

# Internal perfusion of guinea-pig hepatocytes with buffered $\text{Ca}^{2+}$ or inositol 1,4,5-trisphosphate mimics noradrenaline activation of $\text{K}^+$ and $\text{Cl}^-$ conductances

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Received 27 February 1987; revised version received 21 April 1987

External application of noradrenaline to voltage-clamped guinea-pig isolated hepatocytes evoked membrane conductance increases to  $\text{K}^+$  and  $\text{Cl}^-$ . This effect was reproduced by internal perfusion of the cells with 2  $\mu\text{M}$  buffered  $\text{Ca}^{2+}$  and with 20  $\mu\text{M}$  inositol 1,4,5-trisphosphate ( $\text{IP}_3$ ). The kinetic properties of the  $\text{K}^+$  conductance and its selective block by the toxin apamin were the same in each case. Cyclical fluctuations of conductance observed with noradrenaline were reproduced by internal  $\text{IP}_3$  but not by  $\text{Ca}^{2+}$  perfusion, indicating that oscillations of intracellular free  $\text{Ca}^{2+}$  may arise from properties of the  $\text{Ca}^{2+}$  sequestration mechanism at constant  $\text{IP}_3$  concentration.

Hepatocyte; Noradrenaline; Apamin;  $\text{Ca}^{2+}$  activation;  $\text{K}^+$  conductance;  $\text{Cl}^-$  conductance; Inositol 1,4,5-trisphosphate

## 1. INTRODUCTION

Noradrenaline (NA) acting through  $\alpha$ -adrenoceptors produces a large increase in the membrane permeability of guinea-pig hepatocytes to  $\text{K}^+$  and  $\text{Cl}^-$  [1-3]. This is thought to result from activation of  $\text{K}^+$  and  $\text{Cl}^-$  conductances by the rise of cytoplasmic free  $\text{Ca}^{2+}$  concentration which  $\alpha$ -adrenoceptor activation causes [4]. The source of  $\text{Ca}^{2+}$  is

probably the endoplasmic reticulum, since inositol 1,4,5-trisphosphate ( $\text{IP}_3$ ), which appears rapidly after  $\alpha$ -receptor stimulation as a result of the hydrolysis of phosphatidylinositol biphosphate [5,6], can release  $\text{Ca}^{2+}$  from this organelle [7]. We have now applied the whole-cell patch-clamp technique [8] to compare the properties of the conductance increase produced by external NA with those observed when hepatocytes are perfused internally either with BAPTA-buffered  $\text{Ca}^{2+}$  solutions or with  $\text{IP}_3$ . The similarities found strongly suggest that  $\text{IP}_3$  production and a rise of internal free  $\text{Ca}^{2+}$  concentration alone can account for the membrane permeability increase evoked by NA. Moreover, the finding that  $\text{IP}_3$  can elicit cyclical changes in conductance throws fresh light on the observation by Woods et al. [9] that the elevation of internal calcium occurs in a series of transients.

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**Abbreviation:** BAPTA, 1,2-bis(2-aminophenoxy)-ethane-*N,N,N',N'*-tetraacetic acid

## 2. EXPERIMENTAL

Hepatocytes were isolated from the livers of

guinea-pigs or rabbits by perfusion with collagenase followed by mechanical disruption [2]. Cells in suspension were plated onto 35 mm Falcon petri dishes and whole-cell voltage-clamp recordings [8] made after 1–8 h. Patch pipettes of 2–6 M $\Omega$  resistance contained internal solutions given below. Cell conductance was monitored with small voltage pulses applied to the pipette. In experiments with Cl<sup>-</sup> present, the external solution contained (mM) 142 NaCl, 5.4 KCl, 1.8 CaCl<sub>2</sub>, 0.8 MgSO<sub>4</sub>, 8 Hepes, 11 glucose (pH 7.3), and the internal solution 150 KCl, 8 Hepes, 0.5 EGTA (pH 7.3). Cl<sup>-</sup>-free solutions contained: external: 137 Na gluconate, 5.4 K gluconate, 5 CaSO<sub>4</sub>, 8 Hepes, 11 glucose (pH 7.3); internal: 150 K gluconate, 8 Hepes (pH 7.3). Internal solutions contained IP<sub>3</sub> or BAPTA/Ca where indicated. A dissociation constant of 0.1  $\mu$ M was assumed for BAPTA/CaCl<sub>2</sub> or CaSO<sub>4</sub> solutions of specified free Ca<sup>2+</sup>. Propranolol (2 or 5  $\mu$ M) was present throughout to block  $\beta$ -adrenoceptors. IP<sub>3</sub> was purchased from Amersham and BAPTA from BDH. Inositol biphosphate was a generous gift from Dr R.F. Irvine.

