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Noradrenaline activates a calcium-activated chloride conductance and increases the voltage-dependent calcium current in cultured single cells of rat portal vein

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1 Membrane responses were recorded by a patch pipette technique in cultured cells isolated from rat portal vein. Using the whole-cell mode, pressure ejections of noradrenaline evoked depolarization (current clamp) and inward current (voltage clamp) at membrane potentials of −60 to −70 mV. The noradrenaline-induced response was reversibly blocked by prazosin indicating that the response was mediated by α_1-adrenoceptors.

2 The ionic mechanism of the noradrenaline-induced inward current was investigated in potassium-free caesium-containing solutions. Alteration of the chloride equilibrium potential produced similar changes in the reversal potential of the noradrenaline-induced current, indicating that noradrenaline opened chloride-selective channels. There was no evidence implicating sodium or calcium as the charge-carrying ion.

3 Caffeine applied in the bathing solution also induced a transient increase in chloride conductance but the noradrenaline-induced response was lost after application of caffeine. This is interpreted to mean that the increase in chloride conductance induced by noradrenaline and caffeine can occur as a consequence of a rise in intracellular calcium concentration depending on release of calcium from the same intracellular stores.

4 In the presence of caffeine, noradrenaline increased both the voltage-dependent calcium and chloride membrane conductances during application of repetitive depolarizing pulses. It is concluded that in isolated cells of the rat portal vein the depolarization in response to noradrenaline is mediated by an increase in chloride conductance depending on both the calcium release from intracellular stores and the increase of the voltage-dependent calcium current.

Introduction

Stimulation of α_1-adrenoceptors in portal vein smooth muscle produces contraction that can be dependent on an increase in both calcium influx through voltage-dependent channels and release of calcium from intracellular stores (Mironneau & Gargouil, 1979; Dacquet *et al.*, 1987; Pacaud *et al.*, 1987). However, there are recent results concerning the ionic mechanisms underlying the electrophysiological early response to noradrenaline in veins which indicate that the noradrenaline-induced depolarization is mainly mediated by an increase in membrane chloride conductance (Byrne & Large, 1988a, b; Van Helden, 1988). Two other ionic mechanisms involved in noradrenaline action have been described: an increase in a non specific cation channel (Byrne & Large, 1988b) and a decrease in potassium conductance (Suzuki, 1981; Van Helden, 1988). Furthermore, noradrenaline increases the cytoplasmic calcium concentration as measured with fluorescent indicators (Satoh *et al.*, 1987).

In smooth muscle cells isolated from rat portal vein, a chloride current activated by the inward calcium current can modulate the action potential duration (Pacaud *et al.*, 1989). In the present study we have investigated the ionic mechanisms underlying the depolarization to noradrenaline (Nanjo, 1984) in cultured single cells isolated from rat portal vein using whole-cell recordings with patch-pipettes.

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The major finding is that noradrenaline increases the membrane conductance to chloride which is mediated by a rise in intracellular concentration of calcium dependent on both a release of internal calcium and an increase in the amplitude of the voltage-dependent calcium current.

Methods

Single cell isolation and short-term primary culture

Portal veins from Wistar rats (150 g) were dissected free of connective tissue and single cells were obtained by a dispersal procedure similar to that described previously (Loirand *et al.*, 1986). The cells were plated on collagen-coated glass cover slips in medium M199 (Flow Laboratories, Puteaux, France) containing 10% foetal bovine serum, 2 mM glutamine, 20 u ml^{-1} penicillin and 20 $\mu g\,ml^{-1}$ streptomycin (Gibco, Paisley, UK) and kept in an incubator gassed with 95% O_2: 5% CO_2 at 37°C. The cells were transferred in a saline solution (reference solution) 15 min before the electrophysiological experiments.

