Br. J. Pharmac. (1982), **76**, 037–043

FURTHER ANALYSIS OF INHIBITORY EFFECTS OF PROPRANOLOL AND LOCAL ANAESTHETICS ON THE CALCIUM CURRENT IN *Helix* NEURONES

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1 The effects of propranolol and local anaesthetics on Ca^{2+} current (I_{Ca}), individually separated from other ionic currents, in *Helix* neurones were studied under voltage clamp, using a suction pipette technique.

2 Increases in external Ca^{2+} concentrations overcame the inhibitory action of propranolol on I_{Ca}. Double reciprocal plots for peak I_{Ca} versus external Ca^{2+} concentrations in the presence or absence of propranolol did not intersect at the ordinate.

3 Internal application of propranolol (10^{-4} M) inhibited I_{Ca} to about 40–60% of the control in a time-dependent manner.

4 Lignocaine and procaine at concentrations of 10^{-3}–10^{-2} M inhibited I_{Ca} without shifting the threshold in the I-V relationships. Internal application of lignocaine (10^{-3}–10^{-2} M) also inhibited I_{Ca}: the ratio of depression of the I_{Ca} was almost equivalent to that of the agent applied externally.

5 The results provide evidence that propranolol inhibits I_{Ca} in a noncompetitive manner with Ca^{2+} at the cell membrane, and suggest that the agents may occupy the receptor site in the Ca^{2+}-channel somewhere between the outer surface and inner phase of the membrane.

Introduction

In previous studies, we have shown that propranolol inhibits Ca^{2+}-current in the *Helix* neurone at relatively low concentrations (Akaike, Nishi & Oyama, 1981b) and produces relaxations of the mammalian coronary arterial strips contracted by excess K^+ in the external medium (Sakanashi & Nishi, 1981). These observations, together with results obtained from cardiac muscle by other investigators, suggest that propranolol and certain other β-adrenoceptor blocking agents (β-blockers) known to have 'local anaesthetic actions' or 'membrane actions' might impede the translocation or influx of Ca^{2+} in the excitable tissues from the external medium to the cell interior (Fleckenstein, 1964; Parmley & Braunwald, 1967; Hashimoto, Satoh & Imai, 1979). However, at present, the mode of action of propranolol and other β-blockers on Ca^{2+}-movements across the excitable cell membrane is uncertain. The present experiments were designed to analyse the mode of action of propranolol on Ca^{2+}-current (I_{Ca}) in the *Helix* neurone in more detail. We have also compared the effects of the β-blocker on I_{Ca} with those of local anaesthetic agents and organic Ca^{2+}-antagonists, the effects of which have been described in a previous paper (Akaike, Brown, Nishi & Tsuda, 1981a),

Methods

The experimental method was essentially similar to that previously described (Lee, Akaike & Brown, 1978; Akaike *et al.*, 1981a, b). In brief, experiments were performed on single neurones isolated from the circumoesophageal ganglia of *Helix aspersa*. The ganglion was removed and connective tissue was stripped off with fine forceps until clusters of neurones floated free in 'normal' snail Ringer. A part of an individual neurone (30–80 μm diameter) was aspirated under negative pressure of about −300 mmHg so as to occlude the 10–15 μm diameter tip of a suction pipette, and then the cell body was isolated from residual connective tissue and the axon. Internal perfusion was preceded by disruption of part of the neuronal membrane aspirated into the tip of the suction pipette.

The Ca^{2+} current (I_{Ca}) was separated after K^+ current (I_K) and Na^+ current (I_{Na}) were blocked by the substitution of $Tris^+$ for Na^+ and Cs^+ for K^+ in the internal and external solutions. The compositions of all test solutions are listed in Table 1.

Ionic currents were monitored on a storage oscilloscope (Tektronix, 5113), and simultaneously recorded on paper with a fibre optics oscilloscope (Medelec, MS6), or stored on an MF data recorder

0007-1188/82/050037-07 $01.00

Table 1 Ionic composition of snail Ringer solutions

	NaCl	Tris Cl	TEA Cl	KCl	CsCl	$CaCl_2$	$MgCl_2$	4AP	Glucose	pH
External solution										
Normal	85	5		5		10	15		5.5	7.4
I_{Ca}		35	50		5	10	15	5	5.5	7.4

	K aspartate	Cs aspartate	TEA-OH	EGTA acid	pH
Internal solution					
Normal	135			0.1	7.4
I_{Ca}		135	10	0.1	7.4

Tris: tris (hydroxymethyl) aminomethane. Internal solution was buffered by adding Trizma base. All values mM.

