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Vasopressin directly closes ATP-sensitive potassium channels evoking membrane depolarization and an increase in the free intracellular Ca^{2+} concentration in insulin-secreting cells

S.C.Martin*, D.I.Yule*, M.J.Dunne*, D.V.Gallacher and O.H.Petersen

The MRC Secretory Control Research Group, The Physiological Laboratory, The University of Liverpool, Liverpool, L69 3BX, UK

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*To be considered as equal first authors

The effects of arginine-vasopressin (AVP) (0.01-1 μM) on membrane potential, $[Ca^{2+}]_i$ and ATP-sensitive potassium channels have been studied in the insulin-secreting cell line RINm5F. In whole cells, with an average spontaneous cellular transmembrane potential of -64 ± 3 mV (n = 33) and an average basal $[Ca^{2+}]_i$ of 102 ± 6 nM (n = 40), AVP evoked: (i) membrane depolarization, (ii) voltage-dependent Ca^{2+} spike-potentials and (iii) a sharp rise in $[Ca^{2+}]_i$. Single-channel current events recorded from excised outside-out membrane patches show that AVP closes potassium channels that are also closed by tolbutamide (100 μM) and opened by diazoxide (100 μM). AVP acts on K_{ATP} channels specifically from the outside of the membrane and a soluble cytosolic messenger appears not to be involved, since there is no channel activation in cell-attached membrane patches when the peptide is added to the bath solution.

Key words: $[Ca^{2+}]_i$/fura-2/K_{ATP} channels/insulin-secreting cell/RINm5F cell

Introduction

The control of insulin secretion from pancreatic β cells of the islets of Langerhans is mainly determined by changes in the membrane potential of the cell (for reviews see Petersen and Findlay, 1987; Petersen, 1988; Ashcroft, 1988). In intact resting cells open ATP-sensitive potassium channels (Cook and Hales, 1984) dominate the permeability of the membrane, and maintain a transmembrane potential close to the equilibrium potential for K^+ (Findlay *et al.*, 1985; Rorsman and Trube, 1985; Dunne *et al.*, 1986). In the presence of carbohydrate secretagogues these channels close evoking depolarization of the membrane (Ashcroft *et al.*, 1984; Dunne *et al.*, 1986; Misler *et al.*, 1986). This depolarization is required for the opening of voltage-gated L-type Ca^{2+} channels (Matthews and Sakamoto, 1975; Velasco *et al.*, 1988, 1989; Rorsman *et al.*, 1988), causing an increase in the free intracellular calcium ion concentration ($[Ca^{2+}]_i$)—a key intracellular regulator of insulin secretion (Wollheim and Biden, 1987).

A number of peptide hormones and neurotransmitters, known to influence secretion from insulin-releasing cells also have effects upon K_{ATP} channels. De Weille *et al.* (1988) first demonstrated that the hyperglycaemia-inducing neuropeptide galanin, hyperpolarizes the cell membrane by a mechanism involving an increase in K^+ permeability due to opening of ATP-sensitive K^+ channels. Later, Dunne *et al.* (1989a) showed that the stimulation of these channels involved the receptor-mediated activation of a pertussis toxin-sensitive G-protein coupled to the channel. Another important regulatory hormone inhibiting insulin secretion is somatostatin. Like galanin, somatostatin specifically opens ATP-sensitive K^+ channels, and intracellular GTP is required for this effect (De Weille *et al.*, 1989).

Since vasopressin has been reported to stimulate insulin-secreting cells (Li *et al.*, 1988; Monaco *et al.*, 1988), the present study was undertaken to investigate the effects of the peptide on membrane potential, ATP-sensitive K^+ channels and single cell $[Ca^{2+}]_i$. Vasopressin-induced depolarization, Ca^{2+} spike-potential generation and increase in $[Ca^{2+}]_i$ have been demonstrated using patch-clamp and single cell microfluorimetry techniques. In excised outside-out membrane patches vasopressin is shown to specifically close ATP-sensitive K^+ channels.