Electrophysiological recordings in single cells

Isolated cells in short-term primary culture were fully viable for whole-cell recordings for up to 2 days. Cells attached on glass cover slips were fixed in a small experimental chamber which was mounted on the stage of an inverted microscope (Nikon Diaphot, Tokyo, Japan). Whole-cell membrane currents were measured by use of standard patch-clamp technique (Hamill *et al.*, 1981). Calcium current characteristics such as the potentials for threshold and maximal activation, the apparent reversal potential and the time to half-inactivation remained unchanged from 5 to 30 h of culture ($n = 10$). Patch pipettes had resistances of 2–5 MΩ and series resistances of 5–6 MΩ were measured. Thus, even at the maximal membrane current (300–600 pA), errors in potential measurement due to series resistance were within 5% and no correction was applied. Data were filtered at 1 kHz (−3 dB frequency) by a 8 pole Bessel low pass filter (Frequency Devices, Haverhill, USA) and analyzed with a Plessey 6220 microcomputer; illustrations were drawn with an X–Y plotter.

The extracellular medium (reference solution) contained (mM): NaCl 130, KCl 5.6, $CaCl_2$ 5, $MgCl_2$ 0.24, glucose 11, HEPES 8, pH 7.4 at 35 ± 1°C. The basic pipette solution contained (mM): KCl 130, HEPES 10, pH 7.3 with KOH. In order to block outward potassium currents, CsCl or NaCl were used instead of KCl in both external and pipette solutions and pH was adjusted with CsOH or NaOH, respectively. The following test solutions were used: barium solution, prepared by substituting $BaCl_2$ for $CaCl_2$; low chloride solution, prepared by substituting Na-aspartate for NaCl. In some experiments, 10 mM EGTA was added to the pipette solution in order to control the free calcium concentration.

Cobalt chloride was used as an inhibitor of calcium conductance. Chemicals used were: caffeine, noradrenaline, prazosin and propranolol. Noradrenaline (10^{-5} M) was applied from a glass pipette for 100–500 ms with a pressure ejector.

The values given in the text are the mean ± s.e. of mean with n the sample size.

Results

Responses to noradrenaline in potassium-containing solution

In isolated cells of the rat portal vein maintained in short-term primary culture, the resting membrane potential was usually between −60 and −70 mV when recorded with patch pipettes filled with KCl. At these holding potentials the common response to micro-ejections of noradrenaline (10^{-5} M) in a solution containing 2.5 mM cobalt was a transient depolarization (Figure 1a), the membrane potential reaching a value of -30.8 ± 3.4 mV ($n = 6$). In potassium-containing solution, noradrenaline-induced responses were observed in 90% of the cells ($n = 23$). In responding cells, the amplitude of the noradrenaline-induced depolarization was slightly reduced on repeated administration but the effect was small and accurate estimates of reversal potential were obtained readily (e.g. Figure 2a). At depolarized holding potentials (Figure 1a), the noradrenaline-induced response was composed of an early hyperpolarizing component followed by a reduced depolarization. At holding potentials between −10 and 0 mV, the depolarization progressively decreased whereas the amplitude of the hyperpolarization increased. In the absence of external cobalt, action potentials were generally superimposed on the noradrenaline-induced depolarization (Figure 1c). In the same cell the noradrenaline-induced currents were recorded under voltage-clamp conditions (Figure 1b). Two components of currents were clearly identified the amplitude and direction of which were dependent on the holding potential. The time course and duration of both the membrane potential variations and the underlying membrane currents were similar when recorded from the same cell. The action of noradrenaline was blocked in the presence of prazosin (10^{-6} M) for 5 min while it remained unchanged in the presence of propranolol