### 3. RESULTS

Fig.1 shows the main characteristics of the conductance increase evoked by NA in hepatocytes voltage-clamped with the whole-cell patch-clamp technique. The response at -20 mV membrane potential, with 5 mM [K] externally and 150 mM internally (equilibrium potential  $E_K = -85$  mV) and 150 mM [Cl] in both external and internal solutions ( $E_{Cl} = 0$  mV), comprised 3 phases. A transient net outward current was followed by a large inward current which reversed to an outward current before finally declining to control level on washout of NA (upper trace). This sequence of currents suggests that conductance changes to both K<sup>+</sup> (outward) and Cl<sup>-</sup> (inward current) occurred. Responses obtained when internal and external Cl<sup>-</sup> were replaced with the impermeant anion gluconate showed only outward current at potentials positive to -80 mV. Current/voltage relations in both Cl<sup>-</sup> and Cl<sup>-</sup>-free conditions were linear from -75 to 40 mV with mean conductance increases of 51 nS in Cl<sup>-</sup>-containing and 3.5 nS in Cl<sup>-</sup>-free conditions (row 1, table 1). Reversal potentials of -19 and -80 mV were obtained in

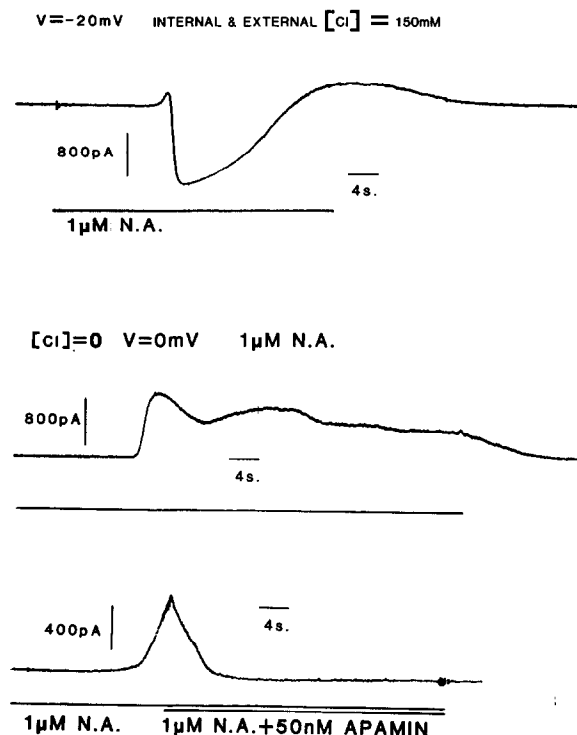


Fig.1. Membrane currents evoked by NA in isolated guinea-pig hepatocytes. (Upper trace) Cl<sup>-</sup>-containing solutions; potential -20 mV. (Middle trace) Cl<sup>-</sup>-free gluconate solutions; potential 0 mV. (Lower trace) Cl<sup>-</sup>-free solutions; apamin (50 nM) was applied during initial part of outward current. Potential 0 mV.

Temperature 30°C. 5  $\mu$ M propranolol present.

Cl<sup>-</sup> and Cl<sup>-</sup>-free conditions, respectively, the latter value being close to the equilibrium potential for K<sup>+</sup>. The response obtained at 0 mV in Cl<sup>-</sup>-free solution, a net outward K<sup>+</sup> current, is shown in the

Table 1

Conductance increase (nS, mean  $\pm$  SE) of hepatocytes evoked by external NA (1  $\mu$ M with 2  $\mu$ M propranolol) and either internal IP<sub>3</sub> (20  $\mu$ M) or internal Ca<sup>2+</sup> (0.7–2.0  $\mu$ M/10 mM BAPTA)

	Normal Cl <sup>-</sup>	Cl <sup>-</sup> -free
NA	51.2 $\pm$ 4.6	3.5 $\pm$ 0.8
IP <sub>3</sub>	42.9 $\pm$ 2.5	3.4 $\pm$ 0.4
0.7–2.0 $\mu$ M Ca <sup>2+</sup>	46.6 $\pm$ 16.0 <sup>a</sup>	2.2 $\pm$ 0.6

Temperature, 30°C or 22°C<sup>a</sup>. Values were corrected for the effects of series resistance between pipette and cell interior

middle trace and the lower trace shows suppression of this  $K^+$  current by apamin, a peptide toxin from bee venom which selectively blocks  $K^+$  flux through a  $Ca^{2+}$ -activated  $K^+$  conductance in hepatocytes [2,10].