Results

The effects of arginine-vasopressin on membrane potential and $[Ca^{2+}]_i$

The action of arginine-vasopressin (AVP) (0.01-1 μM) on the transmembrane potential of single RINm5F cells has been studied using the whole-cell current-clamp variation of the patch-clamp technique. In total 33 separate cells were investigated. The average spontaneous zero-current membrane potential, estimated within seconds of forming the whole-cell was found to be -64 ± 3 mV (n = 33) (mean ± SEM).

Figure 1A shows the effects of AVP (10 nM) on the membrane potential in a single RINm5F cell. In the absence of secretagogue, the cell potential was stable at ~ −70 mV. When challenged with AVP, added to the bath solution in contact with the extracellular membrane surface, a marked depolarization was initiated. This fall in membrane potential appeared to be biphasic, consisting of an initial large transient depolarization, associated with the generation of spike-potentials, followed by a more sustained level. Finally, when AVP is removed the membrane repolarizes to ~ −70 mV. The data shown in Figure 1A was found to be typical of 31 out of 33 applications of AVP to 33 separate cells. On average, AVP depolarized the membrane by 24 ± 6 mV (n = 31) and evoked spike-potentials on 26 out of 31 occasions. The period of Ca^{2+} spike generation was found to last between 10 and 120 s.

Since the spike-potentials result from the influx of Ca^{2+} through voltage-gated calcium channels (Matthews and Sakamoto, 1975), changes in the free intracellular Ca^{2+} concentration of single cells have been monitored using dual-excitation microfluorimetry with fura-2. The effects of AVP (10 nM) on $[Ca^{2+}]_i$ are shown in Figure 1B. In agreement with its action on the membrane potential, AVP caused a

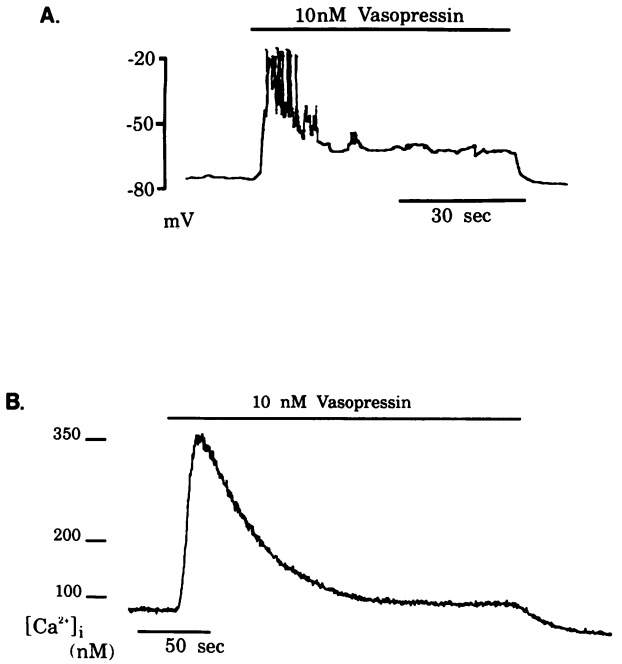

Fig. 1. The effects of vasopressin (10 nM) on the membrane potential (**Panel A**) and $[Ca^{2+}]_i$ (**Panel B**). Figure 1A was obtained using the patch-clamp whole cell recording configuration and begins 1 min after formation of the whole cell. $[Ca^{2+}]_i$ has been assessed using dual excitation microfluorimetry with fura-2. The records come from two separate cells. Panel A has been photographed directly from the pen-recording trace, filtered at 20 Hz (low pass).

