A diacylglycerol-activated Ca^{2+} channel in PC12 cells (an adrenal chromaffin cell line) correlates with expression of the TRP-6 (transient receptor potential) protein

Yordanos TESFAI, Helen M. BRERETON and Greg J. BARRITT¹

Department of Medical Biochemistry, School of Medicine, Faculty of Health Sciences, Flinders University, G.P.O. Box 2100, Adelaide, South Australia, 5001, Australia

The structures, and mechanisms of activation, of plasma membrane intracellular-messenger-activated, non-selective cation channels in animal cells are not well understood. The PC12 adrenal chromaffin cell line is a well-characterized example of a nerve cell. In PC12 cells, 1-oleoyl-2-acetyl-*sn*-glycerol (OAG), a membrane-permeant analogue of diacylglycerol, initiated the inflow of Ca^{2+} , Mn^{2+} and Sr^{2+} . Acetylcholine and thapsigargin initiated the inflow of Ca^{2+} and Mn^{2+} , but not of Sr^{2+} . The activation of bivalent cation inflow by OAG: (i) was mimicked by another membrane-permeant diacylglycerol analogue, 1,2-dioctanoyl-*sn*-glycerol, but not by the membrane-impermeant analogue 1-stearoyl-2-arachidonoyl-*sn*-glycerol; (ii) was not blocked by staurosporin or chelerythrine, inhibitors of protein kinase C; (iii) was enhanced by RHC80267 and R50922, inhibitors of diacylglycerol lipase and diacylglycerol kinase respectively; and (iv) was inhibited by extracellular Ca^{2+} . When OAG was added after acetylcholine, the effect of OAG on Ca^{2+} inflow was over-and-above that induced by acetylcholine. 2-Aminoethyl diphenylborate (2-APB) inhibited Ca^{2+} inflow

initiated by either acetylcholine or thapsigargin, but not that initiated by OAG. Flufenamic acid inhibited OAG-initiated, but not acetylcholine-initiated, Ca^{2+} and Mn^{2+} inflow. OAG-initiated Ca^{2+} inflow was less sensitive to inhibition by SK&F96365 than acetylcholine-initiated Ca^{2+} inflow. In polyadenylated RNA prepared from PC12 cells, mRNA encoding TRP (transient receptor potential) proteins 1–6 was detected by reverse transcriptase (RT)-PCR, and in lysates of PC12 cells the endogenous TRP-6 protein was detected by Western blot analysis. It is concluded that PC12 cells express a diacylglycerol-activated, non-selective cation channel. Expression of this channel function correlates with expression of the TRP-3 and TRP-6 proteins, which have been shown previously to be activated by diacylglycerol when expressed heterologously in animal cells [Hofmann, Obukhov, Schaefer, Harteneck, Gudermann, and Schultz (1999) *Nature* (London) 397, 259–263].

Key words: acetylcholine, 2-aminoethyl diphenylborate, manganese inflow, protein kinase C, thapsigargin.

INTRODUCTION

Store-operated Ca^{2+} channels (SOCs) and intracellular-messenger-activated, non-selective cation channels (NSCCs) have a major role in regulating the concentration and spatial distribution of hormone- and neurotransmitter-induced increases in the cytoplasmic free Ca^{2+} concentration ($[\text{Ca}^{2+}]_{\text{cyt}}$) in nerve cells and in other animal cells [1–4]. PC12 cells, an adrenal chromaffin cell line, are a well-characterized model of nerve cells [2,3,5]. PC12 cells possess SOCs, which admit Ca^{2+} and Mn^{2+} ions and can be activated by thapsigargin, an inhibitor of the $\text{Ca}^{2+}/\text{Mg}^{2+}$ -ATPase of the endoplasmic reticulum, and caffeine, which binds to ryanodine receptors in the endoplasmic reticulum [2,3]. NSCCs in PC12 cells have not been well defined or characterized, although these channels have important roles in the physiology of nerve cells [2,3,5,6].

Recent studies of SOCs in a variety of animal cells have increased knowledge of the nature, mechanism(s) of activation and possible structures of these plasma membrane Ca^{2+} channels (for a review, see [7]). However, the subtypes, structures and mechanisms of activation of NSCCs are not well understood (reviewed in [4]). The results of experiments conducted with the TRP (transient receptor potential) family of proteins have suggested that some TRP proteins may be the molecular counter-

parts of SOCs and NSCCs in animal cells [8–12]. The TRP protein is expressed in the photoreceptor cell of *Drosophila melanogaster*, where it acts as an NSCC in the pathway of phototransduction. Seven TRP-related proteins have been identified in animal cells (reviewed in [8–10]). These are expressed at the highest concentrations in nerve cells, but also appear to be expressed at low levels in several other cell types [9,10]. When expressed heterologously, most TRP proteins are NSCCs with a relatively low selectivity for Ca^{2+} compared with Na^{+} [9,10]. A distinctive feature of TRP proteins 3 and 6 is their activation by diacylglycerol via a mechanism that is independent of the activation of protein kinase C. This has been demonstrated using TRP-3 and TRP-6 heterologously expressed in animal cells [13,14]. These observations suggested that TRP-3 and/or TRP-6 may be the molecular counterparts of endogenous diacylglycerol-activated NSCCs in animal cells.