(Sony, PFM-15) or a digital tape recorder (Kennedy model 9700C). At steps from the usual holding potential (V_H) of -50 mV for I_{Ca} to $+100$ mV, the capacitative current, transient and leakage current associated with the separated I_{Ca} was subtracted by a signal averager (Medelec, DAV 62), using values obtained from equivalent hyperpolarizing voltage steps.

Drugs employed in the present experiments were: (±)-propranolol hydrochloride (ICI), lignocaine (lidocaine) hydrochloride (Merk) and procaine hydrochloride. They were directly dissolved in test solution just before use. All experiments were carried out at room temperatures of 20–25°C.

Results

After the Ca^{2+} current (I_{Ca}) was separated by blocking Na^+ current (I_{Na}) and K^+ current (I_K) by external and internal perfusion with test solutions described in Methods, depolarizing voltage steps of 10 mV from the holding potential (V_H) of -50 mV were applied. An inward current started to appear at depolarizing voltage steps of 10–20 mV from V_H of -50 mV, and its peak current continued to increase along with an increase in depolarizing voltage steps to a level of +20 to +25 mV of the membrane potential. At larger depolarizing voltages beyond this level, I_{Ca} started to decline (Akaike, Lee & Brown, 1978; Akaike *et al.*, 1981a, b). Actual current records were reversed at +80 ~ +100 mV, since non-specific outward current (I_{NS}) appeared at high voltages more than +50 mV and contaminated the true I_{Ca}. However, after correction of the currents for I_{NS}, a null potential rather than a reversal potential for I_{Ca} was usually observed and occurred at potentials between +100 ~ +160 mV (Nishi, Akaike, Oyama, Ito & Brown, 1982). In the present experiments correction for I_{NS} from actual records of currents evoked at voltages above +50 mV was not made and eliminated from the figure, since in previous experiments we have shown that propranolol depresses the I-V relationship for I_{Ca} without shifting the threshold and peak potentials of I_{Ca} along the voltage axis (Akaike *et al.*, 1981b).

Effects of various $[Ca^{2+}]_o$ upon actions of propranolol

In order to characterize the nature of the inhibitory action of propranolol on I_{Ca}, effects of various concentrations of $[Ca^{2+}]_o$ on the action of the agent were examined. In the present series of experiments, propranolol at a concentration of 10^{-5} M was used. $[Ca^{2+}]_o$ was varied between 2.5 and 20 mM. Measurements were first performed in 10 mM $[Ca^{2+}]_o$ for 10 min, and the preparation was superfused with a test solution containing 10 mM $[Ca^{2+}]_o$ and propranolol for 3 min. At the end of a 3 min period of superfusion, the peak I_{Ca} was recorded. Thereafter, the preparation was washed with a solution containing 10 mM Ca^{2+} for 10 min. After ensuring that there was no appreciable change in the peak I_{Ca} in the pre- and post-control periods, the solution was changed to one containing 20 mM Ca^{2+}. In this manner, the peak I_{Ca} was successively recorded in different concentrations of $[Ca^{2+}]_o$ in the presence or absence of propranolol. In each experiment, at least three to four different concentrations were tested on a preparation. Four satisfactory experiments were obtained from different preparations. In all cases the depressant effect of propranolol on the peak I_{Ca} was dependent on $[Ca^{2+}]_o$; the effect of propranolol in 10 mM $[Ca^{2+}]_o$ was partially reversed, but not completely overcome in 20 mM $[Ca^{2+}]_o$, while the depression became more prominent in 5 mM $[Ca^{2+}]_o$ than in 10 mM $[Ca^{2+}]_o$ (Figure 1a). The effects of various concentrations of $[Ca^{2+}]_o$ on the inhibitory action of propranolol on I_{Ca} was different from those observed in organic Ca^{2+}-antagonists (see Figure 6, Akaike *et al.*, 1981a). Figure 1b shows Lineweaver-Burk plots demonstrating the inhibition of I_{Ca} by propranolol, in which double reciprocal plots for peak I_{Ca} and external Ca^{2+} concentration in the presence or absence of propranolol intersected at different points on the ordinate scale. It is, therefore, reasonable to assume