biphasic rise in $[Ca^{2+}]_i$: a large transient rise being followed by a smaller sustained increase in $[Ca^{2+}]_i$. On average, AVP enhanced the internal Ca^{2+} concentration of these cells from a basal value of 102 ± 6 to 327 ± 14 nM (n = 6), during the transient phase and 187 ± 11 nM (n = 6) during the sustained rise. In the complete absence of external Ca^{2+} (by chelation with EGTA) both the generation of Ca^{2+} spike-potentials (n = 6) and the smaller sustained rise in $[Ca^{2+}]_i$ were abolished (n = 3), whereas the initial transient rise in $[Ca^{2+}]_i$ was retained (Figure 2). The experiment shown in Figure 3 demonstrates that readmission of Ca^{2+} to the bathing solution in the continued presence of AVP (10 nM) re-establishes the sustained rise in $[Ca^{2+}]_i$.

Following the initial stimulation of RINm5F cells with AVP, further additions of the peptide produced mixed results. Of the 31 cells found to respond to AVP only 30 out of 68 additional exposures caused the cells to depolarize and initiate Ca^{2+} spike-potentials. Similar results were found with AVP-induced changes in $[Ca^{2+}]_i$ (n = 3). At higher concentrations (0.1 − 1 μM) the effects of AVP were somewhat weaker. In 10 separate whole cells, 6 out of 10 applications of 0.1 μM AVP depolarized the membrane by, on average, 18 ± 2 mV, whereas 1 μM AVP caused an 11 ± 2 mV drop in potential in 19 cells (18 out of 19 applications) and a small transient rise in $[Ca^{2+}]_i$ (n = 3).

The effects of AVP on single ATP-sensitive potassium channel currents

Insulin-secreting cells possess both ATP-sensitive and Ca^{2+}- and voltage-activated potassium channels (Petersen and Findlay, 1987). All single-channel current recordings presented in this paper are derived exclusively from currents passing through ATP-sensitive K^+ channels. Openings

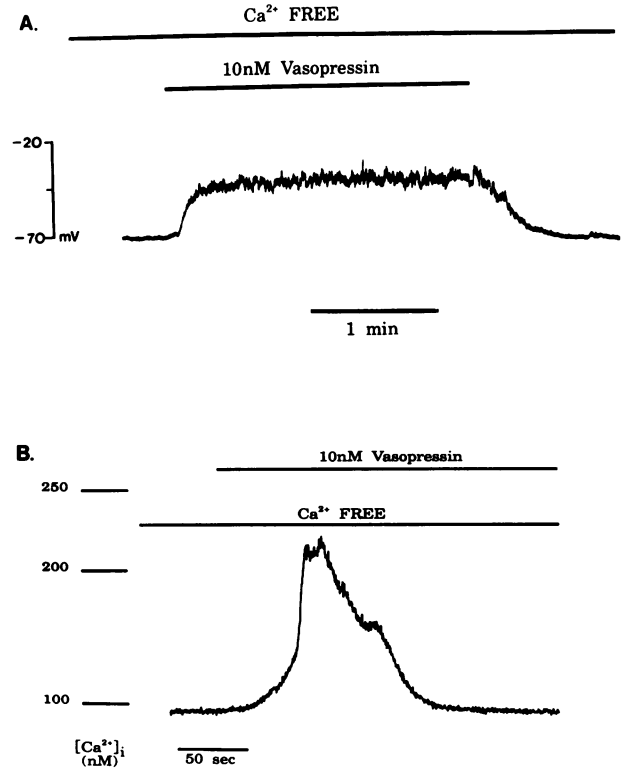

Fig. 2. Vasopressin-induced Ca^{2+} spike-potentials (**Panel A**) and the smaller sustained increase in $[Ca^{2+}]_i$ (**Panel B**) are abolished, whereas the initial transient rise is retained when Ca^{2+} is removed (by EGTA chelation) from the bathing solution. The records come from two separate cells.