The aims of the present study were to determine whether PC12 cells possess diacylglycerol-activated bivalent cation channels, and whether the presence of these functional channels correlates with expression of the TRP-3 and/or TRP-6 proteins. The results have shown that PC12 cells exhibit diacylglycerol-activated bivalent cation channels which, on the basis of bivalent cation selectivity and susceptibility to pharmacological inhibitors,

Abbreviations used: 2-APB, 2-aminoethyl diphenylborate; $[\text{Ca}^{2+}]_{\text{cyt}}$, cytoplasmic free Ca^{2+} concentration; DOG, 1,2-dioctanoyl-*sn*-glycerol; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; NGF, nerve growth factor; NSCC, non-selective cation channel; OAG, 1-oleoyl-2-acetyl-*sn*-glycerol; RT-PCR, reverse transcriptase-PCR; SAG, 1-stearoyl-2-arachidonoyl-*sn*-glycerol; SOC, store-operated Ca^{2+} channel; (m/h)TRP, (mouse/human) transient receptor potential.

¹ To whom correspondence should be addressed (e-mail Greg.Barritt@flinders.edu.au).

are different from acetylcholine- and thapsigargin-activated SOC. Expression of these diacylglycerol-activated Ca^{2+} channels correlated with the expression of mRNA encoding the TRP-3 and TRP-6 proteins, and with the presence of the TRP-6 protein.

MATERIALS AND METHODS

Materials

1-Oleoyl-2-acetyl-*sn*-glycerol (OAG), 1,2-dioctanoyl-*sn*-glycerol (DOG), 1-stearoyl-2-arachidonoyl-*sn*-glycerol (SAG), staurosporin, sulphinyprazole and 2.5 S nerve growth factor (NGF) were obtained from the Sigma Chemical Co., St Louis, MO, U.S.A., and RHC80267 and R50922 were from Alexis Biochemicals, San Diego, CA, U.S.A. All other reagents were obtained from the sources described previously [15,16].

Cell culture

PC12 cells (A.T.C.C. CRL 1721) were cultured at 37 °C in 5% CO_2 in Dulbecco's modified Eagle's medium supplemented with 10% (v/v) horse serum, 5% (v/v) fetal bovine serum, penicillin (100 units/ml) and streptomycin (100 $\mu\text{g}/\text{ml}$), as described previously [5]. Cell lines were subcultured for a maximum of 30 passages. The neuronal phenotype of PC12 cells was induced by incubation of the cells for 48 h in the presence of NGF (100 ng/ml) [17].

Measurement of Ca^{2+} , Sr^{2+} and Mn^{2+} inflow

The inflow of Ca^{2+} , Sr^{2+} and Mn^{2+} to PC12 cells was measured using the intracellular fluorescent indicator fura-2 to measure changes in the cytoplasmic concentration of the cations, following the procedures described previously by Clementi et al. [5]. Cells were grown to 60–80% confluence in 75 cm^2 plastic flat-bottomed flasks, and then detached by applying a gentle flow of 10 ml of Krebs–Ringer–Hepes medium [125 mM NaCl/5 mM KCl/1.2 mM KH_2PO_4 /1.2 mM MgSO_4 /1.2 mM CaCl_2 /6 mM glucose/25 mM Hepes/NaOH buffer (pH 7.4)]. The cell suspension was centrifuged at 300 g for 5 min at room temperature and the cells washed once with Krebs–Ringer–Hepes medium. The cells were then resuspended (2×10^6 cells/ml) in 2 ml aliquots, and incubated with shaking (90 cycles/min) in Krebs–Ringer–Hepes medium in the presence of 3 μM fura-2 acetoxymethyl ester and 250 μM sulphinyprazole (added to inhibit the extrusion of fura-2 from the cytoplasmic space [18]) for 30 min at 37 °C. Ca^{2+} (1.2 mM) was included in the Krebs–Ringer–Hepes medium during the loading of fura-2 and the subsequent wash and resuspension steps in order to prevent the depletion of Ca^{2+} in intracellular stores, which occurs rapidly when PC12 cells are incubated in the absence of extracellular Ca^{2+} [2,5]. Immediately before the measurement of bivalent cation inflow, the cells were washed once by centrifugation at 300 g for 5 min at room temperature, and then resuspended (2×10^6 cells/ml) in Krebs–Ringer–Hepes medium.

Fluorescence measurements were performed at 37 °C using a PerkinElmer LS50B spectrofluorimeter (PerkinElmer Pty. Ltd, Knoxfield, Victoria, Australia) fitted with a 3 ml cuvette, thermostatically controlled cuvette holder and a magnetic stirrer, as described previously [15]. For the measurement of Ca^{2+} and Sr^{2+} inflow, dual excitation wavelengths of 340 and 380 nm and an emission wavelength of 510 nm were employed [19]. The results are expressed as the ratio of the fluorescence obtained at 340 and 380 nm excitation. Mn^{2+} inflow was measured using excitation and emission wavelengths of 359 and 510 nm respectively [19].

Ca^{2+} inflow was measured using the Ca^{2+} add-back assay, in which cells are incubated initially in the absence of added

extracellular Ca^{2+} , before extracellular Ca^{2+} is added and the initial rate of increase in $[\text{Ca}^{2+}]_{\text{cyt}}$ measured [4]. The steps involved were as follows. Cells loaded with fura-2 (2 ml in Krebs–Ringer–Hepes medium, which contained 1.2 mM CaCl_2) were added to the cuvette, and the recording of fluorescence was initiated. EGTA (3 mM final concentration) was added to chelate the extracellular Ca^{2+} (1.2 mM) and reduce the extracellular concentration of free Ca^{2+} to about 0.5 μM . This addition was associated with a rapid decrease in fluorescence ratio (not shown in the Figures), which was attributed to the presence of Ca^{2+} -bound fura-2 in the extracellular medium. (Even in the presence of sulphinyprazole, intracellular fura-2 was found to leak from the PC12 cells into the extracellular space [18].) After the addition of an agonist (indicated in the legends of the Figures and Tables), Ca^{2+} (5 mM) was added to initiate Ca^{2+} inflow. A rapid increase in fluorescence ratio, usually equal in magnitude to the rapid decrease induced by the addition of 3 mM EGTA, was observed following the addition of 5 mM Ca^{2+} (to the medium which contained 1.2 mM Ca^{2+} and 3 mM EGTA). This increase was attributed to the interaction of Ca^{2+} with Ca^{2+} -free fura-2 present in the extracellular medium. The Figures show data obtained after subtracting the value of the rapid EGTA-induced decrease in fluorescence from the fluorescence ratio measurements recorded for all time points after the addition of 5 mM Ca^{2+} . Initial rates of Ca^{2+} inflow (increase in fluorescence ratio/min) were determined from the slopes of the plots of fluorescence as a function of time. The steps involved in the measurement of Sr^{2+} inflow, initiated by the addition of 5 mM Sr^{2+} , were the same as those described above for the measurement of Ca^{2+} inflow, except that 5 mM Sr^{2+} was added in place of 5 mM Ca^{2+} .

