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Differential effects of cocaine and cocaethylene on intracellular Ca^{2+} and myocardial contraction in cardiac myocytes

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1 Isolated cardiac myocytes of the ferret were used to investigate the influence of cocaine and cocaethylene on the intracellular Ca^{2+} transient indicated by the indo-1 405/480 nm ratio signal, and peak cell shortening.

2 Both cocaine and cocaethylene produced significant decreases in peak intracellular Ca^{2+} and peak cell shortening in a dose-dependent manner. Of interest, (1) the minimally effective dose of cocaethylene was ten fold lower (10^{-8} M versus 10^{-7} M) than that of cocaine; (2) the log EC_{50} of cocaethylene was -5.99 ± 0.13 (1.0×10^{-6} M), which was about ten fold lower than that of cocaine (-5.02 ± 0.11, 9.6×10^{-6} M); and (3) 1×10^{-4} M cocaethylene decreased the contraction amplitude by $71 \pm 7\%$, while the same concentration of cocaine decreased the amplitude only by $55 \pm 5\%$, indicating that cocaethylene is more potent than cocaine.

3 The negative inotropic effects of either cocaine or cocaethylene could be overcome by noradrenaline ($\sim 5\,\mu$M) or calcium.

4 In contrast to cocaine, cocaethylene shifted the peak $[Ca^{2+}]_i$-peak shortening relationship downward, indicating that cocaethylene decreased myofilament Ca^{2+}-responsiveness.

5 These data indicate that both cocaine and cocaethylene act directly on cardiac myocytes to produce a negative inotropic effect that is due to decreased Ca^{2+} availability. In contrast to cocaine, cocaethylene produces more potent inhibition by an additional action to decrease myofilament Ca^{2+}-responsiveness.

Keywords: Cocaine, cocaethylene; myocyte; indo-1; intracellular Ca^{2+}; myofilament Ca^{2+}-responsiveness

Introduction

The acute toxic effects of cocaine on the cardiovascular system are widely recognized, and an increased incidence of cardiovascular morbidity and sudden cardiac death has been associated with cocaine abuse (Cregler & Herbert, 1986; Isner *et al.*, 1986; Tazelaar *et al.*, 1987; Karch & Billingham, 1988). Concurrent use of alcohol is common among cocaine abusers, as indicated by recent prevalence studies (Weiss *et al.*, 1988). It has been reported that more than 50% of cocaine abusers provide a history of lifetime alcohol abuse or dependence (Weiss *et al.*, 1988), and about 30% of cocaine users drink ethanol on almost every occasion of cocaine use (Jones, 1987). Co-abuse of these two drugs may be explained by the observation that ethanol can potentiate the stimulant effects of cocaine in some patients (Masur *et al.*, 1989). However, co-abuse of ethanol and cocaine increases the cardiovascular manifestation of cocaine, as suggested by a three to five fold increase in heart rate over that observed after administration of either drug alone (Foltin & Fischman, 1989). Moreover, an epidemiological study reported that concurrent use of ethanol produces an eighteen fold increase in the risk of cocaine-related sudden death (Rose *et al.*, 1990).

Cocaine is rapidly hydrolyzed by plasma and liver esterases to its metabolites (Stewart *et al.*, 1979; Wilkinson *et al.*, 1980; Barnett *et al.*, 1981). Some cocaine metabolites have pharmacological activity in animals. Cocaethylene, the ethyl homologue of cocaine, is a pharmacologically-active cocaine metabolite found in cocaine users who simultaneously consume cocaine and ethanol (Elsworth *et al.*, 1990; Rose *et al.*, 1990; Hearn *et al.*, 1991a). The transesterification of cocaine and ethanol to cocaethylene takes place in the liver and represents a novel metabolic reaction. Cocaethylene has been detected in postmortem blood, liver, and neurological tissues, in concentrations equal to and sometimes exceeding those of cocaine (Rafla *et al.*, 1979; Smith, 1984; Rose *et al.*, 1990; Hearn *et al.*, 1991a). Comparison of the LD_{50} in Swiss-Webster mice suggests that cocaethylene is more potent in mediating lethality than cocaine (Hearn *et al.*, 1991b). However, the direct effects of cocaethylene on mammalian cardiac myocytes have not yet been described.