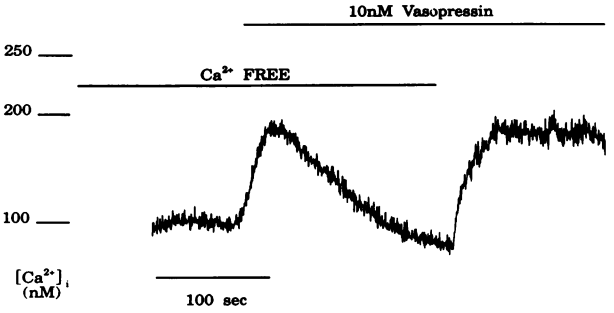

Fig. 3. Readmitting Ca^{2+} to a Ca^{2+}-free solution in the continued presence of vasopressin results in a sustained increase in $[Ca^{2+}]_i$.

from Ca^{2+}- and voltage-activated K^+ channels have been eliminated by keeping the $[Ca^{2+}]_i$ low, $<10^{-9}$ M, by omitting Ca^{2+} and adding EGTA (1 mM) to the solution bathing the inside of the plasma membrane.

Figure 4 shows the effects of diazoxide (100 μM), AVP (10 nM) and tolbutamide (100 μM) on ATP-sensitive K^+ channels in the same excised outside-out patch, recorded with 1 mM ATP added to the cytosolic face of the membrane. The record shows that the sulphonamide diazoxide, a specific K_{ATP} channel opener (Trube *et al.*, 1986; Dunne *et al.*, 1987), opens the channels (upper panel), whereas both AVP (middle panel) and tolbutamide (lower panel) suppress the opening of the ATP-sensitive K^+ channels. AVP-evoked channel closure was seen in 13 separate outside-out patches (17 out of 17 applications), whereas the actions of diazoxide (26 out of 26 applications) and tolbutamide (34 out of 34 applications) were confirmed in 18 and 9 outside-out patches

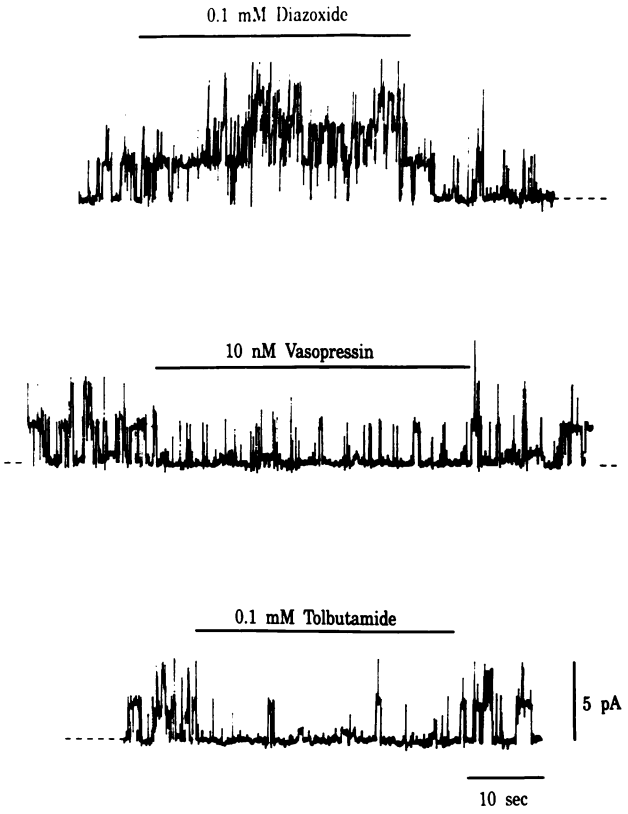

Fig. 4. The direct effects of diazoxide (0.1 mM), vasopressin (10 nM) and tolbutamide (0.1 mM) on K_{ATP} channels in the same excised outside-out patch of membrane. The interval between the end of the upper panel and the beginning of the middle one is 10 s whereas the interval between the end of the middle panel and the beginning of the lower one is 5 s. It may seem surprising that in this series of experiments 1 mM ATP (present in the pipette solution) did not abolish channel activity. The density of K_{ATP} channels is, however, high (Petersen and Findlay, 1987) and outside-out patches are larger than inside-out patches. The control situation in these experiments therefore represents a state where the vast majority of the channels have been closed by ATP. In permeabilized cells we have often found that 1 mM ATP, whilst severely inhibiting channel openings was insufficient to cause complete block, which could only be achieved by 5 mM ATP (Dunne *et al.*, 1986).