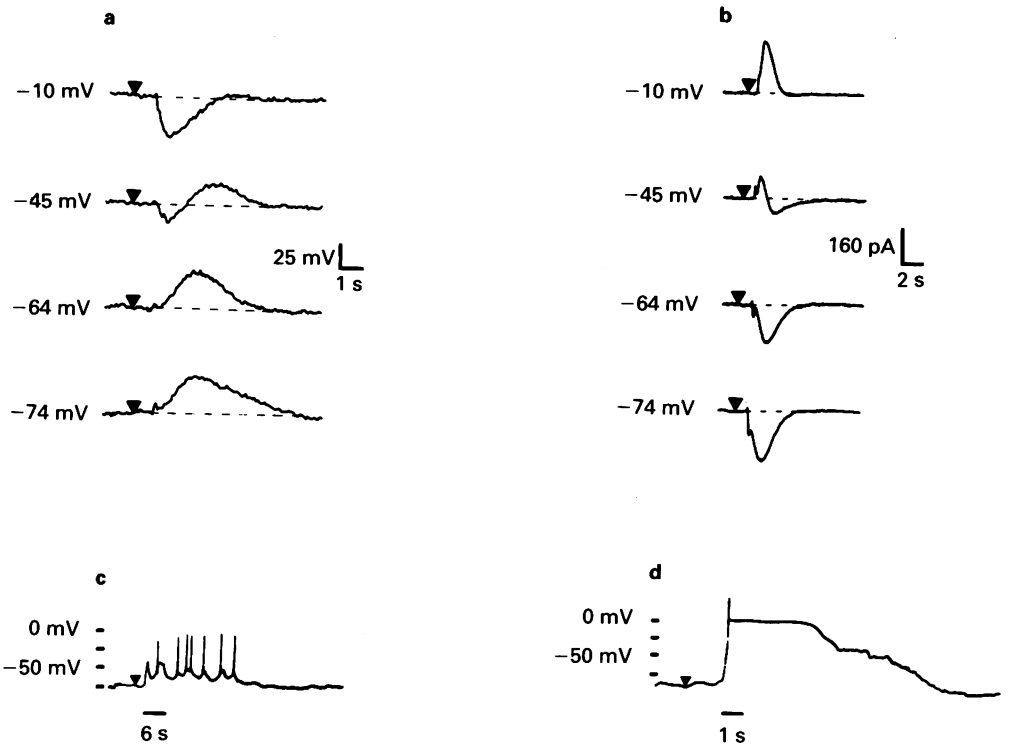

Figure 1 Membrane responses to noradrenaline (10^{-5} M) in isolated cells of rat portal vein in primary culture. (a) Depolarizations and hyperpolarizations to micro-ejections of noradrenaline (▼) recorded using current-clamp at different holding potentials in potassium-containing solution. (b) Membrane currents to noradrenaline recorded using voltage-clamp at different holding potentials in potassium-containing solution. (c) Burst of action potentials superimposed on the noradrenaline-induced depolarization in potassium-containing solution. (d) In potassium-free caesium-containing solution, noradrenaline induced a membrane depolarization to 0 mV which was maintained for 2–4 s. Noradrenaline (▼) was ejected for 100–500 ms at a distance of about 100 μm from the cell. The value of the holding potential (mV) is noted near the baseline.

(10^{-6} M). After washing out the α_1-adrenoceptor antagonist the noradrenaline-evoked current was similar to control. These results suggest that the responses to noradrenaline are mediated by α_1-adrenoceptors.

As hyperpolarization and outward membrane current induced by noradrenaline were abolished in potassium-free caesium-containing solution (Figure 2) it would seem that noradrenaline can increase the potassium conductance in addition to the membrane mechanism responsible for depolarization and inward current. Consequently, experiments to identify the ions responsible for the inward current were carried out in potassium-free caesium-containing bathing and pipette solutions. Under these experimental conditions, noradrenaline induced a membrane depolarization to 0 mV which was maintained for 2–4 s before repolarization occurred (Figure 1d).

Ionic basis of the noradrenaline-induced inward current

In order to determine the ionic basis of the noradrenaline-induced inward current, changes in the reversal potential of the current were studied with various ions in the bathing and pipette solutions. The reversal potential was estimated by measuring the responses to noradrenaline at different holding potentials. In caesium-containing solution (Figure 2a), the noradrenaline-evoked current decreased in amplitude with depolarization, was null at 0 mV and reversed at positive membrane potentials. There was a delay between application of noradrenaline and the onset of the response. The mean latency of the first noradrenaline-induced current was 980 ± 260 ms ($n = 27$) and the duration of the response was 1.7 ± 0.7 s ($n = 27$).