The purpose of the present study was to compare the effects of cocaine and cocaethylene on excitation-contraction coupling in single myocytes loaded with the Ca^{2+}-indicator, indo-1.

Methods

Myocyte isolation

Single cardiac myocytes were isolated from the left ventricle of ferret hearts (Copelas *et al.*, 1987). Briefly, the left ventricle, which was separated from the atria and right ventricle, was sliced into 4×4 mm pieces, washed three times with calcium-free solution, and then digested by stirring in a beaker under an oxygen tension of 100 mmHg at 35°C. The composition of the dispersion solution was (mM): NaCl 75, KCl 2.4, $MgCl_2$ 1.0, HEPES 10, sucrose 58, dextrose 10, $NaHCO_3$ 5, L-glutamic acid 2.5, pH 6.96 with 160 u ml^{-1} collagenase II (Worthington Biochemical, Malvern, Pennsylvania, U.S.A.) and 0.6 mg ml^{-1} bovine serum albumin (Sigma Chemical Company, St. Louis, Missouri, U.S.A.). The solution was decanted and replaced four times at 20 min intervals. Myocytes were harvested by filtration through 230 μm nylon mesh, followed by low speed centrifugation, and were then re-suspended in Medium 199. The yield of rod-shaped myocytes (80%) in the cell suspension with clear cross-striations that excluded trypan blue was routinely $>90\%$.

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Intracellular Ca^{2+} measurement

For measurement of intracellular Ca^{2+}, myocytes were loaded with indo-1 by incubation with the acetoxymethyl ester of indo-1 (indo-1/AM) at room temperature. Briefly, 2 ml of cell suspension (approximately 10^6 myocytes ml^{-1}) were incubated with 0.1 ml of a 10 μM indo-1/AM stock solution consisting of 1.0 ml of 1 mM indo-1/AM in dimethylsulphoxide (Sigma Chemical Company) with 0.2 ml 25% (w.t/w.t) Pluronic F-127 (BASF, Wyandotte, Michigan, U.S.A.) in dimethylsulphoxide, and 8.8 ml foetal bovine serum (Hyclone Laboratories, Logan, Utah, U.S.A.) (Spurgeon *et al.*, 1990). The stock solution of indo-1/AM was 100 μM. The final concentration of indo-1/AM in the cell suspension was approximately 5 μM. The measurement of intracellular Ca^{2+} was performed with the fluorescent Ca^{2+} probe indo-1. A 75 watt continuous output xenon arc (Nikon) was used as a source of illumination for epi-fluorescence. An interferences filter was used to select a wavelength of 360 ± 5 nm (Nikon) to excite indo-1 and a 380 long-pass dichroic mirror (Omega) to direct the excitation light to a × 40, 1.3 NA u.v. objective (Nikon). After excitation at 360 nm, a 455 nm dichroic mirror split the fluorescence emission beam, and the emissions at 405 nm (the emission peak of the Ca^{2+}-bound form of the dye) and 480 nm (the emission peak of the Ca^{2+}-free form of the dye) was directed to two photomultiplier tubes (Nikon). Flourescent light was collected from the microscopic field, which contained a single myocyte. Photomultiplier outputs were passed as current to an analogue divider for instantaneous computation of the 405/580 nm ratio (Photoscan; Nikon). Fluorescence signals at 405 nm emission and 480 nm emission were stored in a personal computer (NEC, Tokyo, Japan) for data processing and analysis throughout each run, so that the time course of alteration in the Ca^{2+} transient could be followed. However, for optimal kinetic analysis, postprocessing of data was carried out. The fluorescent data collection rate for all experiments was 100 Hz. This was sufficient to resolve the rising and falling phases of the Ca^{2+} transient. The cytosolic Ca^{2+} transient was represented by the 405/480 nm ratio of the fluorescent signal.