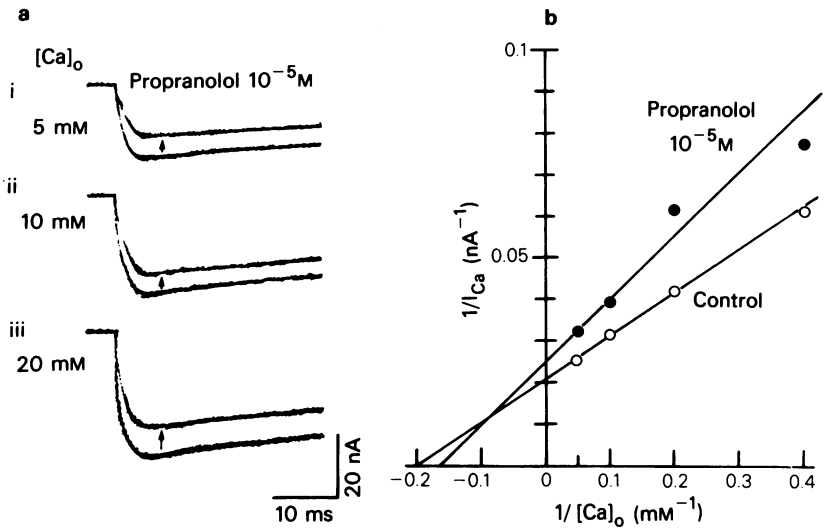

Figure 1 Effects of changes in concentrations of $[Ca^{2+}]_o$ on I_{Ca} in the presence and absence of propranolol 10^{-5} M. (a) Superimposed records of I_{Ca} elicited by a voltage step to +20 mV from the holding voltage of −50 mV before and 10 min after the drug application. The direction of the arrow indicates I_{Ca} after the drug application. (i) 5 mM; (ii) 10 mM; (iii) 20 mM $[Ca^{2+}]_o$. (b) Lineweaver-Burk plots for propranolol. Line obtained by varying $[Ca^{2+}]_o$ at a fixed concentration of propranolol (10^{-5} M). (○) Control; (●) propranolol 10^{-5} M. Straight lines were drawn by eye. Peak I_{Ca} elicited by a fixed voltage step to +20 mV from the holding potential of −50 mV, was measured.

that propranolol depresses I_{Ca} in a non-competitive manner in the snail neurone.

Internal application of propranolol

In a previous study we showed that intracellularly applied organic Ca^{2+}-antagonists depressed I_{Ca} in a dose- and time-dependent manner (Akaike *et al.*, 1981a). To compare the action of propranolol with that of the Ca^{2+}-antagonists, the agent was also applied intracellularly. Internal application of propranolol at concentrations lower than 10^{-6} M did not produce any appreciable changes in I_{Ca}. However, with increases in the dose of propranolol, depression of I_{Ca} was observed. Results are illustrated in Figure 2. Internal application of propranolol at a concentration of 10^{-4} M reduced peak I_{Ca} in a time-dependent manner. The ratio of depression varied with individual neurones, but within 15 min after the start of internal application, I_{Ca} was decreased to 40–60% of the control in 5 cases examined. Depression occurred over the entire voltage range and no shifts of the threshold and the voltage to induce maximum peak I_{Ca} were observed. The inhibitory effects of propranolol applied internally were partially but not completely reversible following a period of inhibition (20–30 min) after the start of perfusion with Cs-asparate solution.