For the measurement of Mn^{2+} inflow, cells loaded with fura-2 (as described above) were resuspended in Krebs–Ringer–Hepes medium, which was modified by omitting Ca^{2+} , reducing the concentration of KH_2PO_4 to 0.6 mM, and replacing MgSO_4 with MgCl_2 . Mn^{2+} (100 μM) was added at the times indicated in Figure 3. Mn^{2+} addition caused a rapid decrease in fluorescence (not shown in Figure 3) that was attributed to quenching of the fluorescence of extracellular fura-2 by Mn^{2+} . Rates of Mn^{2+} inflow (decrease in fluorescence units/min) were determined from the maximum slope of the plot of fluorescence as a function of time following the addition of Mn^{2+} .

Molecular biology techniques

mRNA was isolated from PC12 cells and freshly isolated rat brain (200 mg) by the 'mRNA direct' method, as described previously [15]. Dynabeads® [oligo(dT)₂₅ magnetic beads; Dynal AS, Oslo, Norway] (1 mg) were used according to the manufacturer's instructions. First-strand cDNA was synthesized from mRNA (1 μg) by reverse transcription using a d(T)₁₇ primer [GACTCGAGTCGACATCGA(T₁₇)], as described previously [15]. The amplification of cDNA (2 μl) was conducted using 0.3 μM of each primer (except for MTA 1, which was used at 1 μM) and 0.6 unit of *Taq* DNA polymerase. The amplification of mRNA encoding glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used to compare the quality of the cDNA synthesized from each batch of mRNA prepared from PC12 cells or from brain. PCR products recovered from 1.5% (w/v) agarose gels were directly sequenced using a DyeDeoxy™ Terminator Cycle Sequencing kit and a 373A automated DNA sequencer (Applied Biosystems Prism, Foster City, CA, U.S.A.). Sequence comparisons were performed using MacVector software, Version 5 (Oxford Molecular Inc., Campbell, MD, U.S.A.).

PCR primers were designed as follows. A common 8-fold degenerate antisense primer was constructed on the basis of the

highly conserved base sequence that is located in the predicted cytoplasmic region immediately on the 3' side of the sixth transmembrane segment in the *Drosophila* TRP and TRPL, the human (h) TRP-1, and the mouse (m) TRP-4 sequences. Six specific sense primers (corresponding to TRP-1–TRP-6) were designed on the basis of the sequences of the mTRP fragments identified by Petersen et al. [20] and Zhu et al. [21]. The primer name, sequence, expected size of PCR products (bp) and Genbank® accession number of the source sequence of each of the primers used to amplify mRNA encoding TRP proteins and GAPDH were as follows: mTRP sense primer (MTS) 1 (TRP-1 sense), ATG GGA CAG ATG TTA CAA, 400, U40980 (mTRP-1); MTS 2B (TRP-2 sense), CGC TGG GCA CTC TGC AGA, 370, U40981 (mTRP-2); MTS 3 (TRP-3 sense), AGG ACA TAT TCA AGT TCA, 340, U40982 (mTRP-3); MTS 4 (TRP-4 sense), CAG ATA TCT CTG GGA AGG ATG, 400, X90697 (mTRP-4); MTS 5 (TRP-5 sense), TTC TCT TTA TCT ACT GCC, 360, U40984 (mTRP-5); MTS 6 (TRP-6 sense), TTC ATG GTC ATA TTC ATC, 320, U49069 (mTRP-6); mTRP antisense primer (MTA) 1 (the common degenerate antisense), TGG AGC RAA YTT CCA YTC (where R = G or A and Y = C or T), J04844 and M88185 [*Drosophila* (d)TRP and dTRPL respectively], X90697 (mTRP-4) and U31110 (hTRP-1); GAPDH sense primer, ACC ACA GTC CAT GCC ATC AC, 450, M17701 (rGAPDH); and GAPDH antisense primer, TCC ACC ACC CTG TTG CTG TA.

The relative abundance of mRNA encoding a given TRP protein detected in mRNA isolated from PC12 cells or rat brain was estimated by a limiting dilution PCR method [15]. A sample of cDNA was diluted serially in the range of 1:5 to 1:10 000 000, and was amplified by PCR using the specific primers. The band which corresponded to the highest dilution from which the PCR product could be detected by staining of the gel with ethidium bromide was then identified. The gel was scanned in a FluorImager (Molecular Dynamics Inc., Sunnyvale, CA, U.S.A.) and the intensity of the band was determined by volume integration using ImageQuant software.