Contractility measurement

The cell was illuminated with red (645 nm) light through the normal bright-field illumination optics of the microscope to simultaneously measure cell shortening and intracellular Ca^{2+}. Cardiac myocytes were superfused at 1 ml min^{-1} with medium containing (mM): NaCl 120, KCl 5.9, $MgCl_2$ 1.2, NaH_2PO_4 1.2, $NaHCO_3$ 25, dextrose 11.5, and $CaCl_2$ 2.5 at pH 7.4. The myocytes were maximally stimulated electrically with platinum field electrodes at 0.5 Hz pulses of 5 ms duration. All the experiments were performed at room temperature (23°C) in order to minimize compartmentalization of the Ca^{2+} probe indo-1/AM into intracellular organelles. However, it is important to note that similar results were obtained with 10^{-4} M cocaine at room temperature and at 37°C in the presence of 0.5 mM probenecid (Sigma). Changes in the contractile state of individual myocytes were assessed by the use of a phase-contrast microscope video motion detector system through measuring the amplitude of cell shortening. The cell contractility was expressed as a percentage of the baseline values.

Drugs

A stock solution of cocaine, 10^{-1} M (purchased from Mallinckrodt, St. Louis, Missouri, U.S.A.), was obtained from the Beth Israel Hospital pharmacy dissolved in a diluent containing 0.5% chlorobutanol. In the volumes added to the bath in these experiments, the diluent had no effect on the amplitude or time course of the intracellular Ca^{2+} transient or cell contraction. The metabolite of cocaine and ethanol, (−)-cocaethylene fumarate, was obtained from the National Institute of Drug Abuse, Research Technology Branch of the Division of Preclinical Research, dissolved in physiological salt solution. Solutions were freshly made just before the experiment.

Statistics

Data are expressed as means ± s.e.mean. Results were analyzed by paired Student's t test for data from the same myocytes, and unpaired t test for data from different myocytes. Statistical significance was set at $P<0.05$.

Results

Effects of cocaine on the amplitude of intracellular Ca^{2+} and contraction

Figure 1 shows representative tracings of the intracellular Ca^{2+} transient and cell shortening under the influence of different concentrations of cocaine. Cocaine decreased the amplitude of contraction (peak cell shortening), as well as peak intracellular Ca^{2+}, in a dose-dependent manner. Maximal rates of cell shortening and relaxation were also significantly reduced by cocaine in a dose-dependent manner (Table 1). These results suggest that cocaine inhibits myocardial contractility, presumably by reducing the amplitude of the intracellular Ca^{2+} transient. To test this hypothesis, myocytes were exposed to the Ca^{2+}-dependent inotropic drug noradrenaline (5 μM) during exposure to cocaine. Noradrenaline overcame the inhibitory effects of 1×10^{-4} M cocaine by increasing the peak intracellular Ca^{2+}, thereby enhancing the amplitude of contraction (Figure 2, right-hand panel), and indirectly supporting the hypothesis above.

Comparison of the effects of cocaine with those of cocaethylene

Like cocaine, cocaethylene produced a marked decrease in peak shortening that was associated with a similar decrease in peak intracellular Ca^{2+} (Figure 3a). The inhibitory action on cardiac myocytes of both cocaine and cocaethylene (data not shown) could be overcome by the use of either noradrenaline or calcium. Of interest, (1) the minimally effective dose of cocaethylene was ten fold less (10^{-8} M versus 10^{-7} M) than that of cocaine; (2) the log EC_{50} (Carpenter, 1986) of cocaethylene was -5.99 ± 0.13 (1.0×10^{-6} M), which was about ten fold lower than that of cocaine (-5.02 ± 0.11, 9.6×10^{-6} M) ($P<0.001$, Figure 3c), and (3) at the same concentration (1×10^{-4} M), cocaethylene produced more pronounced negative inotropic effects than cocaine (Figure 3b, and Table 1). Typical continuous recordings of cell shortening under the influence of cocaine and/or cocaethylene from the same myocyte (Figure 3b) clearly show that cocaethylene produces a much more pronounced negative inotropic effect than cocaine at equimolar concentrations, suggesting that cocaethylene is more potent than cocaine as a myocardial depressant. In Figure 3c, the slopes of the concentration-response curves are unusually shallow, and the explanations for this remain unknown.