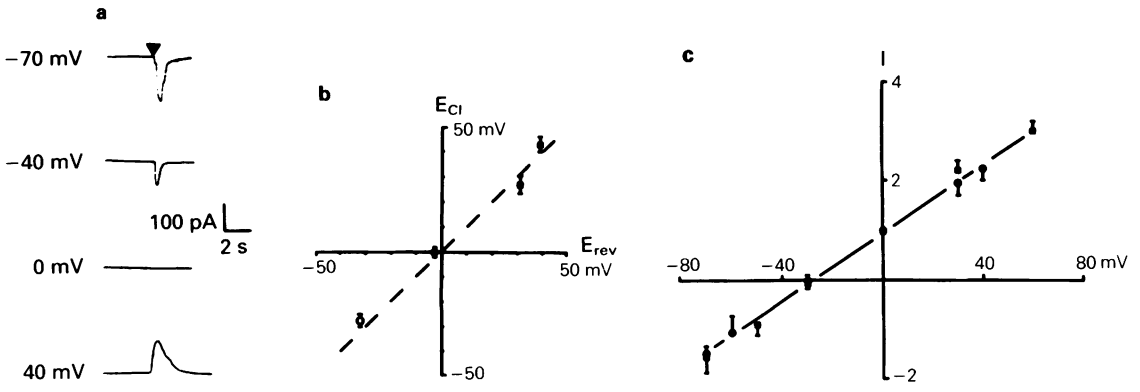

Figure 2 Effects of ionic substitution on the noradrenaline-induced current in caesium-containing solution. (a) Membrane current responses to ejections of noradrenaline (▼) at different holding potentials (noted near the current traces). The external chloride concentration was 146 mM, and the pipette concentration was 130 mM. (b) Reversal potential (E_{rev}) of the noradrenaline-induced current as a function of the equilibrium potential (E_{Cl}), for reference chloride (146 mM : 130 mM) gradient (●), low external chloride (■) and low pipette chloride concentrations (○). Dashed line predicts the reversal potential for a pure chloride conductance. Each point represents the mean of 3–15 cells; vertical lines show s.e.mean. (c) Current-voltage relationship of the noradrenaline-induced currents in caesium-containing solutions (■) and in sodium-containing solutions (●) with similar chloride (146 mM : 40 mM) gradients (substituted with aspartate). In caesium-containing solutions, the external Cs and Na concentrations were 5.6 and 130 mM, respectively. In sodium-containing solutions, the external and internal Na concentrations were 130 mM. Each point represents the mean of 7–10 cells; vertical lines show s.e.mean.

As the relationship between the amplitude of the noradrenaline-induced current and clamp potential was linear (Figure 2c), the reversal potentials were determined when the chloride electrochemical gradient was altered by replacing chloride with aspartate. Figure 2b illustrates the plot of the mean reversal potential against the calculated equilibrium potential for chloride ions. The experimental points were closely distributed along a straight line with a slope of 1, as expected for a pure chloride conductance. Figure 2c shows experiments in which the reversal potentials were determined in caesium-containing solutions (■, -27.5 ± 2.3 mV, $n = 7$) and in sodium-containing solutions (●, -29.2 ± 3.5 mV, $n = 10$). These values are close to the calculated E_{Cl} (−32 mV) and far from E_{Na} (respectively 0 and +67 mV). The absence of change in reversal potential of the noradrenaline-induced current produced by replacing caesium by sodium ions with similar anion gradients suggests that noradrenaline does not open a cation channel in smooth muscle cells isolated from rat portal vein.

It has to be noted that noradrenaline-induced currents were obtained in 45% of the cells in caesium-containing solution ($n = 16$). The percentage of responding cells was reduced to 36% in sodium-containing solution ($n = 11$) although there was no apparent difference in the response to noradrenaline in both cases.