Generation and purification of an anti-(TRP-6) antibody and Western blotting

An anti-(TRP-6) polyclonal antibody was generated against the peptide CLGERLSLEPKLEESRR, which corresponds to the predicted sequence of the 16 amino acids at the C-terminus of the mTRP-6 protein (Genbank® accession no. U49069). The synthetic peptide was conjugated to diphtheria toxoid (Chiron Technologies, Clayton, Victoria, Australia). An initial dose of 500 μg of peptide was injected subcutaneously into two rabbits, followed by three boosts of 200 μg each at fortnightly intervals after the initial peptide injection, and a fourth boost at 18 weeks. Serum was collected after the second, third and fourth boosts, and was affinity-purified using a column of the immunizing peptide bound to epoxy-activated Sepharose 6B (Pharmacia) before eluting with 4.9 M MgCl_2 .

Subcellular fractions enriched in microsomes derived from the plasma membrane and intracellular membranes were prepared from rat brain and cultured PC12 cells as follows [22]. A freshly isolated rat brain (about 10 mg) or a pellet of PC12 cells (2×10^7 cells) was suspended in extraction buffer [50 mM Tris/HCl (pH 7.5)/1 mM EDTA/10 μM leupeptin/10 μM pepstatin A/100 μM PMSF] at 4 °C, homogenized using a glass–glass homogenizer (brain) or an Ultra-turrax homogenizer (PC12 cells) for 30 s at 4 °C, and the extract was then centrifuged at 750 g for 10 min at 4 °C. The resulting supernatant was centrifuged at 50 000 g for 30 min at 4 °C in order to precipitate

the microsomes. The pellet was resuspended in extraction buffer, which also contained 1% (v/v) Triton X-100, and aliquots were stored at –20 °C until required. Protein concentrations were determined using the Bradford method.

For Western blot analysis of the TRP-6 protein, samples of microsomes (40 μg of protein) were separated by SDS/PAGE using a 9% (w/v) gel, and then were transferred to nitrocellulose membranes (Hybond C+) by semi-dry blotting (Horiz Blot; Atto Corporation, Tokyo, Japan). The membranes were blocked for 2 h at room temperature with TBS-T buffer [20 mM Tris/HCl (pH 8)/500 mM NaCl/0.1% (v/v) Tween 20], which contained 5% (w/v) skimmed-milk powder. The membranes were then incubated overnight at 4 °C with the anti-(TRP-6) antibody (1 $\mu\text{g}/\text{ml}$ in TBS-T buffer), before washing four times with TBS-T buffer. The secondary antibody (anti-rabbit IgG–peroxidase conjugate diluted 1:2000 in TBS-T buffer) was applied for 2 h at room temperature, followed by four washes with TBS-T buffer. Proteins were detected by enhanced chemiluminescence (ECL® Western Blotting Detection Reagents; Amersham Pharmacia Biotech, Piscataway, NJ, U.S.A.), according to the manufacturer's instructions, and exposed to X-Omat Blue XB-1 film for periods of 30 s to 5 min. Where indicated, the anti-(TRP-6) antibody (1 $\mu\text{g}/\mu\text{l}$) was blocked by incubation with the antigenic peptide (1 $\mu\text{g}/\text{ml}$) for 2 h at room temperature, followed by centrifugation at 14 000 g for 10 min at 4 °C immediately before use in order to remove immune complexes.

RESULTS

Activation of bivalent cation inflow by OAG and DOG

Ca^{2+} inflow to PC12 cells was measured using fura-2 and a Ca^{2+} add-back assay in which the agonist was added to cells incubated in the absence of added extracellular Ca^{2+} , before adding Ca^{2+}

Figure 1 OAG, a membrane-permeant analogue of diacylglycerol, initiates the activation of plasma membrane Ca^{2+} inflow in PC12 cells comparable in magnitude with that initiated by acetylcholine and thapsigargin

The loading of PC12 cells with fura-2 and measurement of cell fluorescence were performed as described in the Materials and methods section. At the beginning of the experiment, the cell incubation medium contained 1.2 mM Ca^{2+} plus 3 mM EGTA to give a free Ca^{2+} concentration of approx. 0.5 μM . The times of addition of OAG (100 μM), acetylcholine (Ach) (100 μM), thapsigargin (Tg) (10 μM) and Ca^{2+} (5 mM) are indicated by the arrows. The results shown are those obtained for one of 15 experiments, which each gave similar results.

Table 1 Membrane-permeant analogues of diacylglycerol initiate the activation of Ca^{2+} and Mn^{2+} inflow in PC12 cells

Rates of Ca^{2+} and Mn^{2+} inflow were determined as described in the Materials and methods section and in the legends of Figures 1–3 and 5. N.D. represents 'not determined'. Values in parentheses indicate the number of times that the experiment was performed (*n*). *The degree of significance (calculated using Student's *t* test for unpaired samples) for a comparison of the rate of bivalent cation inflow in the presence of an agonist and in the absence of an inhibitor with the rate in the absence of agonist and inhibitor is $P \leq 0.001$ [i.e. all lines marked with an asterisk refer to a comparison with the rate in the first row of data (no agonist, no inhibitor)]. The degrees of significance for a comparison of the rate of bivalent cation inflow in the presence of an inhibitor with that in the absence of the inhibitor are: †, $P \leq 0.01$; and ‡, $P \leq 0.005$.

