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SUBSTANCE P AND BOMBESIN ELEVATE CYTOSOLIC Ca^{2+} BY DIFFERENT MOLECULAR MECHANISMS IN A RAT PANCREATIC ACINAR CELL LINE

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SUMMARY

1. Dual-excitation microfluorometry (Fura-2 as indicator) was employed to monitor directly changes in the cytosolic calcium concentration ($[Ca^{2+}]_i$) in single cells. We investigated and compared the effects of stimulation of AR42J rat pancreatic acinar cells by two peptide agonists, substance P and bombesin.

2. Substance P (10^{-7} M) and bombesin (10^{-8} M) each gave rise to a marked, but transient, elevation in $[Ca^{2+}]_i$. The calcium signals evoked by the two peptides were qualitatively and quantitatively very similar. However, in the absence of extracellular Ca^{2+} the response to substance P, but not bombesin, was abolished. These results suggest that substance P induces calcium influx across the cell surface membrane but does not release calcium from internal stores. Bombesin in marked contrast releases calcium from intracellular stores in the absence of any detectable calcium influx.

3. Depolarization by high-K^+ extracellular solutions evoked a marked, but transient, rise in $[Ca^{2+}]_i$. This elevation in $[Ca^{2+}]_i$ was strictly dependent upon the presence of Ca^{2+} in extracellular media.

4. Nifedipine (5×10^{-6} M), an antagonist of L-type voltage-dependent Ca^{2+} channels, blocked the elevations in $[Ca^{2+}]_i$ induced by either substance P or high-K^+ solutions, but not that evoked by application of bombesin.

5. Patch-clamp, single-channel current recordings from cell-attached patches of membrane confirmed the presence of voltage-dependent calcium channels in the surface membranes of AR42J cells. Whole-cell current recordings demonstrated voltage-dependent inward Ca^{2+} (Ba^{2+}) currents which were increased in amplitude by substance P and blocked by nifedipine.

6. The protein kinase C (PKC) activators, the phorbol diester, phorbol 1,2-myristate 13-acetate (PMA, 10^{-7} M), and cell-permeable diacylglycerol analogues, 1-oleoyl-2-acetyl-*sn*-glycerol (OAG, $2{\cdot}5 \times 10^{-6}$ M) and *sn*-2-dioctanoyl glycerol (DiC_8,

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$2{\cdot}5 \times 10^{-6}$ M), mimicked the effect of substance P, but not bombesin, in elevating $[Ca^{2+}]_i$ in a manner that was blocked by removal of extracellular Ca^{2+} or application of nifedipine.

7. The PKC inhibitor, polymyxin B ($2{\cdot}5 \times 10^{-6}$ M), applied 2 min prior to stimulation blocked the effects of substance P and PKC activators, but not bombesin, in elevating $[Ca^{2+}]_i$.

8. The calcium signals evoked by substance P and bombesin are achieved by activation of different molecular mechanisms. Substance P, the evidence suggests, activates PKC which in turn stimulates calcium influx by opening voltage-dependent Ca^{2+} channels in the cell surface membranes. Bombesin on the other hand releases calcium from internal stores, an effect presumably mediated by inositol 1,4,5-trisphosphate. The implication is that individual peptide receptors can regulate separately the activity of the PKC and inositol polyphosphate limbs of what to date has been considered to be an essentially obligate dual-signal pathway.

INTRODUCTION

It is now recognized that the calcium-mobilizing effects of a variety of neurotransmitters and hormones are due to the activation of a common transduction pathway which operates through hydrolysis of inositol lipids in cell membranes (see Berridge, 1987). In what has become known as 'the dual-signalling hypothesis', receptor activation, via a G-protein, stimulates the activity of a phosphatidylinositol-specific phospholipase C to hydrolyse phosphatidylinositol 4,5-bisphosphate (PIP_2) to yield concomitantly the two second messengers diacylglycerol (DAG) and inositol 1,4,5-trisphosphate ($Ins1,4,5P_3$). In electrically non-excitable cells, which lack any voltage-dependent calcium influx pathway (see Gallacher, 1988), the principal interest has been in establishing the role of $Ins1,4,5P_3$ as an intracellular regulator of the release of calcium from non-mitochondrial stores. It is now unequivocally established in a variety of tissues (Berridge, 1987) that $Ins1,4,5P_3$ activates a calcium release pathway in the membranes of calcium-sequestering organelles thereby elevating the cytosolic calcium concentration ($[Ca^{2+}]_i$). More recent evidence suggests that $Ins1,4,5P_3$ can on its own (Kuno & Gardner, 1987; Llano, Marty & Tanguy, 1987) or in combination with its phosphorylated derivative inositol 1,3,4,5-tetrakisphosphate ($Ins1,3,4,5P_4$) (Irvine & Moor, 1987; Morris, Gallacher, Irvine & Petersen, 1987; Changya, Gallacher, Irvine & Petersen, 1989*a*; Changya, Gallacher, Irvine, Potter & Petersen, 1989*b*), promote calcium entry across the surface membrane of such cells. The role of the other messenger DAG, an endogenous activator of protein kinase C (PKC), is less well understood in these cellular systems and is suggested to operate as a modulator of the inositol polyphosphate-induced calcium signals (see Berridge, 1987) or as a direct regulator of secretion (Wooten & Wrenn, 1984). By contrast, in electrically excitable systems in which calcium influx is achieved by the opening of voltage-dependent calcium channels (see Tsien, Lipscombe, Madison, Bley & Fox, 1988) the effect of DAG and PKC activation attracts most interest. This follows upon the demonstration of a direct modulatory effect of PKC activation on the opening (Lacerda, Rampe & Brown, 1988) or number (Strong, Fox, Tsien & Kaczmarek, 1987) of functional voltage-dependent calcium

channels. It would appear then that in the activation of 'the dual-signalling' pathway the relative significance of each of the two second messengers with regard to calcium homeostasis will be determined by several factors including the availability of the molecular substrates for $Ins1,4,5P_3$ and PKC activity, i.e. internal stores and/or voltage-dependent calcium channels respectively.