Effects of local anaesthetics on I_{Ca}

Extensive studies have been made of the actions of local anaesthetics on sodium current (I_{Na}) in the excitable cell membrane, using giant axons and other nerve fibres (see Ritchie, 1979). However, the absolute specificity of several of these agents, especially at the high concentrations used, is uncertain. In fact, in 1967 Feinstein & Palmer showed that local anaesthetics might act directly on the movements of Ca^{2+} across the cell membrane of smooth muscle (Feinstein & Palmer, 1967). Therefore, effects of local anaesthetics on I_{Ca} were examined in the present experiments. Figure 3a shows I-V relationships for I_{Ca} measured in test solutions containing various concentrations of lignocaine. Lignocaine at concentrations lower than 10^{-5} M did not affect the I_{Ca}. At 10^{-4} M, the ratio of inhibition of I_{Ca} was about 15% of the control 5 min after exposure to the agent. The I_{Ca} was depressed in a dose- and time-dependent manner. Lignocaine did not alter the I-V relationship for I_{Ca}. Qualitatively similar results were obtained when procaine was applied at various concentrations: the threshold dose of procaine that depressed the I_{Ca} was 10^{-3} M. Dose-response curves for the depressant effects of these agents on the peak I_{Ca} are illustrated in Figure 3b.

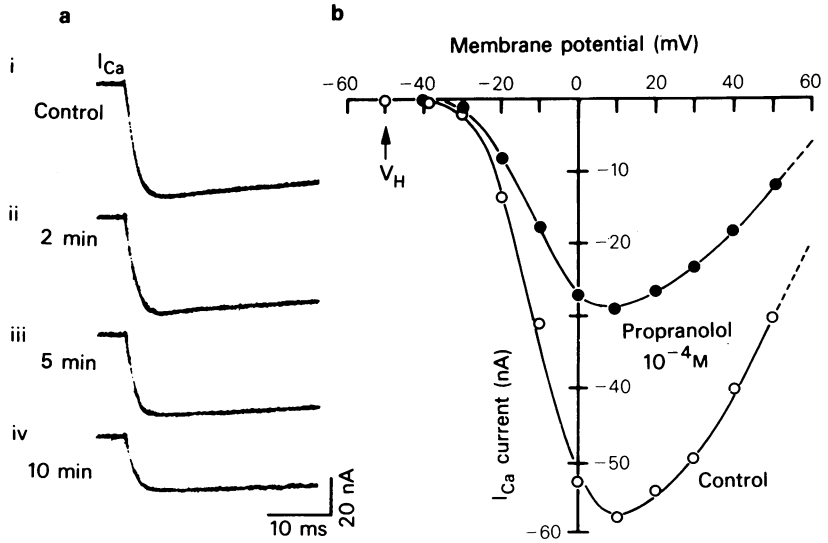

Figure 2 Effects of internal perfusion of propranolol at a constant external Ca^{2+} concentration (10 mM) on I_{Ca}. (a) Actual records of I_{Ca} elicited by a fixed voltage step to + 20 mV from the holding potential of − 50 mV. (i) Control; (ii) 2 min after the start of internal perfusion with solution containing propranolol 10^{-5} M; (iii) 5 min and (iv) 10 min. (b) Current-voltage relationship of I_{Ca}: (○) control; (●) 10 min after internal application of propranolol at 10^{-5} M. (a) and (b) are from different experiments.

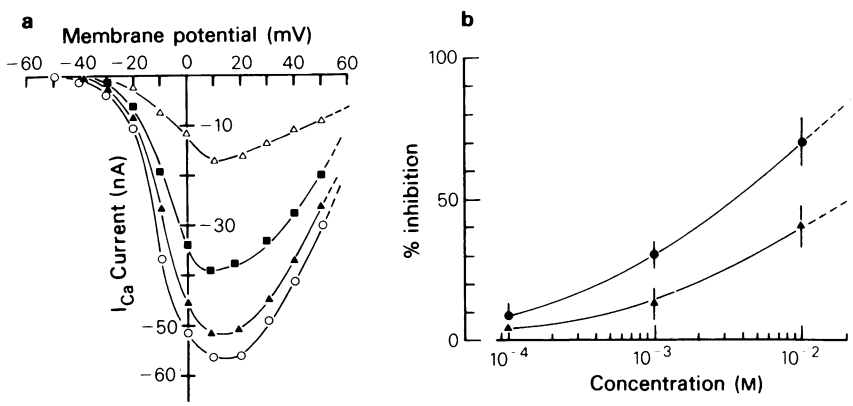

Figure 3 Effects of changes in concentrations of lignocaine on current-voltage relationship of I_{Ca}. (○) Control, (▲) 5 min after application of lignocaine at 10^{-4} M, (■) 10^{-3} M and (△) 10^{-2} M. (b) Dose-response curves for % inhibition of peak I_{Ca} after application of lignocaine (●) and procaine (▲). The % inhibition of the I_{Ca} at 5 min exposure is described in the text. Each point indicates the average value of 5–6 experiments, and vertical bars show s.e.mean.