A further characteristic of the outward  $K^+$  current evoked by NA in  $Cl^-$ -free solution was an increase in current noise, shown in fig.3a. This had a minimum variance at about  $-70$  mV, near  $E_K$ , and was blocked by apamin. Results of spectral analysis of the noise increase will be given below.

Prolonged application of NA in  $Cl^-$  or  $Cl^-$ -free conditions often resulted in a response which consisted of slow cyclical fluctuations of conductance which lasted several minutes when measured with microelectrode [3] or patch-clamp methods [11].

The results of introducing a high level of internal free  $[Ca^{2+}]$  by means of pipette solutions buffered with 10 mM BAPTA [12] are shown in fig.2a-c. The procedure was as follows. A gigohm seal was obtained and the effects of rupturing the membrane under the pipette tip were recorded while ap-

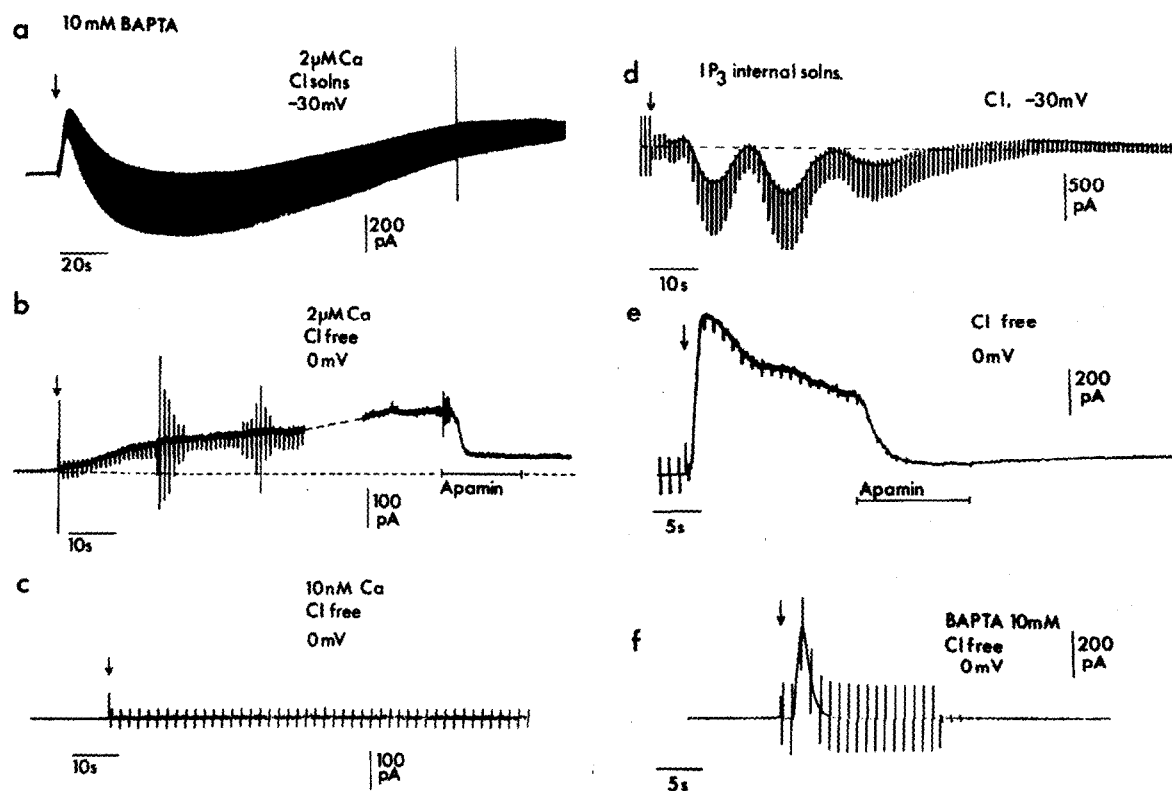


Fig.2. Membrane conductance changes evoked by high free  $Ca^{2+}$  ( $2 \mu M$ ) or  $IP_3$  ( $20 \mu M$ ) in the patch pipette solution. The arrow at the beginning of each trace indicates the point of membrane rupture on going from cell-attached to whole-cell recording condition [8]. Capacity transients partly subtracted. (Left-hand panel) (a)  $Cl^-$ -containing solutions. Potential  $-30$  mV. Temperature  $22^\circ C$ .  $9.4$  mM  $Ca^{2+}$ /10 mM BAPTA ( $2 \mu M$  free  $Ca^{2+}$ ) in the pipette.  $8$  mV potential pulses applied to pipette at  $5$  Hz to measure membrane conductance. (b)  $Cl^-$ -free gluconate solutions. Potential  $0$  mV. Temperature  $30^\circ C$ .  $2 \mu M$  free  $[Ca^{2+}]$  in pipette. Capacity compensation adjusted during record (at approx.  $20$ – $30$  s after membrane rupture), producing large transients on the trace.  $20$  mV pulses at  $1$  Hz applied to monitor cell conductance. Current/voltage relation determined during dashed portion ( $30$  s duration). Apamin ( $50$  nM) reduced the conductance by  $70\%$ . (c) Same conditions as above with free  $[Ca^{2+}]$  buffered to  $10$  nM ( $0.3$  mM  $Ca^{2+}$ /10 mM BAPTA). No capacitance compensation. (Right-hand panel) (d)  $Cl^-$ -containing solutions. Pipette contained  $20 \mu M$   $IP_3$ . Potential  $-20$  mV. Temperature  $30^\circ C$ . (e)  $Cl^-$ -free solutions. Pipette contained  $20 \mu M$   $IP_3$ . Potential  $0$  mV. Apamin ( $50$  nM) applied during the horizontal bar. (f) Conditions as in (e) with the addition of  $10$  mM BAPTA to the pipette solution to buffer cytosolic  $[Ca^{2+}]$ .