Calcium-dependence of the noradrenaline-induced inward current

The role of calcium ions was examined first by comparing the noradrenaline-induced responses in barium and calcium solutions. As shown in Figure 3a, no responses were observed when the cells were perfused for 20 min in 5 mM barium solution. After replacement of barium with calcium for 5 min, inward currents could be evoked by noradrenaline applications. When the pipette solution contained 10 mM EGTA, the responses to noradrenaline were never observed even in the presence of external calcium (Figure 3b). In contrast, the noradrenaline-induced current was little altered (if at all) in the presence of 3 mM cobalt applied for 10 min. These results suggest that the noradrenaline-induced depolarization was not carried by calcium ions flowing through voltage-dependent calcium channels.

It is well known that caffeine displaces calcium from intracellular stores in portal vein smooth muscle (Nanjo, 1984) and we studied the effects of bath-applied caffeine in caesium-containing solution. In an experiment where the external chloride concentration was 130 mM, caffeine (10 mM) evoked a large inward current when the cell was held at −70 mV (Figure 4a). The caffeine-induced current was null near 0 mV and became outward at positive holding potential (Figure 4b). When the extracellular

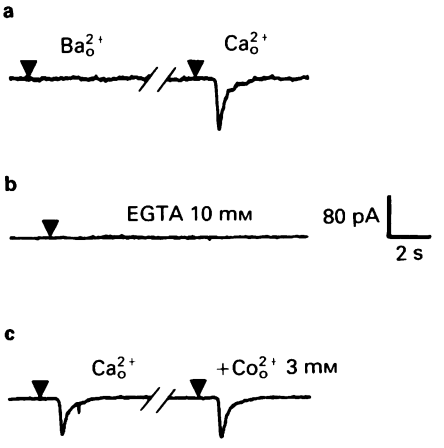

Figure 3 Calcium-dependence of the noradrenaline-induced current in caesium-containing solutions. (a) In 5 mM external barium solution for 20 min, no responses were observed to noradrenaline applications (▼). After reintroduction of 5 mM external Ca for 5 min, an inward current was recorded. (b) When the pipette solution contained 10 mM EGTA, no responses were induced by noradrenaline. (c) In the presence of 3 mM external cobalt for 10 min, the noradrenaline-induced current was unchanged.

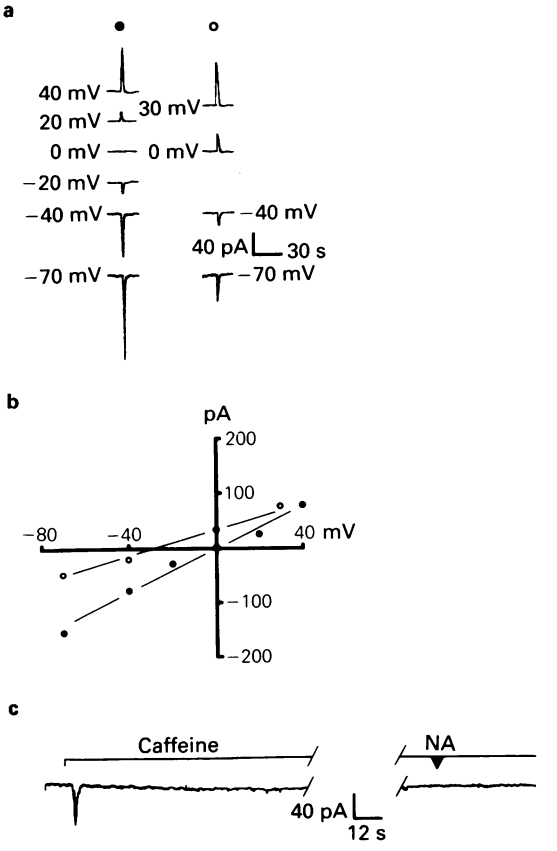

Figure 4 Membrane currents in response to bath-applied caffeine (10 mM) in caesium-containing solutions. (a) The external and pipette chloride concentrations were 146 mM and 130 mM (●) or 40 mM and 130 mM (○), respectively (substituted with aspartate). The value of the holding potential (mV) is noted near the current baseline. (b) Current-voltage relationships of the caffeine-induced current against the membrane potential in conditions as described in (a). (c) In the presence of caffeine (10 mM), there was no detectable action of noradrenaline (NA) (▼).