respectively. A quantitative analysis of the effects of AVP, diazoxide and tolbutamide is shown in Figure 5. Paradoxically, 0.1 and 1 μM concentrations of AVP were apparently less potent at closing channels than 10 nM since they only reduced the open-probability of K^+ channels to 47 ± 9% (n = 5) and 86 ± 6% (n = 6) of the pre-control level of activity respectively. AVP (10 nM − 1 μM) had no effect on the single-channel current amplitude and appears to act specifically from the outside of the plasma membrane, since the peptide had no effect on channel openings in eight separate experiments where the cell-attached recording configuration was used in combination with saponin permeabilization (open cells) and where AVP could be added to the bath solution in contact with the inside of the intact patch membrane (16 out of 16 applications).

In current records obtained from cell-attached membrane patches on intact cells, AVP had no significant effect on the frequency of channel openings in 17 separate cells when it was added to the bath solution (53 out of 55 applications). However, a slight decrease in the single-channel current amplitude was observed, which is commensurate with a

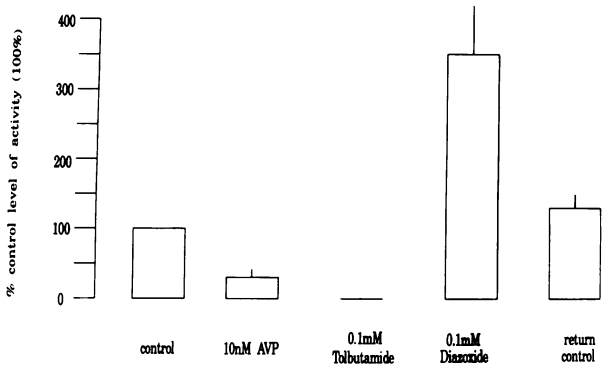

Fig. 5. A comparison between the effects of vasopressin (AVP) (10 nM), tolbutamide (0.1 mM) and diazoxide (0.1 mM) on the average (mean ± SEM) current due to open K_{ATP} channels recorded from excised outside-out membrane patches. All values are expressed as a percentage of the control level of activity (100%) from 4, 4 and 8 separate outside-out patches for AVP, tolbutamide and diazoxide respectively.

depolarization of the membrane (Dunne *et al.*, 1986; Wollheim *et al.*, 1988).

Discussion

In this study we have used patch-clamp and single cell $[Ca^{2+}]_i$ measurements to investigate the effects of AVP on the insulin-secreting cell line RINm5F. These cells are a particularly good model system for the study of mammalian β cells since it has been shown that they respond to a variety of compounds, including the carbohydrate secretagogues glucose (Ribalet *et al.*, 1988; Dunne *et al.*, 1989b) and glyceraldehyde (Praz *et al.*, 1983; Dunne *et al.*, 1986), the neurotransmitters or hormones galanin (De Weille *et al.*, 1988; Dunne *et al.*, 1989a) and somatostatin (De Weille *et al.*, 1989) as well as antidiabetic drugs (Dunne *et al.*, 1987; Schmid-Antomarchi *et al.*, 1987a,b; Dunne, 1989). The link between several of these compounds and their influence on insulin secretion is provided by changes in the gating of ATP-sensitive K^+ channels. Closure of channels in intact insulin-secreting cells allows the membrane to depolarize, whereas opening of channels hyperpolarizes the membrane and prevents the activation of voltage-gated Ca^{2+} channels (Petersen and Findlay, 1987; Petersen, 1988; Petersen and Dunne, 1989).