Agonist	Inhibitor	Initial rate of Ca^{2+} inflow (fluorescence ratio units/min)	Rate of Mn^{2+} inflow (fluorescence units/min)
None	None	0.092 ± 0.018 (20)	4.952 ± 0.042 (20)
	Staurosporin (1 μM)	0.088 ± 0.025 (8)	5.324 ± 0.082 (10)
	RHC80267 (50 μM)	0.079 ± 0.034 (8)	4.512 ± 0.098 (8)
OAG (100 μM)	None	1.260 ± 0.09 (20)*	14.125 ± 0.209 (20)*
	RHC80267 (50 μM)	2.190 ± 0.037 (10)*†	15.721 ± 0.520 (10)*
	R50922 (5 μM)	2.315 ± 0.03 (10)*†	N.D.
	Staurosporin (1 μM)	1.343 ± 0.007 (12)	16.013 ± 0.342 (10)*
	Chelerythrine (5 μM)	1.386 ± 0.008 (10)	N.D.
	SK&F96365 (50 μM)	0.589 ± 0.056 (8)*‡	N.D.
	2-APB (75 μM)	1.935 ± 0.036 (10)	N.D.
	Flufenamic acid (50 μM)	0.523 ± 0.026 (8)*‡	7.072 ± 0.148 (10)*
DOG (100 μM)	None	0.972 ± 0.056 (8)*	10.243 ± 0.342 (10)*
SAG (10 μM)	None	0.124 ± 0.022 (12)	5.382 ± 0.098 (10)
Acetylcholine (100 μM)	None	0.950 ± 0.047 (20)*	12.138 ± 0.186 (20)*
	RHC80267	0.745 ± 0.043 (10)	N.D.
	R50922 (5 μM)	0.323 ± 0.045 (10)*‡	N.D.
	Chelerythrine (5 μM)	0.432 ± 0.018 (8)*‡	N.D.
	SK&F96365 (25 μM)	0.425 ± 0.087 (8)*†	N.D.
	2-APB (75 μM)	0.378 ± 0.092 (10)*†	
	Flufenamic acid	0.842 ± 0.089 (8)	11.438 ± 0.972 (10)*
Thapsigargin (10 μM)	None	0.854 ± 0.08 (12)*	9.612 ± 0.998 (10)*
	2-APB (75 μM)	0.298 ± 0.012 (10)*†

Figure 2 OAG stimulates additional Ca^{2+} inflow in PC12 cells in which acetylcholine-induced Ca^{2+} inflow is already maximal

The loading of PC12 cells with fura-2 and measurement of cell fluorescence were performed as described in the Materials and methods section. The cell incubation medium initially contained 1.2 mM Ca^{2+} and 3 mM EGTA to give a free Ca^{2+} concentration of approx. 0.5 μM . The times of addition of OAG (100 μM), acetylcholine (Ach) (100 μM) and Ca^{2+} (5 mM) are indicated by the arrows. The results shown are those obtained for one of 10 experiments, which each gave similar results.

subsequently to the incubation medium [4]. The membrane-permeant analogues of diacylglycerol, OAG and DOG, each initiated the activation of Ca^{2+} inflow (Figure 1 and Table 1). The magnitudes of OAG-initiated and DOG-initiated Ca^{2+} inflow were comparable with those of Ca^{2+} inflow initiated by acetylcholine and thapsigargin. In contrast, the magnitude of the increase in $[\text{Ca}^{2+}]_{\text{cyt}}$ induced by the addition of extracellular Ca^{2+} in the presence of OAG (the difference between the value of $[\text{Ca}^{2+}]_{\text{cyt}}$ at the plateau and the value before the addition of Ca^{2+})

was greater than that observed in the presence of thapsigargin or acetylcholine (Figure 1). The values for the increase in $[\text{Ca}^{2+}]_{\text{cyt}}$ were 2.7 ± 0.09 , 1.2 ± 0.02 and 1.28 ± 0.04 fluorescence ratio units (means $\pm$ S.E.M., $n = 15$, $P \leq 0.01$), for OAG, thapsigargin and acetylcholine respectively.

1-Stearoyl-2-arachidonoyl-*sn*-glycerol (SAG), a membrane-impermeant analogue of diacylglycerol, did not activate Ca^{2+} inflow (Table 1). RHC80267, an inhibitor of diacylglycerol lipase [23], and R50922, an inhibitor of diacylglycerol kinase [24],

Figure 3 OAG initiates the activation of plasma membrane Mn^{2+} inflow in PC12 cells comparable in magnitude with that initiated by acetylcholine and thapsigargin

The loading of PC12 cells with fura-2 and measurement of Mn^{2+} inflow were performed as described in the Materials and methods section. The cell incubation medium contained no added Ca^{2+} . The times of addition of Mn^{2+} (100 μM), OAG (100 μM), DMSO (0.5%, v/v), acetylcholine (ACh) (100 μM) and thapsigargin (Tg) (10 μM) are indicated by the arrows. The results shown are those obtained for one of 10–20 experiments, which each gave similar results.

enhanced the activation of Ca^{2+} inflow initiated by OAG (Table 1). In order to determine whether the OAG-activated Ca^{2+} -permeable channel differs from the Ca^{2+} -permeable channel activated by acetylcholine, OAG was added after the addition of acetylcholine and Ca^{2+} (Figure 2). A further OAG-induced increase in fura-2 fluorescence was observed (compare Figure 2B with Figures 2A and 2C), indicating that the OAG-activated channel differs from that activated by acetylcholine.

OAG and DOG also activated Mn^{2+} inflow (measured in the absence of added extracellular Ca^{2+} [5]) with a degree of activation comparable with that induced by acetylcholine and thapsigargin [2,5] (Figure 3 and Table 1). The effect of OAG on Mn^{2+} inflow was not mimicked by SAG (Table 1). OAG-activated Mn^{2+} inflow was inhibited by 35% in the presence of 1.2 mM extracellular Ca^{2+} . The rates of OAG-activated Mn^{2+} inflow were 14.1 ± 0.21 and 9.3 ± 0.3 fluorescence units/min (means $\pm$ S.E.M., $n = 6$, $P \leq 0.05$) in the absence and presence of 1.2 mM extracellular Ca^{2+} respectively. The effects of OAG on Sr^{2+} inflow were also investigated. Figure 4 shows that Sr^{2+} inflow was activated by OAG, but not by acetylcholine. The rates of Sr^{2+} inflow were 0.085 ± 0.014 and 0.647 ± 0.085 fluorescence units/min (means $\pm$ S.E.M., $n = 10$, $P \leq 0.01$) in the absence or presence of 100 μM OAG respectively, and 0.102 ± 0.053 (mean $\pm$ S.E.M., $n = 10$) in the presence of 100 μM acetylcholine.