In the present study we investigated the calcium-mobilizing effects of two peptides whose receptors are recognized to operate through phosphoinositide breakdown, substance P and bombesin (Hanley, Lee, Jones & Michell, 1980; Heslop, Blakeley, Brown, Irvine & Berridge, 1988), on a homogenous cell population. The cells are a clonal line of AR42J cells, originally derived from a tumour of the rat exocrine pancreas. These cells have functional receptors for substance P and bombesin which give rise to an elevation in $[Ca^{2+}]_i$ (Womack, Hanley & Jessell, 1985; Horstman, Takemura & Putney, 1988). The study shows for the first time, however, that the mechanism underlying these calcium signals is very different for each of these peptides. The data indicate that substance P regulates, in functional terms, predominantly if not exclusively for calcium influx via voltage-dependent calcium channels opened as the consequence of PKC activation. Bombesin by contrast promotes the release of calcium from internal stores in the absence of any detectable calcium influx, an effect in keeping with its production of $Ins1,4,5P_3$. The implication of the data is that a molecular specialization exists in a single cell population which allows for the discrete and separate regulation of the PKC and $Ins1,4,5P_3$ wings of the presumptive dual-signalling pathway by substance P and bombesin receptors respectively.

METHODS

Cell culture

AR42J cells were grown in L-15 CO_2 medium supplemented with penicillin–streptomycin (50 units/ml), glutamine (2 mM), glucose (44 mM), essential vitamins and 20% fetal calf serum at 37 °C in 95% air, 5% CO_2. This is essentially as previously reported (Womack *et al.* 1985). Cells were fed every day and passaged at 70% confluence with 0·05% trypsin in Ca^{2+}–Mg^{2+}-free media. These cells have been repeatedly subcloned by limiting dilution, and are a stable, clonal population.

Calcium measurements

AR42J cells were gently detached by aspiration and resuspended in a standard saline (mM): NaCl, 140; KCl, 4·7; HEPES, 10; MgCl, 1·13; glucose, 10; and $CaCl_2$, 1·0. Cells were spun down and resuspended in identical media containing 0·5 μM-Fura-2 AM (Molecular Probes, USA) for 30 min at room temperature. At the end of this period the cells were spun down, resuspended in saline and transferred to a chamber (volume 0·15 ml) mounted on the stage of a Nikon Diaphot microscope. The cells were continuously superfused (0·5 ml/min) in a stream of media issuing from any one of six inlet tubes converging into the chamber. The Ca^{2+}-free solution had no Ca^{2+} but 1 mM-EGTA added. All other modifications to control media are indicated in the figures. Solutions were superfused at 30 °C.

$[Ca^{2+}]_i$ was measured in single cells as previously reported (Grynkiewicz, Poenie & Tsien, 1985; Yule & Gallacher, 1988). Briefly, alternating excitation wavelengths, at not less than 10 Hz, of 340 and 380 nm were employed and emission at 505 nm monitored. $[Ca^{2+}]_i$ was calculated from the ratio (R) of the fluorescence at the two different wavelengths according to the formula:

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

where $K_d = 225$ nM, $R_{max} = 8{\cdot}2 \pm 0{\cdot}57$ ($n = 10$), $R_{min} = 0{\cdot}43 \pm 0{\cdot}07$ ($n = 10$) and $\beta = 4{\cdot}2 \pm 0{\cdot}26$ ($n = 10$). All records were corrected for autofluorescence at each wavelength. Values of $[Ca^{2+}]_i$ are expressed as mean ± S.E.M.

Patch-clamp current recording

Whole-cell and single-clamp current recording modes were utilized (Hamill, Marty, Neher, Sakmann & Sigworth, 1981). An LM-EPC5 (List Electronics, Darmstadt, FRG) patch-clamp amplifier was employed and the signal displayed on a storage oscilloscope and simultaneously