plying small voltage pulses to the pipette. An increase of capacitance and current noise (arrows in fig.2) indicated continuity between the pipette and cell interior. The capacity transient was incompletely subtracted and appears in the first few seconds of these records. Changes in membrane conductance resulting from diffusion of components of the pipette solution into the cell were recorded.

Fig.2a shows the result obtained when pipette and external solutions contained  $\text{Cl}^-$  and free  $[\text{Ca}^{2+}]$  in the pipette was buffered to  $2\text{ }\mu\text{M}$ . After rupture of the patch, a net outward current and conductance increase (indicated by the width of the trace) developed over the first 7 s, followed by a net inward current and further increase of conductance to a maximum at about 30 s. This time course is consistent with the diffusive exchange of pipette and cell solutions [13,14] and the change of current polarity from outward to inward indicates the consecutive activation of  $\text{K}^+$  and  $\text{Cl}^-$  conductances. Without  $\text{Ca}^{2+}$  buffer in the pipette no conductance change developed under otherwise similar conditions. In some instances the conductance increase persisted for 10 min or more. A mean maximum conductance increase of 47 nS was observed in  $\text{Cl}^-$  solutions with high internal  $[\text{Ca}^{2+}]$  (table 1, column 1, row 3). Fig.2b shows a similar experiment with  $\text{Cl}^-$  replaced by gluconate anion and free  $[\text{Ca}^{2+}]$  buffered to  $2\text{ }\mu\text{M}$ . An outward current developed with a smaller conductance increase than that with  $\text{Cl}^-$  present. The current/voltage relation (determined during the dashed region of the trace) was linear with a conductance of 1.8 nS and reversal potential of  $-75\text{ mV}$ , close to  $E_{\text{K}}$ . Furthermore, 50–90% of the  $\text{K}^+$  conductance increase in  $\text{Cl}^-$ -free solutions was suppressed on applying 50 nM apamin. In several experiments a smaller component of  $\text{K}^+$  conductance resistant to apamin also developed. This was suppressed by 1 mM quinine and showed different spectral (noise) characteristics. In fig.2c the experiment was repeated with free  $\text{Ca}^{2+}$  in the pipette solution buffered to a low level. No conductance increase was seen at the known resting  $\text{Ca}^{2+}$  level of rat hepatocytes, 200 nM [4], or at a lower level of 10 nM.