chloride concentration was reduced to 40 mM (substituted with aspartate), the caffeine-induced current reversed at more negative membrane potentials (-29.5 ± 2.5 mV, $n = 5$, Figure 4a–b). This value is close to the calculated E_{Cl} (−32 mV). As the reversal potential of the caffeine-induced current was similar to that found for noradrenaline with the same chloride concentrations, it appears that caffeine can induce a chloride conductance increase similar to that evoked by noradrenaline. In order to determine whether noradrenaline and caffeine displaced calcium from the same intracellular stores, microejections of noradrenaline were applied in the presence of 10 mM caffeine for 4 min. As shown in Figure 4c, we observed no detectable action of noradrenaline under these conditions. This observation favours the idea that the chloride conductance is activated by intracellular calcium release and that both noradrenaline and caffeine act on the same intracellular calcium stores.

Chloride conductance is activated by calcium release and calcium influx

It has been previously shown that noradrenaline increases the fast calcium current in rat portal vein smooth muscle cells (Pacaud *et al.*, 1987). To verify that the increased calcium influx could also activate the chloride conductance, we examined the change in membrane currents with time after pressure ejection of noradrenaline (Figure 5). In potassium-free caesium-containing bathing and pipette solutions, the current measured at the end of 150 ms depolarizing pulses was carried by chloride ions. Upon returning to −70 mV the chloride current was seen as a large inward tail current (Pacaud *et al.*, 1989). After stabilization of the membrane current in control for 10 min noradrenaline (10^{-5} M) was ejected in the vicinity of the cell. After the development of the noradrenaline-induced inward current (Figure 5a), repetitive depolarizing pulses from

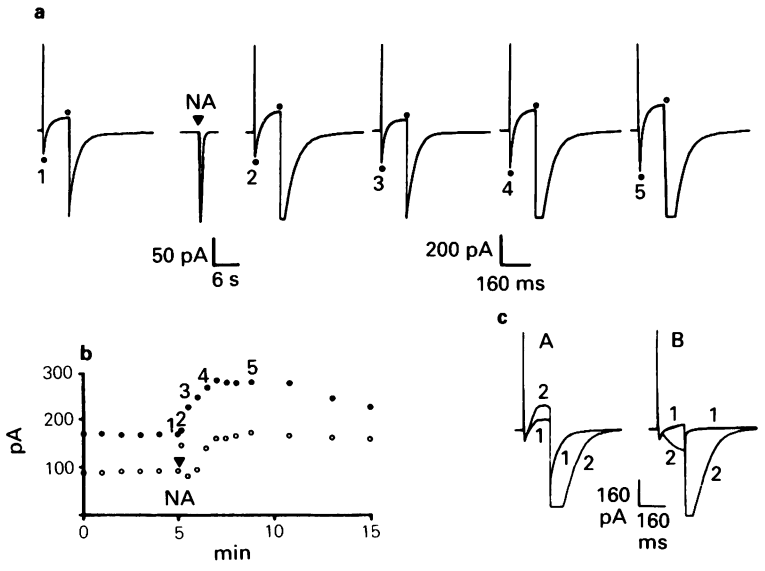

Figure 5 Change in membrane currents with time after pressure ejection of noradrenaline in caesium-containing solutions. (a) Currents were elicited from a holding potential of −70 mV to +10 mV in reference solution for 10 min (1). Ejection of noradrenaline (NA) (▼) produced the development of a transient inward current. Membrane currents in response to depolarizing pulses were recorded after 15 s (2), 30 s (3), 90 s (4) and 4 min (5). (b) Time course of the outward current measured at the end of the pulse (○) and the peak calcium current (●) before and after application of noradrenaline. (c) Superimposition of membrane currents elicited from a holding potential of −70 mV to +10 mV in the absence (1) and presence of noradrenaline (2). The external and pipette chloride concentrations were 146 mM and 130 mM (A) and 36 mM and 130 mM (B), respectively (substituted with aspartate). In potassium-free caesium-containing bathing and pipette solutions, the current measured at the end of the pulse is carried by chloride ions. Upon returning to −70 mV the chloride current is seen as a large inward tail current (Pacaud *et al.*, 1989).