Internal application

It has been suggested that there is a locus of action, namely a receptor site within the sodium channel, at which local anaesthetic molecules in their charged form act (Narahashi & Frazier, 1971; Ritchie, 1975; Hille, 1977), and thus internally applied lignocaine can block I_{Na}. There is the possibility that local anaesthetics could also act on the locus within the 'Ca^{2+} channel' in the same manner as in the case of Na^+ channel. To explore this possibility, lignocaine was applied intracellularly. External application of lignocaine at a concentration of 10^{-3} M which inhibited I_{Ca} by about 30% of the control, depressed the

I_{Ca} in a time-dependent manner. At the end of a 10 min-period of internal application, peak I_{Ca} was reduced to about 60% of the control. There was no appreciable change in the time course of slowly inactivating I_{Ca} (Figure 4). The effects were partially reversible after perfusing the preparation with Cs-aspartate internal solution. Thus, lignocaine applied internally inhibited I_{Ca} to an almost equal extent to lignocaine applied externally.

Discussion

In a previous study, we have shown that propranolol at a relatively low concentration inhibits the I_{Ca}, while the I_K is little affected (Akaike *et al.*, 1981b). The depressant action of propranolol resembled that of an organic Ca^{2+}-antagonist as follows: (1) propranolol inhibited the I_{Ca} dose-dependently over the entire range of the I-V relationship without shifting the threshold and peak potentials of the I-V relationship. (2) An increase in dose of these agents prolonged the time for I_{Ca} to reach its peak, and at the same time, slowed the time course of inactivation, and (3) internal application of propranolol depressed the I_{Ca} (Akaike *et al.*, 1981a). However, the Lineweaver-Burk plots for I_{Ca} and external Ca^{2+} concentration in the presence and absence of organic Ca^{2+}-antagonists intersected on the ordinate scale (Akaike *et al.*, 1981a), while with propranolol it was found that the respective Lineweaver-Burk plots *did not* intersect on the ordinate. This finding that the β-blocker inhibited the I_{Ca} in a non-competitive manner for Ca^{2+} on the membrane site, suggests that the site and mode of inhibitory action of the β-blocker on I_{Ca} is different from that of the organic Ca^{2+}-antagonists. We can speculate that the site of action of propranolol may be within or at the inner surface of the membrane rather than at the outer membrane surface. This idea is supported by observations that internal application of propranolol inhibited I_{Ca} relatively faster than the Ca^{2+}-antagonists did, and that the membrane sensitivity to internal application of the β-blocker was almost equivalent to that to external application. Furthermore, the depressant effects of propranolol on I_{Ca} increased progressively as the time of exposure to the agents was prolonged. In this respect, the mode of action of the β-blocker on I_{Ca} is similar to that of lignocaine, which is known to pass through the membrane from the intracellular space to the internal phase in the uncharged form and then act on the binding site for the local anaesthetics in Na^+-channel (Hille, 1977; and see Ritchie, 1979). Considering the relatively high lipophilicity of propranolol and lignocaine, the membrane may be permeable to these agents and thus the inhibition of I_{Ca} would result. Therefore, we propose, that there are at least 2 types of receptor sites for Ca^{2+}-blocking agents in Ca^{2+}-channel; one is located at the outer surface of the membrane as originally suggested by Hagiwara & Takahashi (Hagiwara & Takahashi, 1967; Akaike *et al.*, 1981a) and the other is within the Ca^{2+}-channel. The local anaesthetic molecule and propranolol would bind in the pore of Ca^{2+}-channel so as to promote I_{Ca}-inhibition. The molecule can reach the binding site with a hydrophobic component from the intracellular solution and from the membrane phase if it is sufficiently lipophilic. With this hypothesis, the apparent differences in the actions of organic Ca^{2+}-antagonists, inorganic Ca^{2+}-blocking substances, certain types of β-blockers and local anaesthetics are largely explained by analogies with the actions of various molecules of local anaesthetics in inactiving Na^+-