A series of experiments was made to see if  $\text{IP}_3$  introduced into the cell via the patch pipette produced conductance changes similar to those with

NA and  $\text{Ca}^{2+}$ . Fig.2d–f shows the effect of  $20\text{ }\mu\text{M}$   $\text{IP}_3$  in the pipette, a concentration producing maximal  $\text{Ca}^{2+}$  release from permeabilized hepatocytes [7]. Fig.2d was obtained with  $\text{Cl}^-$  in both internal and external solutions with no  $\text{Ca}^{2+}$  buffer present in the pipette. After a delay of about 3 s, the conductance increased slightly during a brief period of net outward current, followed by a large conductance increase associated with a substantial inward current. As may be seen, the conductance fluctu-

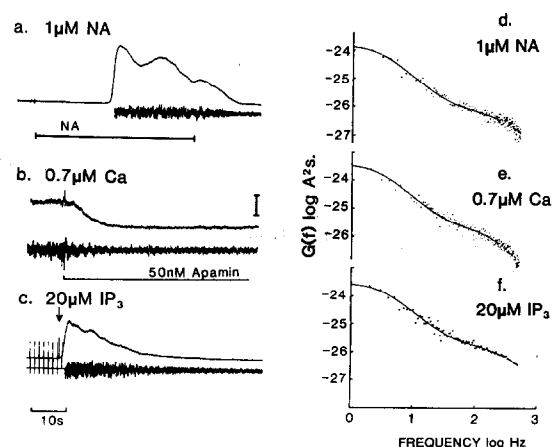


Fig.3. Increase in membrane current noise evoked by external NA, internal  $\text{Ca}^{2+}$  and internal  $\text{IP}_3$ . (Left-hand panel) Currents recorded in  $\text{Cl}^-$ -free gluconate solutions. Potential 0 mV. Temperature  $30^\circ\text{C}$ . (Upper traces) Low-gain d.c. records, (lower traces) high-gain current noise traces band-pass filtered 0.5–500 Hz ( $-3\text{ dB}$ ,  $48\text{ dB/octave}$ ). Initial part of the noise trace was set to zero to prevent ringing of the high-pass filter during the rapid rise of the current. (a) External application of NA ( $1\text{ }\mu\text{M}$ , in the presence of  $2\text{ }\mu\text{M}$  propranolol). (b) High internal  $\text{Ca}^{2+}$  ( $8.5\text{ mM Ca}^{2+}/10\text{ mM BAPTA}$ ; free  $[\text{Ca}^{2+}] = 0.7\text{ }\mu\text{M}$ ). Application of apamin ( $50\text{ nM}$ ) during the horizontal bar abolished the outward current and reduced the current noise. (c) Internal  $\text{IP}_3$  ( $20\text{ }\mu\text{M}$ ) applied via the pipette solution; the arrow indicates rupture of the membrane patch. Calibration: upper traces  $400\text{ pA}$ , lower  $40\text{ pA}$ . (Right-hand panel) Power spectra of current noise variance evoked by NA,  $\text{Ca}^{2+}$  and  $\text{IP}_3$  (traces shown in the left-hand panel a–c). Average of 10–30 1 s samples digitised at  $1024\text{ Hz}$ . Spectra fitted with the sum of 2 Lorentzian functions by least squares. For internal  $\text{Ca}^{2+}$  or  $\text{IP}_3$ , noise recorded in the presence of  $50\text{ nM}$  apamin was subtracted as control. Estimates of single-channel conductance ( $\gamma$ ) were calculated from the mean current ( $I$ ), the variance of the fitted spectral density functions ( $\text{var}$ ) and  $V - E_{\text{K}}$ :  $\gamma = \text{var}/I(V - E_{\text{K}})$

Table 2

Results of noise analysis of NA-evoked conductance increase in  $\text{Cl}^-$ -free (aspartate or gluconate) solutions, compared with noise associated with conductances activated by internal  $\text{IP}_3$  (20  $\mu\text{M}$ ) or high internal  $\text{Ca}^{2+}$  (free  $\text{Ca}^{2+}$  0.7–2  $\mu\text{M}$ /10 mM BAPTA)