−70 mV to +10 mV were applied. Figure 5b illustrates the typical time course of both the outward current measured at the end of the pulse (○) and the peak calcium current (●). About 15 s after noradrenaline ejection, both the outward current measured at the end of the pulse and the inward tail current upon returning to −70 mV were strongly increased (Figure 5a). After 30 s, the amplitude of the calcium current began to increase and finally, after 4–5 min, it reached an almost double stable amplitude (Figure 5b). It is obvious that the amplitude of the outward current measured at the end of the pulse increased with the same time course as that of the calcium current. The stimulation of the currents was not maintained. Obviously, 10 min after application of noradrenaline the calcium current remained increased to about 35% of control. At this time, the outward current measured at the end of the pulse remained almost at its maximal value. Changes in membrane currents obtained in control and after ejection of noradrenaline are illustrated in Figure 5a. The action of noradrenaline was completely blocked in the presence of 10^{-6} M prasozin. In order to verify that the chloride current did not overlap with the inward calcium current, an experiment was done with two different external chloride concentrations (146 and 36 mM). As shown in Figure 5c, the (outward or inward) noradrenaline-activated chloride current developed with a sufficient delay so that it did not interfere with the peak calcium current.

Activation of chloride conductance by internal calcium release and calcium influx can be separated by using successive applications of caffeine and noradrenaline (Figure 6). During bath application of caffeine (10 mM) a transient inward current was first recorded (Figure 4c) and then repetitive depolarizing pulses were applied as in Figure 5b to follow the time course of both the outward current measured at the end of the pulse (○) and the peak calcium current (●, Figure 6b). About 30 s after perfusion with caffeine, both the outward current measured at the end of the pulse and the tail inward current were strongly increased (Figure 6a) but the calcium current remained unchanged. In the presence of caffeine, pressure ejection of noradrenaline induced a strong increase in the amplitude of the calcium

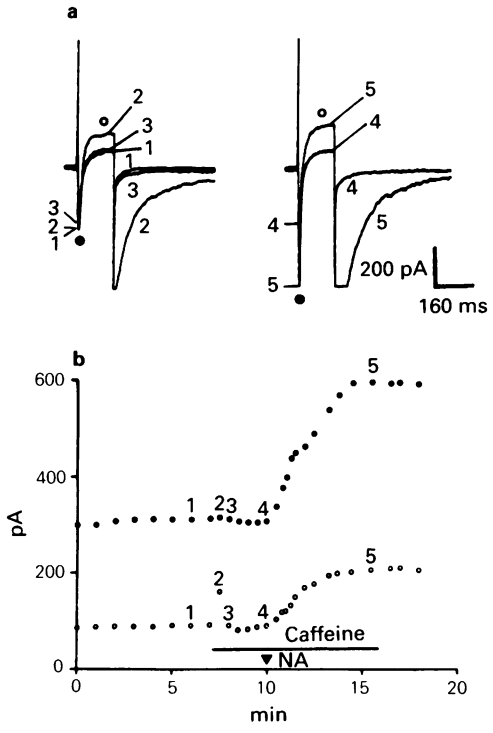

Figure 6 Changes in membrane currents with time in the presence of caffeine and noradrenaline in caesium-containing solutions. (a) Currents were elicited from a holding potential of −70 mV to +10 mV in reference solution (1), and during application of caffeine for 30 s (2), 1 min (3) and 3 min (4). Ejection of noradrenaline stimulated both the peak calcium current, the current measured at the end of the pulse and the tail current upon returning to −70 mV (5). (b) Time course of the outward current measured at the end of the pulse (○) and the peak calcium current (●) before and during application of caffeine and noradrenaline (NA) (▼). In potassium-free caesium-containing bathing and pipette solutions, the current measured at the end of the pulse is carried by chloride ions. Upon returning to −70 mV the chloride current is seen as a large inward tail current (Pacaud *et al.*, 1989).

current. Consequently, the amplitude of the outward current measured at the end of the pulse increased with the same time course as that of the calcium current. Changes in membrane currents obtained in control and after applications of caffeine and noradrenaline are illustrated in Figure 6a.