The role of protein kinase C in the activation of Ca^{2+} and Mn^{2+} inflow initiated by OAG was investigated using two inhibitors of protein kinase C activation, staurosporin [25] and chelerythrine [26]. Neither of these inhibitors blocked the ability of OAG to initiate the activation of Ca^{2+} or Mn^{2+} inflow (Table 1). In

contrast, chelerythrine inhibited both acetylcholine-initiated Ca^{2+} release (results not shown) and Ca^{2+} inflow (Table 1). The inhibition by chelerythrine of the acetylcholine-induced Ca^{2+} release and inflow might be due to effects of protein kinase C (and hence chelerythrine) on SOC [27] and $\text{Ins}(1,4,5)\text{P}_3$ receptors, and/or to effects of chelerythrine that are independent of protein kinase C [28]. Taken together, the results suggest that, in PC12 cells, analogues of diacylglycerol initiate the activation of plasma membrane bivalent cation channels, which differ from the bivalent cation channels (SOCs) activated by acetylcholine and thapsigargin.

Pharmacological inhibition of OAG-activated bivalent cation inflow

Further comparison of the OAG-activated bivalent cation channels with the channels activated by acetylcholine and thapsigargin was made using three plasma membrane Ca^{2+} channel inhibitors, SK&F96365, 2-APB and flufenamic acid. SK&F96365 inhibits SOC and several other types of plasma membrane Ca^{2+} channels [29]. 2-APB was first employed as an inhibitor of the function of $\text{Ins}(1,4,5)\text{P}_3$ receptors [11], but has been shown recently to be a membrane-permeant inhibitor of SOC in some cell types [30,31]. Flufenamic acid is an inhibitor of NSCCs [32].

Ca^{2+} inflow initiated by OAG was inhibited by SK&F96365 (Table 1), with a concentration of 50 μM giving half-maximal inhibition of OAG-initiated Ca^{2+} inflow (results not shown). Acetylcholine-initiated Ca^{2+} inflow was also inhibited

Figure 4 OAG, but not acetylcholine, stimulates Sr^{2+} inflow in PC12 cells

The loading of PC12 cells with fura-2 and measurement of cell fluorescence were performed as described in the Materials and methods section. The cell incubation medium initially contained 1.2 mM Ca^{2+} . The times of addition of OAG (100 μM), acetylcholine (Ach) (100 μM) and Sr^{2+} (5 mM) are indicated by the arrows. The results shown are those obtained for one of 10 experiments, which each gave similar results.

Figure 5 2-APB inhibits acetylcholine (Ach)- and thapsigargin (Tg)-stimulated Ca^{2+} inflow, but not OAG-stimulated Ca^{2+} inflow, in PC12 cells

The loading of PC12 cells with fura-2 and measurement of cell fluorescence were performed as described in the Materials and methods section. The cell incubation medium initially contained 1.2 mM Ca^{2+} . The times of addition of OAG (100 μM), Ach (100 μM), Tg (10 μM) and Ca^{2+} (5 mM) are indicated by the arrows. When present (B, D, and F), 2-APB (75 μM) was added at the beginning of the period of incubation. The results shown are those obtained for one of 10 experiments, which each gave similar results.

by SK&F96365, with 50% inhibition provided by 25 μM SK&F96365 (Table 1) [5]. 2-APB at 75 μM had no effect on OAG-initiated Ca^{2+} inflow (Figures 5E and 5F). In contrast, this concentration of 2-APB inhibited acetylcholine-initiated (Figures 5A and 5B), as well as thapsigargin-initiated (Figure 5C and 5D), Ca^{2+} inflow. There was no inhibition by 2-APB of acetylcholine-induced Ca^{2+} release from intracellular stores (Figures 5A and

5B). The amounts of Ca^{2+} released by acetylcholine (peak of the acetylcholine-induced increase in $[\text{Ca}^{2+}]_{\text{cyl}}$) were 0.622 ± 0.039 and 0.689 ± 0.056 fluorescence ratio units (means $\pm$ S.E.M., $n = 10$) in the presence or absence of 2-APB respectively. Flufenamic acid inhibited both Ca^{2+} and Mn^{2+} inflow initiated by OAG, but did not have any significant effect on acetylcholine-initiated Ca^{2+} and Mn^{2+} inflow (Table 1).

Figure 6 Dose-response curve for the activation of Ca^{2+} inflow by acetylcholine

The loading of PC12 cells with fura-2, measurement of cell fluorescence and determination of the initial rates of Ca^{2+} inflow were performed as described in Figure 1 and Table 1. The results are the means $\pm$ S.E.M. for three determinations.