Our results show that AVP depolarizes RINm5F cells, thereby generating Ca^{2+} spike-potentials and an increase in $[Ca^{2+}]_i$. This explains why AVP evokes secretion of insulin from these cells (Li *et al.*, 1988; Monaco *et al.*, 1988). Li *et al.* (1988) also observed that the effects of AVP on insulin secretion were abolished during prolonged exposure to the peptide. These data also fit with our findings, since the actions of AVP on membrane potential and $[Ca^{2+}]_i$ were biphasic, consisting of a large transient depolarization and a marked rise in $[Ca^{2+}]_i$, followed by a more sustained depolarization and increase in $[Ca^{2+}]_i$ (Figure 1). Both the generation of spike-potentials and the sustained rise in $[Ca^{2+}]_i$ were dependent on external Ca^{2+} (Figures 2 and 3). The AVP-evoked increase in $[Ca^{2+}]_i$ in the sustained phase is therefore mainly due to Ca^{2+} flowing into the cells through voltage-gated Ca^{2+} channels. Such channels have recently been characterized directly at the single-channel

current level in both RINm5F cells (Velasco *et al.*, 1988; Velasco and Petersen, 1989) and normal pancreatic β cells (Rorsman *et al.*, 1988). Part of the initial AVP-evoked Ca^{2+} response is also observed in Ca^{2+}-free solutions and is therefore probably due to intracellular Ca^{2+} release mediated by inositol (1,4,5)-trisphosphate ($InsP_3$), since it is known that AVP stimulation results in $InsP_3$ formation (Li *et al.*, 1988).

It has previously been shown that the influences of a number of hormones and neuropeptides on insulin-secreting cells are mediated by their effects on K^+ channels (De Weille *et al.*, 1988, 1989; Dunne *et al.*, 1989a). In our experiments on excised outside-out membrane patches, AVP was found to close single ATP-sensitive K^+ channels (Figures 4 and 5). The pores closed by AVP were identified as K_{ATP} channels since they were also, in the same patch of membrane, closed by tolbutamide and opened by diazoxide, sulphonamides that are known to interact specifically with K_{ATP} channels (Trube *et al.*, 1986; Dunne *et al.*, 1987; Schmid-Antomarchi *et al.*, 1987a,b; Dunne, 1989). The experimental observation (Figure 4) that AVP caused an immediate reduction in channel opening that was immediately reversible in excised cell-free membrane patches, whilst not having any effect in intact cells when added to the bath solution in contact with the cell membrane outside the isolated patch area from which the single-channel current recording was made, shows that AVP interacts directly with the K_{ATP} channel, probably via a G-protein, rather than a soluble cytosolic messenger. Carbohydrate-evoked K_{ATP} channel closure, on the other hand, does depend on internal messenger(s) as both glucose and glyceraldehyde uptake across the plasma membrane can be topologically separated from the site of channel closure under experimental conditions (Ashcroft *et al.*, 1984; Dunne *et al.*, 1986). Although vasopressin stimulation generates diacylglycerol (Li *et al.*, 1988) and it is known that activators of protein kinase C can evoke K_{ATP} channel closure in the cell-attached recording configuration, when added to the bath solution (Wollheim *et al.*, 1988), it is unlikely that the AVP effect is mediated in this way because of the failure of AVP to close channels in this configuration.

It is interesting that two different neuropeptides, galanin and vasopressin can directly influence K_{ATP} channel gating in exactly opposite ways. The control of the K_{ATP} channel is clearly very complex, which is understandable since this channel is central to stimulus-secretion coupling in insulin-secreting cells.

Materials and methods

Cell isolation and maintenance

All experiments were carried out on the clonal insulin-secreting cell line RINm5F, maintained as previously described (Dunne *et al.*, 1988b, 1989a).