Discussion

The results from the present study provide evidence that in cultured single cells of the rat portal vein, noradrenaline activates two separate membrane conductance mechanisms. First, noradrenaline produces an increase in chloride conductance which leads to membrane depolarization and inward current. The reversal potential of the noradrenaline-induced inward current changes with external or internal chloride concentration as predicted by the Nernst relation. These results are in good agreement with those previously observed in short segments of guinea-pig mesenteric vein (Van Helden, 1988) and in isolated cells of rat anococcygeus muscle (Byrne & Large, 1987) and rabbit portal vein muscle (Byrne & Large, 1988b). However, the reversal potential of the noradrenaline-induced current does not change when different cations with similar anion gradients are used, suggesting that in rat portal vein noradrenaline does not open some non specific cation channels as shown in rabbit portal vein (Byrne & Large, 1988b). A mechanism involving only an increase in chloride conductance is consistent with ion flux studies which indicate that only a noradrenaline-induced efflux of chloride is recorded in muscle of rat portal vein (Wahlström, 1973). The finding that the noradrenaline-induced responses are maintained in the presence of external cobalt but can not be recorded in barium-containing solution or after addition of 10 mM EGTA in the pipette solution suggests that these responses can be mediated by intracellular calcium release activated by receptor stimulation. This is supported by a characteristic latency of about 1 s between the pressure ejection of noradrenaline and the onset of response. This long delay is not seen when external ATP is applied to isolated smooth muscle cells with the same pressure ejecter (P. Pacaud, G. Loirand, C. Mironneau and J. Mironneau, unpublished results). It is thought that the latency represents the time taken for intracellular mediators to link α-receptors binding to membrane channel opening. The involvement of internal calcium release is consistent with the observation that caffeine stimulates the chloride conductance in a manner similar to noradrenaline. A chloride conductance activated by the inward calcium current has also been observed in the smooth muscle cells of the rat portal vein (Pacaud *et al.*, 1989). The chloride current can be measured as either an outward current at the end of 150 ms depolarizing pulses or an inward tail current upon returning to −70 mV. The increase in chloride conductance produced by noradrenaline does not result from specific activation of a specialized subset of chloride channels as (i) the noradrenaline-induced response is lost after application of caffeine, and (ii) the increase in both depolarization-induced calcium and chloride currents during depolarizing pulses in response to noradrenaline is maintained in the presence of caffeine. Our results indicate that the chloride channels acti-

vated by noradrenaline belong to the general class of calcium-activated chloride channels.

Secondly, noradrenaline appears to open channels selective for potassium. Removal of potassium from the bathing and pipette solutions suppresses the noradrenaline-induced hyperpolarization and outward current. It is noted that, at the resting membrane potential, the increase in potassium conductance produced by noradrenaline is overwhelmed by the chloride inward current. From the present experiments it is not clear whether the outward current produced by noradrenaline is related to the opening of receptor-operated potassium channels or calcium-activated potassium channels.

After ejection of noradrenaline there is a discrepancy between the brief time course of chloride conductance activation (1–2 s) and the delayed increase in calcium current amplitude (5–10 min). As a similar observation is not seen during caffeine application, a possible membrane effect related to the increase in cytoplasmic calcium concentration may be discounted. A likely possibility is that the phospholipase C-protein kinase C pathway may be involved in the generation of the effects of noradrenaline on the calcium channels. More detailed experiments are needed to confirm this hypothesis.

In conclusion, both chloride and potassium channels in smooth muscle cells from rat portal vein are opened by noradrenaline in physiological conditions. This would result in the generation of an inward depolarizing current allowing repetitive electrical activity and contraction to occur.

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