Evaluation of the role of acetylcholine in initiating activation of the OAG-activated Ca^{2+} channel

Since acetylcholine induces the hydrolysis of $\text{PtdIns}(4,5)\text{P}_2$, it would be expected that acetylcholine would also induce the formation of diacylglycerol ([33], but see [34,35]), which could

activate the OAG-activated Ca^{2+} -permeable channel. However, the results shown in Figure 1 and Table 1 suggest that acetylcholine induces little activation of the OAG-activated Ca^{2+} -permeable channel. To evaluate further the action of acetylcholine, a dose-response curve for the activation of Ca^{2+} inflow by acetylcholine was determined (Figure 6). The concentration of acetylcholine that gave half-maximal activation was $8 \mu\text{M}$, and maximal activation was achieved at $100 \mu\text{M}$ acetylcholine, the concentration employed in the experiments described in Figure 1 and Table 1. The nature of the receptors involved in the action of acetylcholine was determined using hexamethonium chloride, a nicotinic acetylcholine receptor antagonist, and atropine, a muscarinic acetylcholine receptor antagonist. Hexamethonium chloride had no effect on acetylcholine-stimulated Ca^{2+} inflow, whereas atropine caused almost complete inhibition. The rates of acetylcholine-induced Ca^{2+} inflow in the absence of inhibitor, in the presence of hexamethonium chloride, and in the presence of atropine were 0.894 ± 0.086 ($n = 6$), 0.838 ± 0.058 ($n = 6$) and 0.112 ± 0.023 ($n = 6$) fluorescence ratio units/min (means $\pm$ S.E.M.) compared with a value of 0.09 ($n = 6$) obtained in the absence of acetylcholine and inhibitor (basal Ca^{2+} inflow). These results indicate that the actions of acetylcholine in inducing the release of Ca^{2+} from intracellular stores and Ca^{2+} inflow are mediated by muscarinic acetylcholine receptors.

Expression of mRNA encoding TRP proteins 1–6 and the TRP-6 protein in PC12 cells

Since a distinguishing feature of heterologously expressed TRP-3 and TRP-6 cation channels is activation by analogues of

Figure 7 Amplification by RT-PCR of mRNA encoding the TRP proteins 1–6 (panels A–F respectively) in mRNA isolated from PC12 cells

The culture of PC12 cells in the presence or absence of NGF, preparation of mRNA, and RT-PCR were conducted as described in the Materials and methods section. Dilutions of cDNA ranging from 10^{-1} – 10^{-6} were employed. The first lane for each gel represents *Hpa*II-digested pUC19 DNA size markers, and the size (bp) of each PCR product is indicated. No PCR bands were observed with any of the primer sets when reverse transcriptase was omitted from the reaction mixture for cDNA synthesis, thus excluding the possibility that the PCR products were amplified from contaminating DNA. The results shown are those obtained in one of three experiments, employing two cDNA preparations, which each gave similar results.

Table 2 Size and limits of detection of the PCR products obtained by amplifying cDNA derived from mRNA encoding TRP proteins 1–6, and the relative abundance of TRP mRNA in PC12 cells and rat brain

The culture of PC12 cells in the presence and absence of NGF, extraction of mRNA, RT-PCR, and measurement of the relative intensity of the PCR bands were performed as described in the Materials and methods section. The values shown are the highest dilution of cDNA from which PCR product could be detected as a visible band by ethidium-bromide staining in agarose gels. The results are representative of those obtained in two or three experiments, which gave similar results. Comparisons can be made horizontally between cell types, but not vertically between TRP types, because the products were amplified using different primer sets.

TRP protein	Observed size of PCR product (bp)	Highest dilution of cDNA from which PCR product was detected			Intensity of TRP PCR band relative to the intensity of the GAPDH PCR band		
		PC12 cells		Rat brain	PC12 cells		Rat brain
		NGF absent	NGF present		NGF absent	NGF present	
TRP-1	400	1/10 000	1/10 000	1/10 000	1.4×10^{-2}	9.2×10^{-2}	4.0×10^{-2}
TRP-2	370	1/100	1/100	1/100	3.0×10^{-4}	8.0×10^{-5}	1.1×10^{-4}
TRP-3	340	1/1000	1/1000	1/1000	2.5×10^{-3}	1.8×10^{-3}	7.7×10^{-3}
TRP-4	400	1/100	1/100	1/1000	1.3×10^{-4}	9.4×10^{-4}	1.8×10^{-3}
TRP-5	360	1/1000	1/100	1/1 000 000	6.4×10^{-4}	2.4×10^{-5}	1.2×10^{-1}
TRP-6	320	1/1000	1/100 000	1/1 000 000	1.7×10^{-3}	4.7×10^{-1}	2.8×10^0

diacylglycerol via a mechanism which does not involve protein kinase C [13], experiments were conducted to determine whether TRP-3 and TRP-6 proteins are expressed in PC12 cells. These experiments employed reverse transcriptase (RT)-PCR and primers corresponding to mRNA encoding the TRP proteins 1–6, which were designed as described in the Materials and methods section. PCR bands corresponding to the expected sizes of the amplified sequences for TRP-1–TRP-6 were detected in mRNA from PC12 cells and, as shown previously [10,36,37], in mRNA from rat brain (Figure 7 and Table 2). Sequencing showed that for each the TRP proteins TRP-1–TRP-6, the PCR product corresponding to the TRP mRNA in PC12 cells showed sequence similarity (in the range 90–100%) with the same region of the corresponding mTRP mRNA [21] (results not shown). Comparison of the values obtained for the highest dilution of the cDNA for which the PCR product for a given TRP mRNA was detected indicating that PC12 cells express mRNA encoding TRP proteins 1–3 at levels comparable with those in brain (Table 2). The level of expression of mRNA encoding TRP proteins 4, 5 and 6 is lower than that found in brain. These conclusions are consistent with the results obtained by quantifying the intensity of the bands (at the highest dilution) and using mRNA encoding GAPDH as a reference (Table 2).

Since NGF induces the differentiation of PC12 cells, and this is accompanied by major phenotypic changes [17], the effects of NGF on the expression of mRNA encoding the TRP proteins was investigated. NGF caused a substantial increase in the amount of TRP-6 mRNA (Table 2). No substantial NGF-induced increase in the expression of the mRNAs encoding TRP proteins 1–5 was detected (Table 2).

