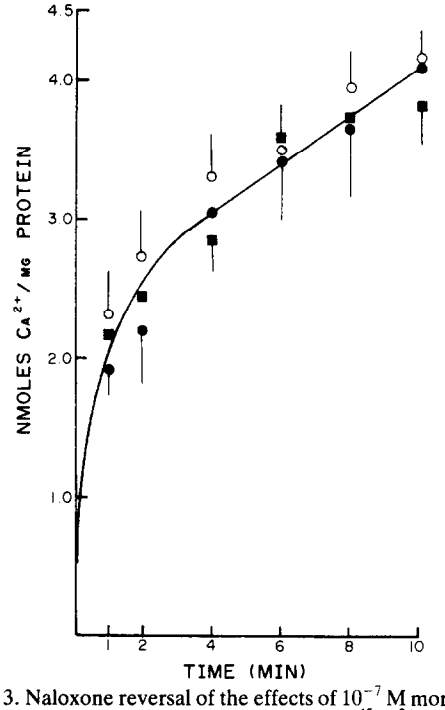

Fig. 3. Naloxone reversal of the effects of 10^{-7} M morphine on depolarization-induced synaptosomal $^{45}Ca^{2+}$ uptake, and the lack of inhibition with the nonanalgesic (+) isomer of the analgesic levorphanol, dextrorphan (10^{-5} M). Values are means ± S.D. (N = 3). Key: (■) 60 mM KCl-stimulated control; (●) 60 mM KCl with 10^{-7} M morphine plus 10^{-8} M naloxone; and (○) 60 mM KCl plus 10^{-5} M dextrorphan.

Acknowledgements—Supported, in part, by grants from the National Institute of Drug Abuse, DA-00326, DA-00490, and DA-01647. D. W. E. was a Postdoctoral Fellow of the National Science Foundation (SMI-17622901). R.A.C. is a recipient of RCDA No. AM 00565.

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Biochemical Pharmacology, Vol. 30, pp. 676–678.

0006-2952/81/060676-03 $02.00/0

Lack of inhibition of glutathione reductase by anthracycline antibiotics*

(*Received* 3 *July* 1980; *accepted* 3 *October* 1980)

Although the mechanism by which the anthracycline antibiotics produce their cumulative dose-dependent cardiomyopathy remains to be fully elucidated, recent evidence suggests that reactive oxygen species may play an etiological role. Doxorubicin and daunorubicin undergo conversion to free radical semiquinones via the acceptance of one electron from NADPH–cytochrome P-450 reductase (EC 1.6.2.4), and the free radical thus formed may subsequently transfer an electron to molecular oxygen thereby generating superoxide [1, 2]. Superoxide and its decomposition product hydrogen peroxide have also been generated in an NADH-dependent system containing submitochondrial particles isolated from bovine heart [3], and hydrogen peroxide was identified as a product formed during incubations of doxorubicin with human erythrocytes [4].

Enzymes for the detoxification of superoxide and hydrogen peroxide are ubiquitous in mammalian tissues. Superoxide dismutase (EC 1.15.1.1) catalyses the conversion of superoxide to hydrogen peroxide. The latter may be converted to water either by glutathione peroxidase (glutathione : hydrogen peroxide oxidoreductase, EC 1.11.1.9) using reduced glutathione (GSH)† as a source of reducing equivalents, or by catalase (EC 1.11.1.6). The reaction of glutathione peroxidase with hydrogen peroxide results in the oxidation of GSH to GSSG which is normally reduced back to GSH by glutathione reductase (NADPH : oxidized-glutathione oxidoreductase, EC 1.6.4.2) and NADPH.

Cardiac tissue is ill-equipped to metabolize reactive oxygen species. It contains much less catalase than the liver—about 2–4 per cent of the hepatic activity in rabbits and rats [3] and less than 1 per cent in mice [5]. Additionally, superoxide dismutase activity in mouse heart was shown to be about one-fourth that found in mouse liver [5]. Although glutathione peroxidase activity in heart is similar

* Supported, in part, by the American Cancer Society, Illinois Division Grant 80-46 to R. F. N. and by NIH Training Grant GM07263 to the Department of Pharmacology.

† Abbreviations: GSH, reduced glutathione; and GSSG, oxidized glutathione.