Effects of cocaine and cocaethylene on myofilament Ca^{2+} responsiveness

Besides the amplitude of intracellular Ca^{2+}, the myofilament Ca^{2+}-responsiveness also contributes to myocardial contractility. Any agent that decreases the myofilament Ca^{2+}-responsiveness would reduce the amplitude of contraction. Therefore, the more pronounced negative inotropic effects of cocaethylene (Figure 3) may possibly be caused by cocaethylene-induced decrease of the myofilament Ca^{2+}-responsiveness. To test this hypothesis, peak $[Ca^{2+}]_i$-peak

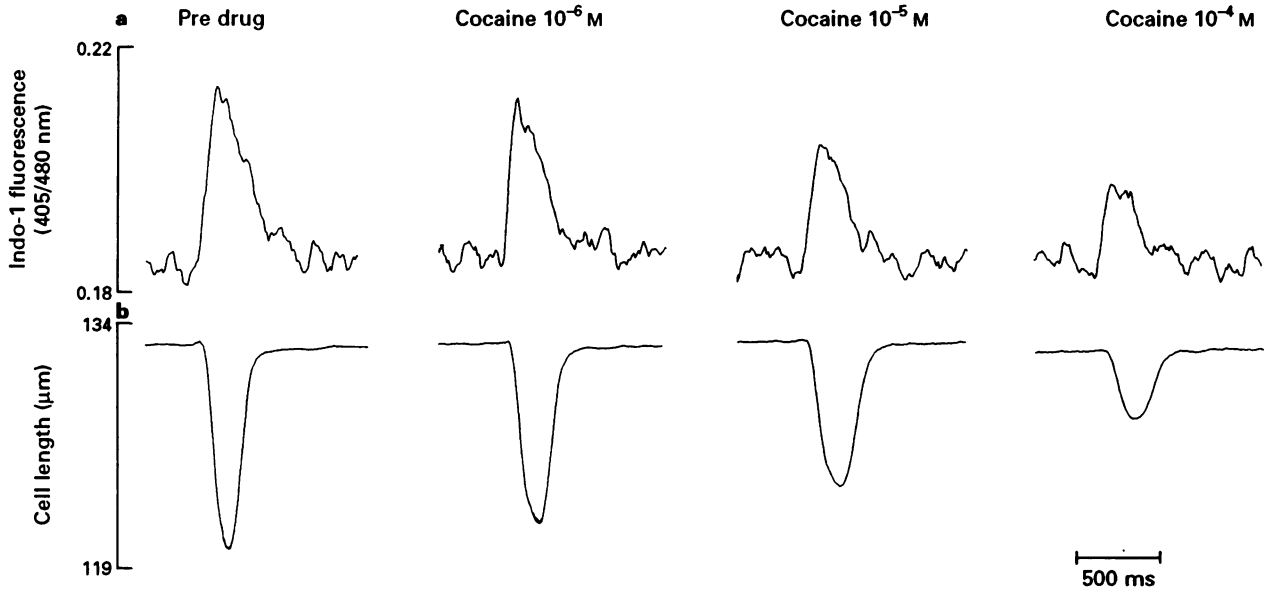

Figure 1 Tracings showing the effects of different concentrations of cocaine on the intracellular Ca^{2+} transient (a) and peak cell shortening (b). The concentrations were increased in a manner of accumulation. Tracings were from the same myocyte, and are representative of 12 similar experiments. Cell was stimulated at 0.5 Hz and at room temperature.