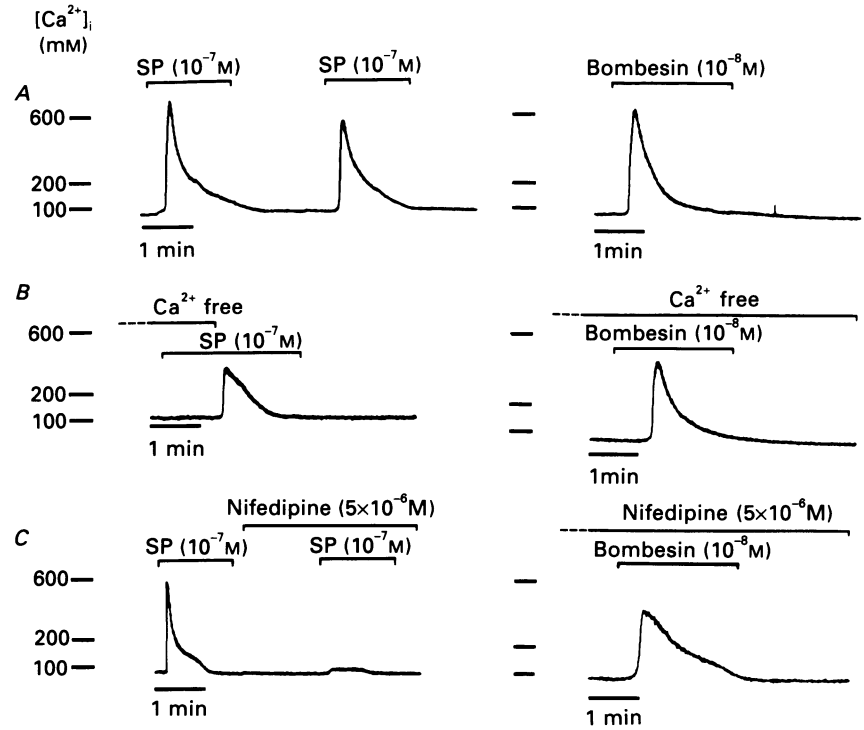

Fig. 1. The traces show the changes in cytosolic $[Ca^{2+}]_i$ (nM) in single AR42J acinar cells evoked by application of substance P (SP, on the left) and bombesin (on the right). *A*, typical transient responses to SP and bombesin in AR42J cells bathed in control saline. *B*, the trace on the left shows the failure of SP to evoke a response in a cell bathed in Ca^{2+}-free extracellular solution. The SP response is restored when Ca^{2+} is readmitted to the extracellular solution in the continued presence of the agonist. The trace on the right shows the persistence of the response to bombesin in Ca^{2+}-free media. *C*, nifedipine blocks the SP but not the bombesin response.

recorded for analysis on magnetic tape (Racal 4DS, Southampton, UK). For analysis the current recordings were digitized at 8 kHz (CED 1401, Cambridge, UK) for input to a Tandon microcomputer which displayed and processed digitized data for substraction of leakage and capacitive currents (softwear, CED Cambridge, UK). The fabrication and characteristics of the patch pipettes are as previously reported (Gallacher, Maruyama & Petersen, 1984; Gallacher & Morris, 1986). The standard extracellular solution (indicated as solution E_a in the figure legends) contained the following (mM): NaCl, 140; KCl, 4·8, $MgCl_2$, 1·13; HEPES, 10; glucose, 10; EGTA, 1·0; tetraethylammonium (TEA), 10 at pH 7·2. In addition extracellular solutions containing 10 mM-$BaCl_2$ (indicated E_b), 100 mM-$BaCl_2$ (indicated E_c) or 140 mM-KCl (indicated E_d) have been employed in certain experiments as detailed in the figure legends.

Solution E_b = E_a + 10 mM-$BaCl_2$.

Solution E_c: 110 mM-$BaCl_2$ replaces all NaCl.

Solution E_d: 140 mM-KCl replaces all NaCl, no TEA.

The control intracellular solution (indicated as solution I_a in the figure legends) was of the

following composition (mM): potassium glutamate, 99; KCl, 40; $MgCl_2$, 1·13; HEPES, 10; glutamate, 10; EGTA, 10; ATP, 1·0; ATP-γ-S, 1·0; GTP, 0·1 at pH 7·2. To facilitate recording of small currents due to calcium channel activation a high-Ba^{2+} solution (indicated as solution I_b in the figure legends) which contained Na^+ and K^+ channel blockers was employed in certain

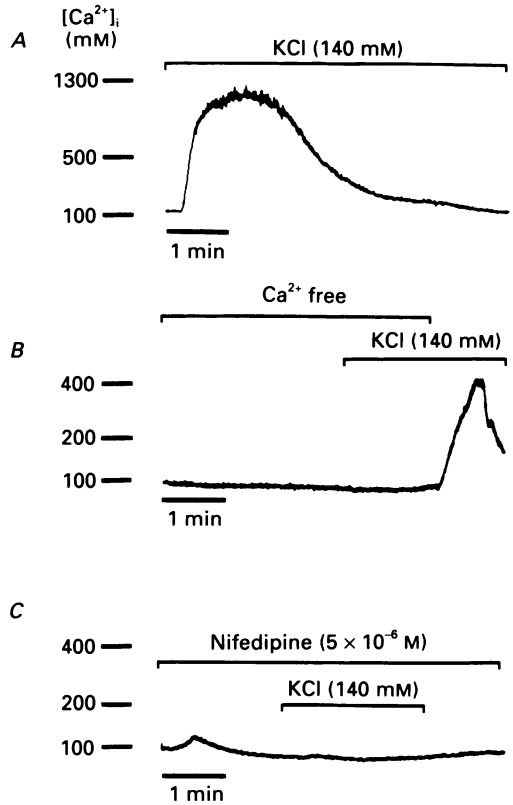

Fig. 2. *A* shows the increase in $[Ca^{2+}]_i$ induced upon changing the superfusate from control saline to a solution in which K^+ (140 mM) replaces Na^+. *B*, the high-K^+ solution fails to evoke any change in $[Ca^{2+}]_i$ in a Ca^{2+}-free solution. If Ca^{2+} is readmitted in the presence, but not absence, of the high-K^+ solution the increase in $[Ca^{2+}]_i$ is restored. *C*, nifedipine blocks the increase in $[Ca^{2+}]_i$ in response to high-K^+ solutions.

protocols. Solution I_b had the following composition (mM): $BaCl_2$, 105; HEPES, 10; tetrodotoxin (TTX), 0·01; TEA, 10 at pH 7·2. All experiments were carried out at between 22 and 24 °C.