	Gluconate, 30°C			Aspartate, 22°C	
	NA	$\text{IP}_3$	$\text{Ca}^{2+}$	NA	$\text{Ca}^{2+}$
Single-channel conductance (pS)	1.69 $\pm$ 0.21	1.65 $\pm$ 0.27	1.74 $\pm$ 0.52	1.04 $\pm$ 0.51	0.75 $\pm$ 0.21
$f_{c1}$ (Hz)	2.13 $\pm$ 0.33	2.60 $\pm$ 0.17	4.27 $\pm$ 1.64	1.82 $\pm$ 0.48	4.72 $\pm$ 0.86
$f_{c2}$ (Hz)	189 $\pm$ 11	192 $\pm$ 9.0	179 $\pm$ 39	138 $\pm$ 34	233 $\pm$ 35
Low-frequency variance (%) of total	71 $\pm$ 2	69 $\pm$ 3	69 $\pm$ 9	64 $\pm$ 11	47 $\pm$ 9
<i>n</i>	7	4	4	3	6

Power spectra were fitted by the sum of 2 Lorentzian curves. Estimates of single-channel conductance (from variance and mean current), half power frequencies for both components ( $f_{c1}$  and  $f_{c2}$ ) and proportion of variance associated with the low-frequency component are given. Means  $\pm$  SE

tuated slowly before declining and reversing to a net outward current. Similar responses were observed with 5  $\mu\text{M}$   $\text{IP}_3$ . Fluctuations of conductance of this kind were often seen following NA application and may reflect fluctuations of internal  $[\text{Ca}^{2+}]$  similar to those described by Woods et al. [9]. Cyclical changes of  $\text{Ca}^{2+}$ -activated conductance with intracellular  $\text{IP}_3$  application have also been reported in lacrimal gland cells [15] and amphibian oocytes [17].

A record obtained with internal  $\text{IP}_3$  in  $\text{Cl}^-$ -free solutions is shown in fig.2e. As with NA or high  $[\text{Ca}^{2+}]$ , 20  $\mu\text{M}$   $\text{IP}_3$  gave an outward current after a short delay and a smaller conductance increase than was seen with  $\text{Cl}^-$  present. The current showed an initial peak, as seen in the response to NA under similar conditions, and was abolished by 50 nM apamin. When free  $[\text{Ca}^{2+}]$  in the pipette was buffered to 10 nM with 10 mM BAPTA (fig.2f), 20  $\mu\text{M}$   $\text{IP}_3$  produced a transient outward current which rapidly declined to zero, suggesting that  $\text{Ca}^{2+}$  released by the high  $\text{IP}_3$  concentration was buffered as the concentration of BAPTA in the cell rose.

Inositol 1,4-bisphosphate ( $\text{IP}_2$ ) is formed in hepatocytes following breakdown of  $\text{IP}_3$  by its 5'-phosphomonoesterase. However, when  $\text{IP}_2$  (20  $\mu\text{M}$ ) was introduced into hepatocytes via the patch pipette, the cell conductance remained low.

The peak conductances recorded in  $\text{Cl}^-$  and  $\text{Cl}^-$ -free conditions with external NA, internal  $\text{IP}_3$

or BAPTA-buffered  $\text{Ca}^{2+}$ , may be compared in table 1. These were similar in size for  $\text{Cl}^-$  solution in each case and were reduced in  $\text{Cl}^-$ -free conditions to give similar  $\text{K}^+$  conductances.

As may be seen in fig.3b and c, a current noise increase was observed when internal  $\text{IP}_3$  or  $\text{Ca}^{2+}$  evoked the  $\text{K}^+$  conductance, as had been found with NA ([17]; fig.3a). The single-channel conductance and spectral characteristics of the noise increase were calculated in the former cases by subtraction of noise in the presence of apamin as control. Power spectra obtained with NA,  $\text{IP}_3$  and  $\text{Ca}^{2+}$  are shown in fig.3d–f. These were fitted well by the sum of two Lorentzian components. The parameters of the curves and the estimated single-channel conductances may be compared in table 2. Within the limits of experimental error, the properties of the ion channels were the same with regard both to conductances estimated for the open channel (1.7 pS at 30°C, 1.0 pS at 22°C) and to the half power frequencies and amplitudes of the spectral components.