Table 1 Cocaine (Coc)- and cocaethylene (CEt)-induced changes in the intracellular Ca^{2+} transient and contraction (% of control) in single cardiac myocytes from the ferret

	T_{50}	S_{50}	*ROR*	*ROS*
Coc 10^{-6} M ($n = 17$)	90 ± 3	104 ± 3	78 ± 7	84 ± 8
CEt 10^{-6} M ($n = 7$)	93 ± 9	96 ± 3	72 ± 25	71 ± 4*
Coc 10^{-5} M ($n = 13$)	93 ± 4	106 ± 4	62 ± 9	72 ± 13
CEt 10^{-5} M ($n = 7$)	98 ± 8	99 ± 3	55 ± 4	54 ± 2*
Coc 10^{-4} M ($n = 13$)	101 ± 7	105 ± 4	39 ± 5	41 ± 6
CEt 10^{-4} M ($n = 7$)	106 ± 10	106 ± 6	30 ± 4*	27 ± 3*

Values are means ± s.e.mean. T_{50}, time from the beginning of the stimulation to 50% decline from peak intracellular Ca^{2+}; S_{50}, time from the beginning of the stimulation to 50% decline from peak shortening; ROR, maximum rate of relaxation of cell shortening; ROS, maximum rate of shortening of cell shortening.
*$P < 0.05$ compared to the corresponding effects of cocaine.

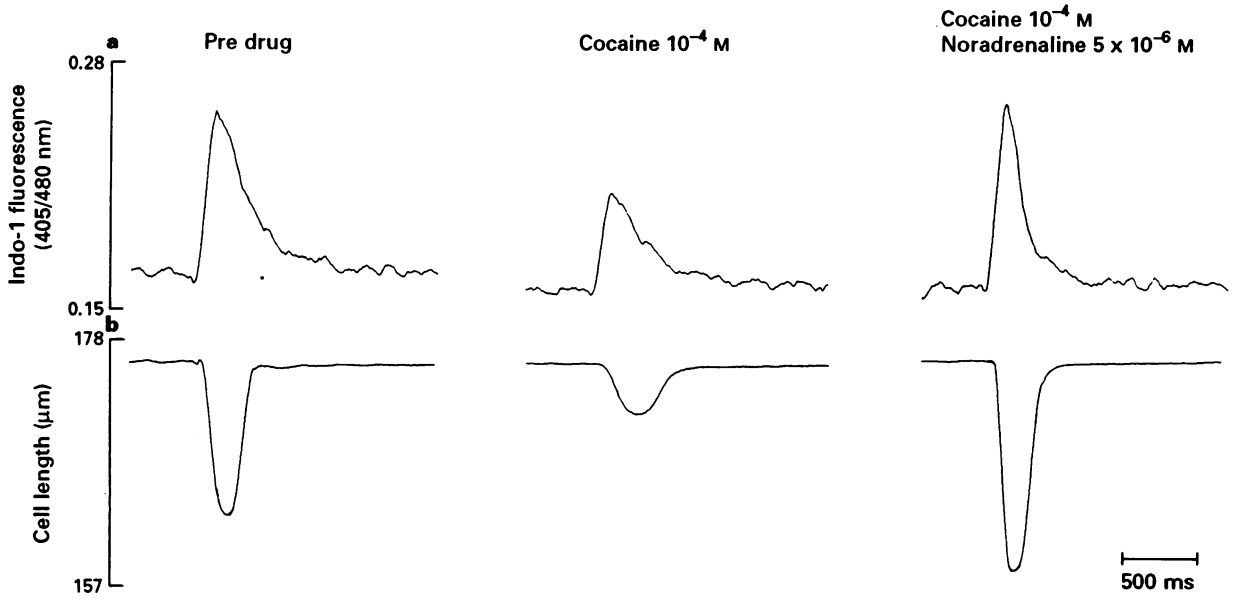

Figure 2 Tracings showing effect of noradrenaline on the intracellular Ca^{2+} transient (a) and cell peak shortening (b) in the presence of cocaine. Left panel, control; middle panel, in the presence of 1×10^{-4} M cocaine; right panel, in the presence of 1×10^{-4} M cocaine plus 5×10^{-6} M noradrenaline. Tracings are representative of five similar experiments. Cell was stimulated at 0.5 Hz and at room temperature.