RESULTS

Elevations in $[Ca^{2+}]_i$ *in single AR42J cells stimulated by substance P and bombesin are mediated by separate mechanisms*

The mean basal $[Ca^{2+}]_i$ in unstimulated AR42J cells was 105 ± 5 nM ($n = 46$), a value very close to that reported for enzymatically isolated mature rodent pancreatic acinar cells (Yule & Gallacher, 1988). Figure 1 shows the changes in $[Ca^{2+}]_i$ induced in single AR42J cells upon stimulation by either of the peptide agonists, substance P or bombesin. The responses to both agonists are marked but transient and within the first few minutes of stimulation, in the continued presence of the agonists, $[Ca^{2+}]_i$

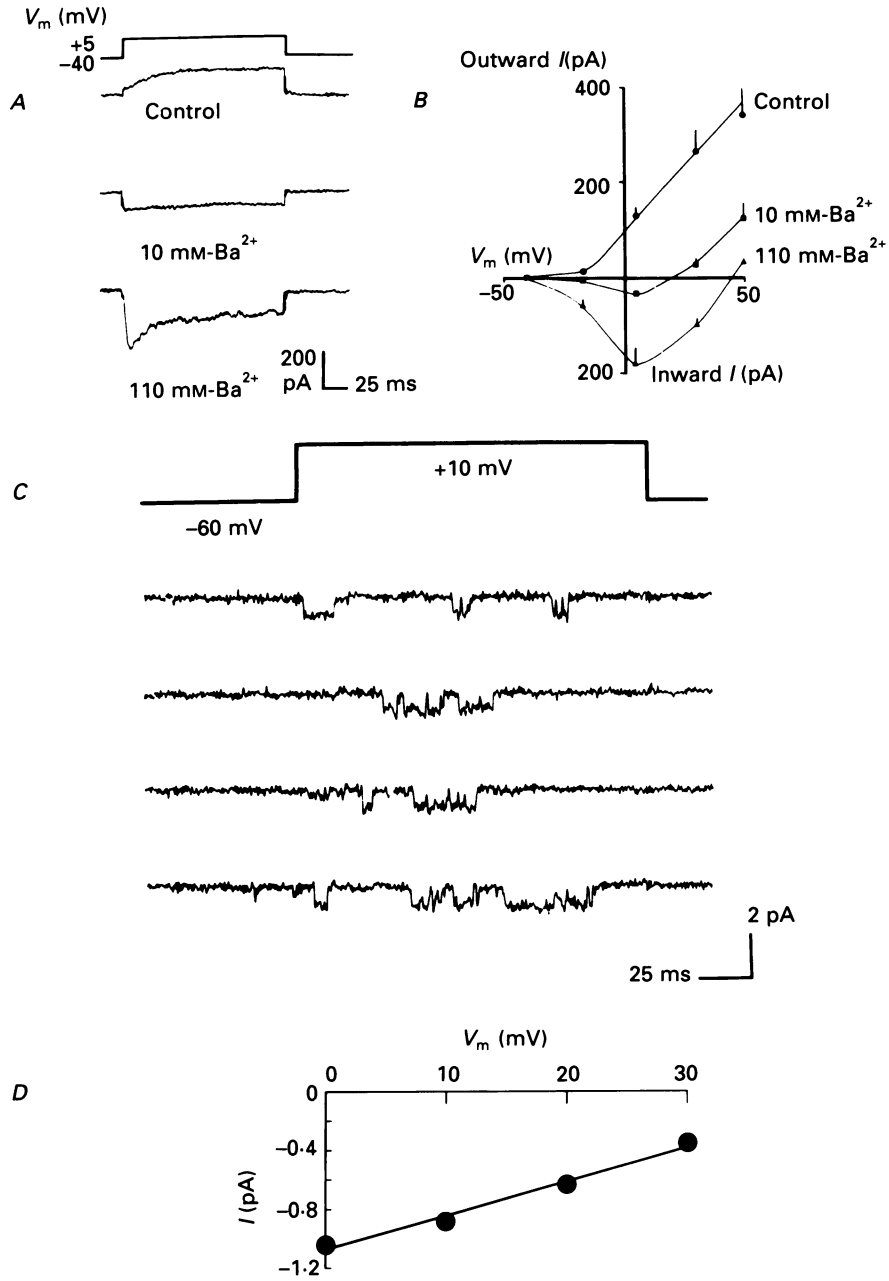

Fig. 3. *A*, whole-cell current recording from a single AR42J cell. The recording pipette is filled with a high-K^+ solution (solution I_a, see Methods). Depolarizing voltage steps of 45 mV are imposed from a holding potential of −40 mV as indicated by the top trace. The three traces below show the whole-cell currents evoked. The first (top) of these traces shows the current evoked by depolarization in a cell bathed in a control saline (solution E_a, see below). The middle trace is obtained after the external solution has been changed to one containing 10 mM-Ba^{2+} (solution E_b). The lower trace is for the cell now bathed in a solution in which 110 mM-Ba^{2+} replaces Na^+ (solution E_c). The introduction of 10 mM-Ba^{2+} is associated with the appearance of an inward current and this is greatly augmented upon increasing [Ba^{2+}] to 110 mM. *B*, whole-cell current–voltage relationship