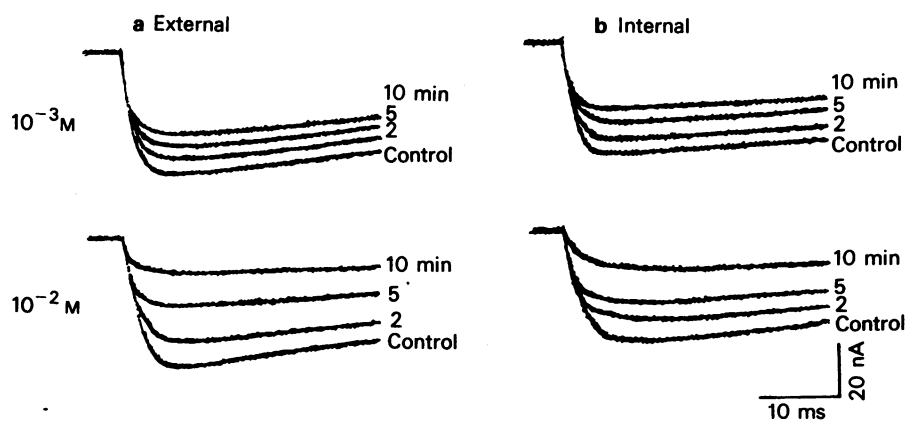

Figure 4 Effects of internal perfusion of lignocaine at 10^{-3} M and 10^{-2} M on I_{Ca}. Data obtained from 4 different experiments. (a) External application of lignocaine at 10^{-3} M and 10^{-2} M; (b) internal application of lignocaine at 10^{-3} M and 10^{-2} M. I_{Ca} was elicited by a voltage step to + 20 mV from the holding potential of − 50 mV.

channel (Ritchie, 1979). At the moment, however, further characterization of both Ca^{2+}-channels and effects of Ca^{2+}-blocking agents on the drug-receptor reaction in the Ca^{2+}-channel is required.

From previous studies, local anaesthetics have been shown to inhibit the early transient (I_{Na}) and late steady state (I_K) currents in the nerve membrane (Taylor, 1959; Shanes, Freygang, Grunfest & Amatniek, 1959; Blaustein & Goldman, 1967; Narahashi, Moore & Piston, 1969; Hille, 1977; see Ritchie, 1979). In addition to the inhibitory actions of local anaesthetics on both I_{Na} and I_K, the present results show that lignocaine and procaine also depress I_{Ca} in *Helix* neurones at concentrations required to block nerve conduction, and thus local anaesthetics possess another pharmacological action different from the known actions of the agents on the excitable membrane. In fact, some unpredictable effects of local anaesthetics reported on mammalian tissues might well be explained by their inhibitory action on I_{Ca}. Feinstein & Paimer (1967) reported that in the rabbit and guinea-pig taenia coli, curves describing the dose-response relationship to carbachol were shifted to the right in the presence of tetracaine, and other smooth muscles contacted by 5-hydroxytryptamine or noradrenaline were also antagonized non-competitively by the agent. These observations suggest that local anaesthetics may impede the influx of Ca^{2+} from the external medium to the internal phase of these tissues. Furthermore, in mammalian cardiac muscle, lignocaine is known to accelerate repolarization of ventricular and Purkinje fibres, an effect explained presumably by increasing outward K^+ current (Davis & Tempte 1969; Bigger & Mandel, 1970). However, there is the possibility that the marked shortening of action potentials might be in part due to the depressant action of lignocaine on the slow inward current mainly carried by Ca^{2+}, since it is known that verapamil and other organic Ca^{2+}-antagonists also depress the plateau phase of myocardial action potentials (Kohlhardt, Bauer, Krause & Fleckenstein, 1972; Kass & Tsien, 1975; Nakajima, Hoshiyama, Yamashita & Kiyomoto, 1975). The question whether lignocaine and other local anaesthetics cause any inhibitory actions on Ca^{2+} currents in mammalian smooth and cardiac muscles similar to those found in *Helix* neurones deserves further study.

The authors wish to thank Miss R. Kobayashi for preparing the manuscript. Propranolol was kindly supplied by ICI.

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(Received June 18, 1980.
Revised January 13, 1982.)