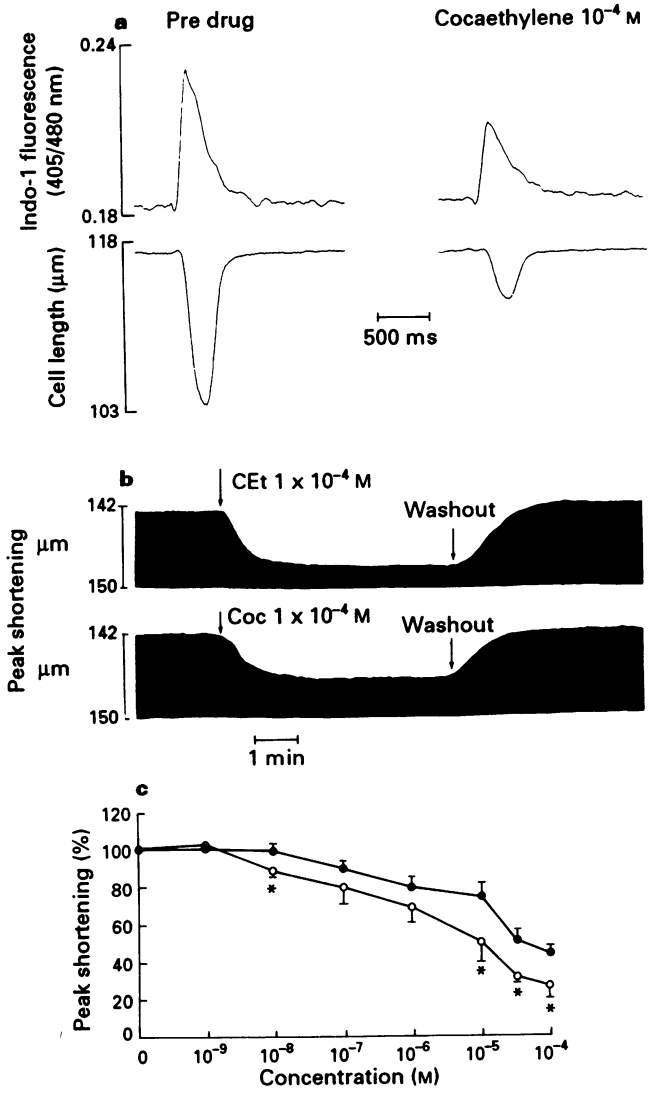

Figure 3 Effect of cocaethylene on the intracellular Ca^{2+} transient and cell contraction. (a) Effects on the intracellular Ca^{2+} transient and peak cell shortening (fron nine similar experiments); (b) comparisons of the effects of the same concentration of cocaine versus cocaethylene on peak cell shortening (data were from the same myocyte); (c) comparisons of the concentration-response curves of cocaine (●) versus cocaethylene (○) on the effect of peak cell shortening ($n = 6$).