returns to prestimulus levels. This is in contrast to the sustained increase in $[Ca^{2+}]_i$ recently demonstrated in single, acutely isolated adult rodent pancreatic acinar cells (Yule & Gallacher, 1988). The changes in $[Ca^{2+}]_i$ evoked by substance P and bombesin were qualitatively and quantitatively very similar, $[Ca^{2+}]_i$ rising to mean maxima of 590 ± 40 nM ($n = 27$) and 540 ± 75 nM ($n = 21$) for maximal doses of substance P (10^{-7} M) and bombesin (10^{-8} M) respectively. As shown in Fig. 1*B* the present study confirms the previous report (Horstmann *et al.* 1988) that the substance P-evoked elevation in $[Ca^{2+}]_i$ is dependent on external Ca^{2+}. In the absence of external Ca^{2+} substance P failed to, or evoked only very small, elevations in $[Ca^{2+}]_i$ ($n = 8$). Removal of extracellular Ca^{2+} revealed the first important difference between the substance P and bombesin effects. The elevations in $[Ca^{2+}]_i$ in response to bombesin in Ca^{2+}-free media were not significantly different from those evoked in control media (mean maximal increase was to 440 ± 30 nM from a basal level of 95 ± 10 nM, $n = 23$) whereas substance P evoked no response. Accordingly the elevations in $[Ca^{2+}]_i$ in response to substance P and bombesin are mediated by different mechanisms. Substance P apparently exclusively controls calcium influx from the external solution whereas bombesin regulates internal calcium release in the absence of influx. This difference in the mode of action of substance P and bombesin is also reinforced by the different effects of the two peptides when applied for a second time after the initial response. Substance P evoked successive increases in $[Ca^{2+}]_i$ with only a few minutes between applications (see figures) whereas bombesin reapplied after the first stimulation ($n = 9$) was never associated with a second response (even when tested 30 min after the first response; $n = 3$). To characterize the nature of the calcium influx induced by substance P we evaluated the effect of the widely used calcium channel (L-type) antagonist, nifedipine (Tsien *et al.* 1988). As Fig. 1 shows nifedipine (5×10^{-6} M) abolished the substance P-evoked $[Ca^{2+}]_i$ increase in the AR42J cells ($n = 9$), whereas the bombesin-induced increase in $[Ca^{2+}]_i$ was unaffected (average maximal increase was to 470 ± 50 nM, $n = 7$). The effect of nifedipine on the substance P-induced calcium influx was surprising in view of the

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for the series of experiments illustrated in *A*. The data plotted are means ± S.E.M. for seven cells. Since E_K and E_{Cl} are both negative (about −80 mV and −30 mV respectively) in all three external solutions the outward currents observed, particularly in the control experiments, could be carried either by K^+ or Cl^-. In a series of experiments where intracellular KCl was replaced by CsCl the outward currents were reduced by 80% (142 ± 16 pA, $n = 7$) in K^+ solution at +5 mV compared to 25 ± 7·5 pA ($n = 11$) for Cs^+ solution at the same potential. The major component of outward current is thus carried by K^+. *C*, recording of single-channel barium currents in cell-attached patch of membrane on AR42J cell. The cell is bathed in 140 mM-KCl solution (solution E_d) containing no added Ca^{2+} and 1 mM-EGTA. The recording pipette was filled with a 105 mM-$BaCl_2$ solution (I_b) containing 1 μM-TTX and 10 mM-TEA. Due to the high-K^+ solution in the bath the cell is depolarized to, or very close to, 0 mV. The recording pipette is voltage clamped to restore a gradient of −60 mV (inside with respect to outside) across the electrically isolated patch of membrane. Depolarizing voltage jumps of 70 mV from this holding potential achieve a membrane potential of +10 mV during the pulse (indicated by top trace). The four traces (from one cell) demonstrate the single-channel barium currents that are recorded during the depolarizing voltage jumps to +10 mV but never seen at the holding potential of −60 mV, i.e. voltage-activated inward (barium) currents. Leakage and capacitive currents have been subtracted. *D*, the quasilinear current–voltage plot obtained for single channel currents recorded in cell-attached patches.

ample literature reporting the absence of voltage-gated calcium channels in mature exocrine acinar cells (see Petersen, 1980; Gallacher, 1988; Petersen & Gallacher, 1988). The effect of voltage activation through depolarizing (high-K^+) solutions on $[Ca^{2+}]_i$ was therefore tested. Figure 2 shows that these depolarizing solutions evoked a dramatic, but transient, elevation in $[Ca^{2+}]_i$ (to a maximum of 1160 ± 45 nM, $n = 6$). As for the substance P-induced responses the increase in $[Ca^{2+}]_i$ in high-K^+ solutions was dependent on extracellular calcium ($n = 4$) and blocked by nifedipine ($n = 4$). These experiments provide consistent evidence that the substance P-induced calcium influx is most likely proceeding through receptor-coupled activation of voltage-gated (L-type) calcium channels in the surface membranes of AR42J cells. This was evaluated directly by patch-clamp methodology.

Expression, and regulation by substance P, of voltage-gated calcium channels in AR42J cells

The patch-clamp electrophysiological techniques of whole-cell and single-channel current recording (Hamill *et al.* 1981) were employed to pursue the identification and regulation of voltage-gated calcium channels in the surface membranes of AR42J cells. Barium (Ba^{2+}) was employed as the charge carrier to enhance the amplitude of the currents through calcium channels and to reduce the outward currents which tend to obscure the smaller inward current component (Reuter, 1983). Figure 3*A* shows the whole-cell current in response to a depolarizing voltage pulse and demonstrates that the introduction of Ba^{2+} to the extracellular fluid was associated with the appearance of an inward current. The amplitude of the inward current was greatly enhanced upon increasing the $[Ba^{2+}]$ in the external solution from 10 to 110 mM. Figure 3*B* shows the current–voltage relationship for whole-cell currents in the absence and in the presence of 10 mM- and 110 mM-Ba^{2+}. Voltage-gated inward currents in the presence of barium could also be resolved at the single-channel level. Figure 3*C* shows examples of single-channel barium currents recorded in cell-attached patches of surface membrane. Such currents were seen in seven of the eighteen patches where this protocol was applied. These large unitary inward currents were only seen during depolarizing voltage jumps and not when the patch membrane was held at more negative potentials. It can be seen that the channels in this, as in the other patches, show little, if any, evidence of inactivation during the 250 ms duration of the depolarizing jumps. Figure 3*D* shows the quasilinear current–voltage relationship of the single-channel currents. The mean single-channel conductance determined from four cell-attached patches was $25{\cdot}8 \pm 1{\cdot}1$ pS. These findings are consistent with activation of L-type voltage-dependent Ca^{2+} channels (see Tsien *et al.* 1988). In the presence of nifedipine (5×10^{-6} M) the voltage-activated whole-cell inward currents were abolished ($n = 3$; Fig. 4). The effect of substance P (10^{-7} M) was to increase the amplitude of the voltage-activated whole-cell inward current ($n = 10$; Fig. 4). The effects of these agents are apparently selective for the inward current component with neither the holding nor outward current amplitude being affected. This does not exclude an effect of substance P on resting K^+ current in intact cells, since this is most probably blocked by the Ba^{2+} employed in whole-cell experiments. Indeed substance P has previously been shown to depolarize intact AR42J cells by an as yet uncharacterized ionic mechanism

(Womack *et al.* 1985). The electrophysiological experiments thus confirm the presence of voltage-gated Ca^{2+} channels and clearly indicate a role for substance P in their modulation.