#### 4. DISCUSSION

The membrane conductance increases evoked by external NA, by intracellular application of  $\text{IP}_3$  or by raised internal  $[\text{Ca}^{2+}]$  show marked similarities in respect of the ion selectivity for  $\text{K}^+$  and  $\text{Cl}^-$ , the sequential activation and magnitude of  $\text{K}^+$  and  $\text{Cl}^-$  components, the suppression of  $\text{K}^+$  conductance

by low concentrations of apamin and the spectral characteristics of the apamin-sensitive conductance. This clearly suggests a common pathway for ion channel activation. The present results provide direct evidence of the activation of the  $K^+$  and  $Cl^-$  conductances of hepatocytes by raised internal  $Ca^{2+}$ , and provide strong support for the chain of events from  $\alpha$ -adrenoreceptor stimulation to ion conductance increase via  $IP_3$  production and  $Ca^{2+}$  release from internal stores. Whatever role the simultaneously formed diacylglycerol may have [18], it is clearly not essential for the responses we have studied. The observation of cyclical changes of conductance with NA and internal  $IP_3$  (see also [15,17]) but not internal high  $[Ca^{2+}]$  is in keeping with the report of cyclical increases in cytosolic  $[Ca^{2+}]$  during NA action [9]. The fact that these occurred in the present experiments with a maintained high concentration of  $IP_3$  suggests that when elicited by NA they do not result from fluctuations in the concentration of  $IP_3$ . The explanation is more likely to be found in processes regulating  $Ca^{2+}$  sequestration and release by intracellular stores.

#### ACKNOWLEDGEMENTS

We thank Professor D.H. Jenkinson for helpful discussions during the course of this work and Dr G.M. Burgess for reading the manuscript. Supported by the MRC and the Wellcome Trust. T.C. was an EMBO Fellow.

#### REFERENCES

- [1] Haylett, D.G. and Jenkinson, D.H. (1972) *J. Physiol.* 225, 721-750.
- [2] Burgess, G.M., Claret, M. and Jenkinson, D.H. (1981) *J. Physiol.* 317, 67-90.
- [3] Field, A.C. and Jenkinson, D.H. (1985) *J. Physiol.* 369, 106P.
- [4] Charest, R., Blackmore, P.F., Berthon, B. and Exton, J.H. (1983) *J. Biol. Chem.* 258, 8769-8733.
- [5] Burgess, G.M., Godfrey, P.P., McKinney, J.S., Berridge, M.J., Irvine, R.F. and Putney, J.W. (1984) *Nature* 309, 63-66.
- [6] Burgess, G.M., McKinney, J.S., Irvine, R.F. and Putney, J.W. (1985) *Biochem. J.* 232, 237-243.
- [7] Burgess, G.M., Irvine, R.F., Berridge, M.J., McKinney, J.S. and Putney, J.W. (1984) *Biochem. J.* 224, 741-746.
- [8] Hamill, O.P., Marty, A., Neher, E., Sakmann, B. and Sigworth, F.J. (1981) *Pflügers Arch.* 391, 85-100.
- [9] Woods, N.M., Cuthbertson, K.S.R. and Cobbold, P.H. *Cell Calcium* 8, 79-100.
- [10] Banks, B.E.C., Brown, C., Burgess, G.M., Burnstock, G., Claret, M., Cocks, T.M. and Jenkinson, D.H. (1979) *Nature* 282, 415-417.
- [11] Capiod, T., Field, A., Jenkinson, D.H. and Ogden, D.C. (1985) in: *Pharmacology of Adrenoceptors* (Szabadi, E. et al. eds) pp. 135-145, Macmillan, New York.
- [12] Tsien, R.Y. (1980) *Biochemistry* 19, 2396-2404.
- [13] Marty, A. and Neher, E. in: (1983) *Single Channel Recording* (Sakmann, B. and Neher, E. eds) chap. 7, pp. 107-122, Plenum, New York.
- [14] Lamb, T.D., Matthews, H.R. and Torre, V. (1986) *J. Physiol.* 372, 315-350.
- [15] Evans, M.G. and Marty, A. (1986) *Proc. Natl. Acad. Sci. USA* 83, 4099-4103.
- [16] Capiod, T. and Ogden, D.C. (1985) *J. Physiol.* 369, 107P.
- [17] Parker, I. and Miledi, R. (1986) *Proc. R. Soc. Lond. B* 228, 307-315.
- [18] Nishizuka, Y. (1984) *Nature* 308, 693-698.