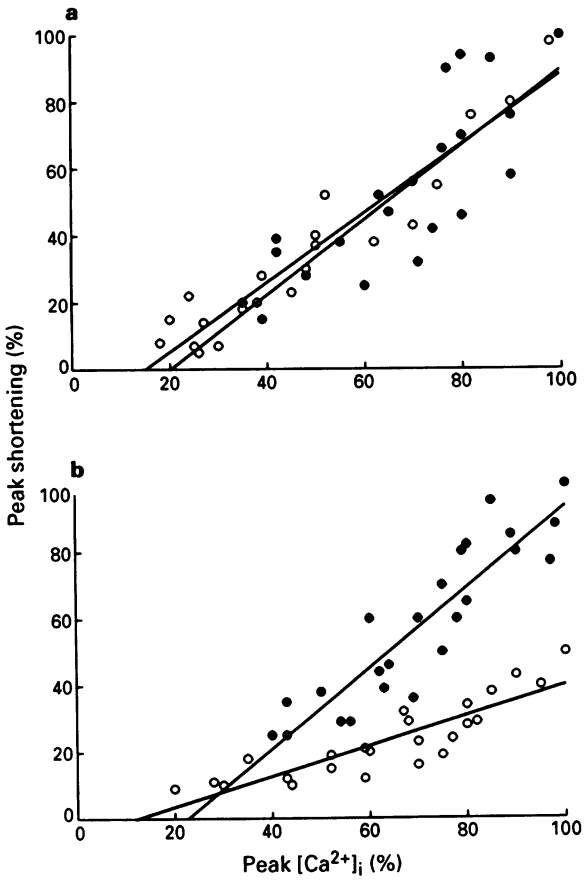

Figure 4 Comparisons of the effects of cocaine or cocaethylene on the peak $[Ca^{2+}]_i$-peak shortening relationship. (a) Effects of 1×10^{-4} M cocaine ($n = 7$); (b) effects of 1×10^{-4} M cocaethylene ($n = 8$). The maximal responses were considered as 100, and all other values were referred to 100. The changes of peak $[Ca^{2+}]_i$ were induced by increasing the $[Ca^{2+}]_o$. (●) Control values; (○) values in the presence of cocaine or cocaethylene.

shortening curves were determined first in the absence, then in the presence, of cocaine or cocaethylene. Peak $[Ca^{2+}]_i$ was increased by raising $[Ca^{2+}]_o$ from 2 to 5 and then to 8–10 mM. Cocaine did not shift the peak $[Ca^{2+}]_i$-peak shortening relationship (Figure 4a), whereas cocaethylene substantially shifted the peak $[Ca^{2+}]_i$-peak shortening relationship downwards (Figure 4b). These results suggest that, in contrast to cocaine, cocaethylene produced Ca^{2+}-desensitizing effects, which may, at least partially explain its more pronounced negative inotropic action.

Discussion

This is the first study comparing the cardiac effects of cocaine to those of its active metabolite, cocaethylene, at the cellular level. Our results indicate that the negative inotropic effect of cocaethylene is due to (1) a decrease of peak intracellular Ca^{2+} availability (Figure 3a), and (2) a decrease in myofilament Ca^{2+}-responsiveness (Figure 4b). In contrast, the negative inotropic effect of cocaine appears to be due to decreased intracellular $[Ca^{2+}]_i$ alone, with no obvious effect on myofilament Ca^{2+}-responsiveness.

The cellular mechanism of cocaethylene appears to reflect the myocardial effects of both cocaine and ethanol. Cocaine has two primary pharmacological actions on the cardiovascular system. First, it blocks the re-uptake of catecholamines at the presynaptic level in the central and peripheral nervous systems (Ritchie & Greene, 1985; Billman, 1990; Isner & Chokshi, 1991). Second, it reduces the inward Na^+ current by inhibiting the sodium channel in the sarcolemma, producing a local anaesthetic effect (Hale *et al.*, 1989; Przywara & Dambach, 1989; Billman, 1990). Reduction of the inward Na^+ current (Matthews & Collins, 1983; Reith *et al.*, 1986; Reiter, 1988) would reduce the subsequent inward Ca^{2+} current (Reiter, 1988; Stewart *et al.*, 1991). The negative inotropic effect of cocaine on cardiac muscle from a variety of different mammalian species appears to be predominantly produced by the local anaesthetic action. In addition, it is likely that cocaine may also directly inhibit the sarcolemmal voltage-sensitive Ca^{2+} channels (Stewart *et al.*, 1991). These actions would lead to the decreases of (1) trans-sarcolemmal Ca^{2+} entry, (2) Ca^{2+} loading of the sarcoplasmic reticulum, and (3) $[Ca^{2+}]_i$ available for activation of the contractile apparatus (Stewart *et al.*, 1991). These mechanisms would explain the results of the present study, since the dose-dependent decrease in contractile function produced by cocaine correlated with a progressive decrease in the amplitude of the $[Ca^{2+}]_i$ transients.