Protein kinase C and the regulation of voltage-gated calcium channels

The failure of substance P to achieve, under these conditions, any significant release of Ca^{2+} from the internal stores would indicate that the substance P effects

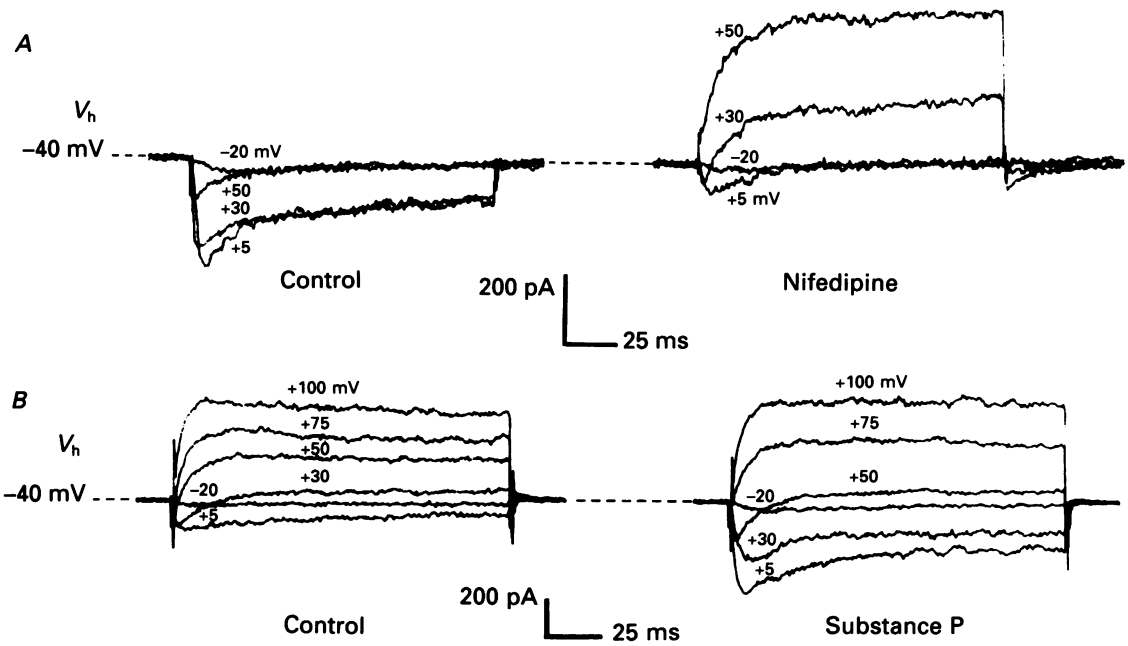

Fig. 4. Whole-cell current recording from two AR42J cells. The recording pipette is filled with a high-K^+ solution (solution I_a). *A*, on the left are shown the whole-cell currents evoked for a range of depolarizing voltage jumps from a holding potential of −40 mV. The cell is bathed in the 110 mM-$BaCl_2$ solution (solution E_c). On the right nifedipine has been added to the solution bathing the cell blocking the inward currents. Typical of three such experiments. *B*, on the left are shown the whole-cell currents evoked for a range of depolarizing jumps from −40 mV. The cell is bathed in the 10 mM-$BaCl_2$ solution (solution E_b). On the right recordings from the same cell after substance P (10^{-7} M) had been added to the external solution. An increase in inward current is seen. Typical of ten such experiments.

on Ca^{2+} channels are unlikely to be mediated through Ins1,4,5P_3. We tested the possible involvement of protein kinase C in the regulation of cell surface membrane permeability of Ca^{2+} ions. As shown in Fig. 5 the phorbol diester phorbol 12-myristate 13-acetate (PMA; 10^{-7} M), an activator of protein kinase C (Nishizuka, 1984), induced a marked, but transient, elevation in $[Ca^{2+}]_i$ to a maximum of 280 ± 35 nM ($n = 6$). Another phorbol diester, 4-phorbol, 12,13-didecanoate (4α-PDD), which does not activate protein kinase C, did not give rise to any change in $[Ca^{2+}]_i$ (Fig. 5*B*; $n = 6$). The elevation of $[Ca^{2+}]_i$ induced by PMA was abolished in