Media

The standard extracellular Na^+-rich solution used throughout these experiments, contained (mM): 140 NaCl, 4.7 KCl, 1.13 $MgCl_2$, 2.5 $CaCl_2$ and 10 HEPES. The pH was set at 7.2 using NaOH. The standard K^+-rich solution contained (mM): 140 KCl, 10 NaCl, 1.13 $MgCl_2$, 10 HEPES, 1 EGTA and 1 ATP. No $CaCl_2$ was added and the pH was set at 7.2 using KOH. Stock solutions of tolbutamide (Sigma, UK) and diazoxide (Glaxo, UK) were prepared in dimethylsulphoxide (DMSO), which at maximal concentrations of 1% had no effect on the gating of K^+ channels (Dunne *et al.*, 1987). The osmolality of all solutions was 290 ± 5 mosm/kg. All experiments were carried out at room temperature 22–25°C.

Patch-clamp experiments and analysis

Electrophysiological investigations of RINm5F cells were carried out using the patch-clamp technique (Hamill *et al.*, 1981). Single-channel current recordings from outside-out membrane patches, cell-attached membrane patches and whole-cell current-clamp records were made with the K^+-rich solution in the pipette and the Na^+-rich solution in the bath. Open cell experiments were carried out with the Na^+-rich solution in the pipette and the K^+-rich solution (with ATP omitted) in the bath. Patch-clamp pipettes (Type 101PB, Ceebee Glass, Denmark) had a final resistance of between 5 and 10 MΩ when filled. Exchange from control to test solutions was achieved manually through a series of outlet pipes, under visual control (Dunne *et al.*, 1987, 1988a). Whole cell recordings of membrane potential (zero-current) and single-channel current data were stored on FM tape (Racal 4DS recorder) for subsequent replay and analysis.

Analysis of single-channel current records was performed on continuous stretches of data [filtered at 1 kHz (low pass)], lasting between 10 and 20 s. All data were digitized at 8 kHz [CED 1401 (Cambridge, UK)] and analysed using a Tandon microcomputer in conjunction with a software package supplied by CED (Cambridge, UK). Idealized current traces were obtained from computerized threshold analysis of data, using pre-selected current levels. Comparative changes in K^+ channel open-state probability have been presented as a fraction of the pre-stimulus level of activity (100%). This particular method of quantification was preferred to that of expressing an absolute value of open probability, since the finite number of operational channels in a particular patch of membrane is often unknown (Dunne, 1989; Dunne *et al.*, 1989a). All data presented has been photographed directly from the pen-recording trace, filtered at 20 Hz (low pass). In all single-channel current traces shown upward deflections represent outward current flow (i.e. from the inside to the outside of the membrane patch).

Measurements of single cell $[Ca^{2+}]_i$

RINm5F cells were loaded with the Ca^{2+} fluorophore fura-2 by a 30 min pre-incubation in the Na^+-rich solution with 3 μM fura-2-acetoxymethyl ester, at room temperature. Changes in single cell $[Ca^{2+}]_i$ were assessed using dual excitation microfluorimetry (Grynkiewicz *et al.*, 1985), with a Spex (Glen spectra) DM 3000 CM system, as previously described (Yule and Gallacher, 1988). $[Ca^{2+}]_i$ was estimated from the ratio (R) of the fluorescence at 340 and 380 nm according to the formula (Schlegel *et al.*, 1987):

$$[Ca^{2+}]_i = K_d\,\beta(R-R_{min})/(R_{max}-R)$$

where K_d = 225 nM (Grynkiewicz *et al.*, 1985), R_{max}, R_{min} and β are constants: 7.9 ± 0.4 (n = 6), 0.49 ± 0.08 (n = 6) and 3.9 ± 0.4 (n = 6) respectively. These constants were determined using the *in situ* calibration procedures described by Schlegel *et al.* (1987, 1988). All records have been corrected for autofluorescence at each wavelength (determined in unloaded cells) before the ratio was calculated.

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