It has been established that ethanol depresses myocardial contractility in man and experimental animals, both *in situ* and in isolated heart muscle. Recent studies indicate that (1) the effects of ethanol in amounts routinely ingested by man, i.e., those leading to clinically relevant ethanol plasma concentrations (0.1–0.15%, vol/vol), produce a negative inotropic effect that is predominantly due to decreased myofilament Ca^{2+}-responsiveness (Schulman *et al.*, 1989; Danziger *et al.*, 1991). Higher concentrations (1–5%, vol/vol) depress both the amplitude of the Ca^{2+}-transient as well as alter myofilament Ca^{2+}-responsiveness (Katz, 1982; Polimeni *et al.*, 1983; Taraschi & Rubin 1985). Our results suggest that the negative inotropic effect of cocaethylene was due to both actions, in contrast to cocaine, which may explain its greater potency as a negative inotropic agent.

In addition to effects on intracellular Ca^{2+} availability and on myofilament Ca^{2+} responsiveness, drugs may produce negative inotropic effects through a direct action on sarcoplasmic reticular Ca^{2+} handling. Such an effect did not appear to be important with regard to the actions of either cocaine or cocaethylene in our experiments. All drugs studied to date that affect Ca^{2+} handling by the sarcoplasmic reticulum have been shown to alter the duration of the Ca^{2+} transient. As shown in Figures 1 and 3, and Table 1, neither drug affected the time course of the $[Ca^{2+}]_i$ transient; therefore, it does not appear likely that cocaine or cocaethylene directly affect sarcoplasmic reticular Ca^{2+} handling. However, both drugs may decrease Ca^{2+} loading of this organelle if the inward calcium current is inhibited.

Most of the previous studies of the effects of cocaine on cardiac function have been carried out *in vivo* (Hardman *et al.*, 1965; Levy & Blattberg, 1978) or with multicellular cardiac muscle (Chang & Lee, 1973; McCulloch *et al.*, 1974; Masuda & Levy, 1985) or Purkinje fibre preparations (Weidmann, 1955). The direct cardiovascular effects of cocaine are difficult to evaluate in these studies because the drug acts in part through its effects on catecholamine re-uptake and metabolism. The present study represents the first direct evidence that cocaine and cocaethylene can alter the Ca^{2+}-dependent steps of excitation-contraction coupling in adult mammalian cardiomyocytes. We previously reported that cocaine produced positive inotropic effects at concentrations of 10^{-6} to 10^{-5} M, and negative inotropic effects only at concentrations $\geq 1 \times 10^{-4}$ M in ferret papillary muscles with intact nerve endings (Perreault *et al.*, 1990). In the present study, we found negative inotropic actions on single myocytes over the entire effective dose-range, indicating that the direct action of cocaine on myocardial cells is to decrease contractility. The result that noradrenaline can reverse the negative inotropic effects of cocaine (Figure 2) and cocaethylene (data not shown) suggests that, in multicellular preparations, the negative inotropic effects of cocaine may be overcome by cocaine-induced release of catecholamines. Therefore, the local concentrations of cocaine and catecholamine determine whether the net inotropic effect is negative or positive. In the case of cocaethylene, a higher incidence of clinical cardiac toxicity might be expected due to its greater potency as a negative inotropic agent.

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