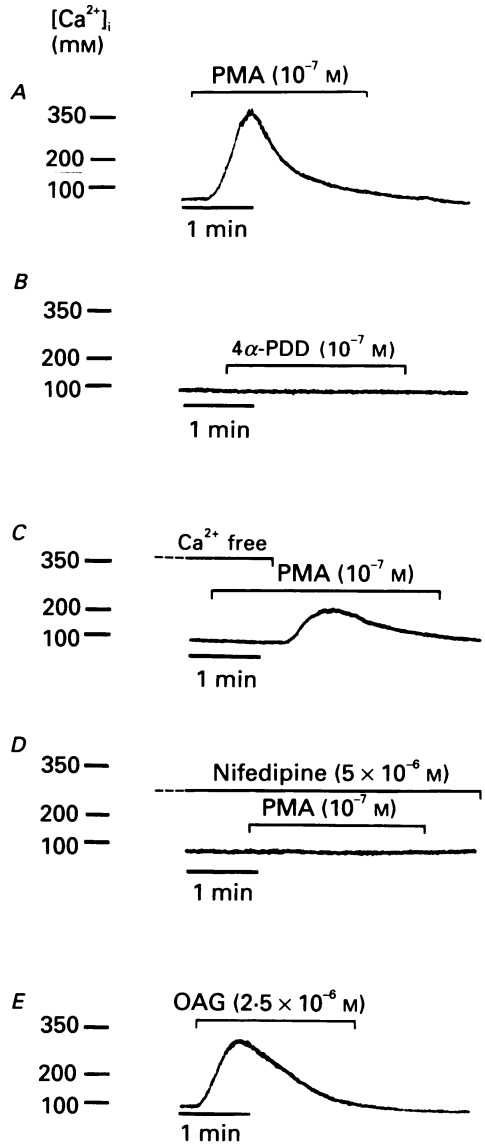

Fig. 5. Measurements of $[Ca^{2+}]_i$ in single AR42J cells. *A*, increase in $[Ca^{2+}]_i$ induced by the protein kinase C activator, the phorbol ester PMA. *B*, 4α-PDD cannot mimic the effect of PMA. *C*, the effect of PMA is dependent on the presence of Ca^{2+} in the extracellular solution. *D*, the effect of PMA on $[Ca^{2+}]_i$ is blocked by nifedipine. *E*, the effect of PMA is mimicked by OAG, a permeable analogue of diacylglycerol. All traces obtained in cells bathed in control saline. Any modifications are indicated on the traces.

the absence of extracellular Ca^{2+} (Fig. 4*C*; $n = 5$) or in the presence of nifedipine (Fig. 4*D*; $n = 4$). The cell-permeable analogues of diacylglycerol, 1-oleoyl-2-acetyl-*sn*-glycerol (OAG) and *sn*-2-dioctanoyl glycerol (DiC_8; Davis, Ganong, Bell & Czech, 1985) which mimic endogenous diacylglycerol in activation of protein kinase C both evoked increases in $[Ca^{2+}]_i$. The maximal increases were to 255 ± 25 nM ($n = 59$) and

250 ± 30 nM ($n = 5$) for OAG (Fig. 5*E*) and DiC_8 (not shown) respectively. Polymyxin B is reported to inhibit selectively the activation of protein kinase C in intact cells, including secretory cell types such as adrenal chromaffin cells (Wakade, Malhotra & Wakade, 1986), pancreatic islet cells (Stuchfield, Jones & Howell, 1986) and

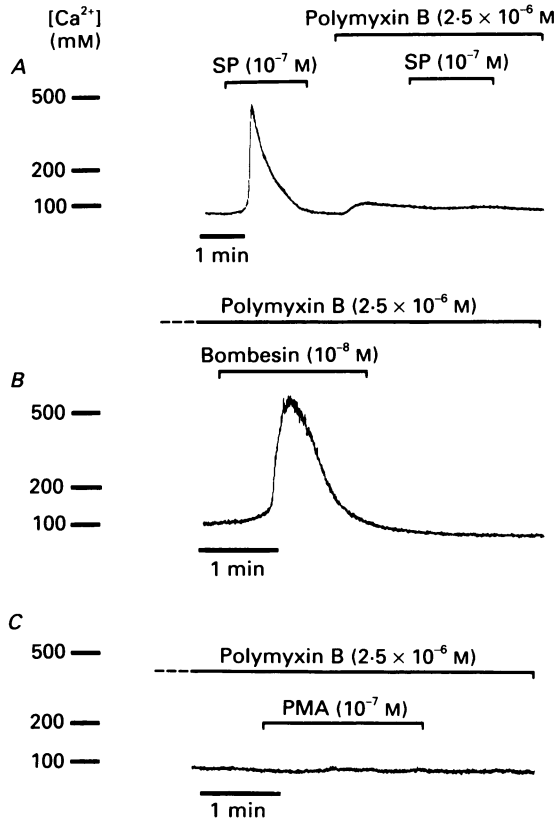

Fig. 6. $[Ca^{2+}]_i$ in single AR42J cells. *A*, the effect of pre-incubation (2 min prior to stimulation) with polymyxin B. The response to substance P (SP) is absent. *B*, in identical protocols polymyxin B failed to block the increase in $[Ca^{2+}]_i$ induced by bombesin. *C*, polymyxin B blocks the effect of PMA on $[Ca^{2+}]_i$.

pancreatic acinar cells (Wooten & Wrenn, 1984). As shown in Fig. 6 polymyxin B applied 2 min prior to stimulation blocked totally the increases in $[Ca^{2+}]_i$ evoked by substance P or PMA. In identical protocols bombesin in the presence of polymyxin B evoked increases in $[Ca^{2+}]_i$ (maximum 455 ± 35 nM, $n = 5$) that were not significantly different from controls.

DISCUSSION

The AR42J cell line maintains many differentiated properties of pancreatic acinar cells, but it is recognized to differ significantly from the normal parent population in several aspects. For example, the present study indicates that the AR42J cells ectopically express a classical gene product of excitable cells: voltage-dependent

calcium channels. Neither the substance P nor voltage-dependent calcium channels are to be found in the adult rodent pancreatic acinar cells (Petersen, 1980; Petersen & Gallacher, 1988). The study establishes a clear link between the substance P receptors and the regulation of the voltage-dependent calcium channel and this raises the possibility that the ectopic co-expressions of substance P receptors and L-type calcium channels are co-ordinate phenomena in this, and possibly other, cell systems. In particular the AR42J cells may mimic a neuronal phenotype and suggest possible mechanisms of substance P action in normal neurones (Womack, MacDermott & Jessell, 1988). The evidence of this study strongly suggests, but does not unequivocally establish, that the substance P receptor regulation of the L-type calcium channels is mediated by activation of PKC. This is not a novel hypothesis and effects of PKC activation on voltage-dependent calcium channels have been reported in recent studies (Strong *et al.* 1987; Lacerda *et al.* 1988). Of particular interest is the minimal effect of substance P to promote the release of calcium from intracellular stores in this AR42J, pancreatic acinar cell clone. The existence of receptor-regulated internal calcium stores in the AR42J cells is clearly indicated by the ability of bombesin to markedly elevate $[Ca^{2+}]_i$ in the absence of extracellular calcium. The store is then accessed by bombesin but not by substance P. Since Ins1,4,5P_3 is the most effective endogenous activator of calcium release in exocrine acinar cells (Streb, Irvine, Berridge & Schulz, 1983; see also Putney, Takemura, Hughes, Horstman & Thastrup, 1989) the effect of bombesin on intracellular calcium release is likely to be secondary to the production of this cytosolic messenger. The separation of the effects of the two peptides is not, however, consistent with a simple scheme whereby both substance P and bombesin activate an obligate 'dual-signalling' pathway that is initiated upon hydrolysis of PIP_2. The data indicate that other regulatory mechanisms must come into play to elicit biasing towards one or other wing of the bifurcating pathway: substance P receptors biased towards PKC activation, and bombesin receptors biased towards inositol polyphosphate production and intracellular calcium discharge. The manner in which this is achieved is not at all clear though there is a report that bombesin is four to five times more effective than substance P in stimulating inositol phosphate metabolism in the AR42J cells (Hanley & Too, 1987). It is also possible that substance P receptors regulate for the production of DAG, to activate PKC, from a source other than by hydrolysis of PIP_2 and one source could be the choline phospholipids (see Loffelholz, 1989). This would imply that the substance P receptors regulate the activity of a phospholipase other than phospholipase C. The mechanism is not clear from the present study but the characterization of the AR42J cells as a clonal system exhibiting marked functional polarization in the substance P and bombesin transduction pathways suggests that this exocrine acinar cell line will be a valuable model to investigate the manner in which different phospholipase-coupled receptors are specialized to give distinctive cellular responses.

There is one further aspect of the AR42J cells which is most pertinent to the present study. A recent report (Horstman *et al.* 1988) has described the calcium influx in AR42J cells stimulated by substance P. This study also reported the absence in these cells of the Ins1,4,5P_3-3-kinase which converts Ins1,4,5P_3 to Ins1,3,4,5P_4, a candidate mediator of calcium influx in acinar cells (Morris *et al.* 1987;

Changya *et al.* 1989*a*, *b*). These authors (Horstman *et al.* 1988) assumed that the calcium influx they had demonstrated would be via the second messenger-operated (inositol polyphosphate-induced) pathway that is typical of electrically non-excitable cellular systems, such as exocrine acinar cells. Horstman *et al.*'s conclusion was that in demonstrating a calcium influx in the absence of Ins1,3,4,5P_4 they had excluded for any essential or universal role for Ins1,3,4,5P_4 in the gating of calcium influx in these cellular systems. Our study does not confirm or exclude for the presence of Ins1,4,5P_3-3-kinase in the AR42J cells. However, in establishing that the substance P receptor-regulated calcium influx is via voltage-dependent calcium channels, and not the second messenger-operated mechanism assumed by Horstman *et al.* (1988), it is clear that the conclusions made by these authors with regard to any role of Ins1,3,4,5P_4 in that pathway are invalid. Importantly it is also to be noted that bombesin can release calcium from intracellular stores in AR42J cells but that it does this is in the absence of the sustained calcium influx which is a characteristic of the mature rodent acinar cell (Yule & Gallacher, 1988). This ability of bombesin to promote a marked release of calcium in the absence of influx of calcium indicates, at least for the AR42J cells, (a) that the activator of calcium release does not promote calcium influx, and (b) that the release of calcium from internal pools is not in itself a stimulus or effector of calcium entry. This is a clear demonstration that there is no obligatory coupling of the calcium release and calcium entry mechanisms as has been suggested (Putney, 1986). Also of relevance is the observation that in the time course of the experiments performed in the present study (up to 30 min), a second application of bombesin failed to evoke any further calcium release. This was the situation even when calcium was present in the extracellular fluid throughout and $[Ca^{2+}]_i$ had returned to basal levels between stimuli. In mature pancreatic acinar cells the refilling of the internal stores is automatic in the presence of external calcium, upon removal of the agonist (Yule & Gallacher, 1988). It appears then that the AR42J cells lack both a second messenger-operated calcium influx and also a mechanism to refill the internal calcium stores depleted by bombesin. A role for Ins1,3,4,5P_4 in the refilling of calcium stores has been reported in permeabilized hepatocytes (Hill, Dean & Boynton, 1988) and Changya *et al.* (1989*a*, *b*) have suggested that Ins1,3,4,5P_4, in addition to calcium influx, has a role to play in communication of Ins1,4,5P_3-sensitive and insensitive calcium stores in lacrimal acinar cells. Whatever the deficit, it is clear that the bombesin receptors on the AR42J cells have lost the ability to regulate calcium influx across the cell surface membrane for entry into both the cytosol and Ins1,4,5P_3-sensitive store. The same defect may explain both phenomena if calcium entry to the cytosol is achieved via the internal stores (Putney, 1986; Gallacher, 1988).

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