In Western blot analysis of rat brain and PC12 cells, an anti-(TRP-6) polyclonal antibody detected several proteins, including one with an approximate size of 98 kDa (Figure 8A). The appearance of the 98 kDa band was blocked by pretreatment of the antibody with the immunizing peptide (compare Figure 8B with 8A). The size of the endogenous TRP-6 band (98 kDa) matches that of the rTRP-6B isoform of TRP-6 expressed in COS cells [38]. Taken together, the results of the RT-PCR and Western blot experiments provide evidence that the TRP-3 and TRP-6 proteins (as well as TRP proteins 1, 2, 4 and 5) are expressed in PC12 cells. Moreover, the size of the TRP-6 protein band (98 kDa) suggests that the isoform of TRP-6 expressed in PC12 cells is the rTRP-6B isoform [38].

Figure 8 Detection of the TRP-6 protein in PC12 cells and rat brain by Western blot analysis using an anti-(TRP-6) antibody

(A) Membranes treated with the anti-(TRP-6) antibody. (B) Membranes treated with the anti-(TRP-6) antibody after incubation of the antibody with the antigenic peptide. The preparation of fractions enriched in microsomal membranes, Western blot analysis, and treatment of the anti-(TRP-6) antibody with the immunizing peptide were conducted as described in the Materials and methods section. The results shown are those obtained in one of seven experiments, which each gave similar results.

DISCUSSION

The results obtained with analogues of diacylglycerol and inhibitors of diacylglycerol metabolism have shown that PC12 cells possess diacylglycerol-activated NSCCs. The observation that inhibitors of the activation of protein kinase C did not block the OAG- and DOG-initiated activation of bivalent cation inflow indicates that the activation of bivalent cation inflow by diacylglycerol does not involve protein kinase C. Several observations indicate that the diacylglycerol-activated bivalent cation channels

are different from the acetylcholine- and thapsigargin-activated channels described in detail by others previously [2,3,5]: (i) OAG caused an increase in Ca^{2+} inflow in the presence of maximal activation of Ca^{2+} inflow by acetylcholine; (ii) the OAG-activated channels admit the entry of Ca^{2+} , Mn^{2+} and Sr^{2+} ions, whereas the acetylcholine-activated channels admit Ca^{2+} and Mn^{2+} , but not Sr^{2+} ; (iii) the OAG-activated channels are not inhibited by 2-APB, but are inhibited by flufenamic acid, whereas the acetylcholine-activated channels are inhibited by 2-APB but not by flufenamic acid; (iv) the OAG-activated channels have a lower sensitivity to inhibition by SK&F96365 than the acetylcholine-activated channels; and (v) the maximal increase in $[\text{Ca}^{2+}]_{\text{cyt}}$ following extracellular Ca^{2+} addition in the presence of OAG is greater than that observed in the presence of acetylcholine or thapsigargin. Thus it can be concluded that the present study has identified a diacylglycerol-activated bivalent cation channel in PC12 cells (compare with the diacylglycerol-activated NSCC identified previously in rabbit portal vein [39]).

The binding of acetylcholine to muscarinic receptors induces the hydrolysis of $\text{PtdIns}(4,5)\text{P}_2$ in PC12 cells, and hence is likely to lead to the formation of diacylglycerol [33], which, in turn, might be expected to activate diacylglycerol-activated NSCCs. However, the results (e.g. those obtained in the presence of 2-APB, which completely inhibited acetylcholine-initiated Ca^{2+} inflow, but did not inhibit OAG-initiated Ca^{2+} inflow) provide no evidence for an acetylcholine-induced activation of the diacylglycerol-activated NSCCs under the conditions employed in the present study. Previous studies suggest that, in PC12 cells, acetylcholine induces the accumulation of detectable increases in diacylglycerol only in cells incubated in the presence of NGF [35]. Nevertheless, it is possible that smaller amounts of diacylglycerol are formed under the conditions of the present experiments. The inability of this diacylglycerol to activate the diacylglycerol-activated NSCCs might be due to rapid metabolism and/or inappropriate location of the diacylglycerol. Moreover, it has been suggested that the activation of NSCCs by diacylglycerol analogues is due to a metabolite of diacylglycerol [40]. Although the nature of the metabolite is not yet known, its formation might depend on the fatty acid composition of the diacylglycerol involved. Thus OAG and DOG might lead to the generation of the metabolite, whereas diacylglycerol derived from acetylcholine might not lead to its generation, and hence to activation of the NSCC. Other agonists, such as pituitary adenyl cyclase-activating peptide, might generate diacylglycerols [41] with appropriate fatty acid compositions and/or intracellular locations, which allow them to be physiological activators of the diacylglycerol-activated NSCCs.

The results obtained using RT-PCR and limiting-dilution PCR have shown that mRNA encoding each of TRP proteins 1–6 is expressed in PC12 cells at levels comparable with (TRP1–3), or somewhat lower than (TRP4–6), those found in brain. Western blot analysis confirmed that the TRP-6 protein is expressed in PC12 cells.

Previous studies of the properties of the TRP-3 and TRP-6 proteins heterologously expressed in CHO, COS, bovine pulmonary artery endothelial cells and HEK-293 cells have shown that these channels: (i) are activated by diacylglycerol in a protein-kinase-C-independent manner [13]; (ii) are not activated by the depletion of Ca^{2+} from intracellular stores [42,43], but see [44,45]; (iii) require cytoplasmic Ca^{2+} for activation [43]; (iv) are suppressed by an increase in the extracellular Ca^{2+} concentration (TRP-3) [14,43]; (v) have a broad specificity for uni- and bivalent cations [13,38,43] (vi) admit Mn^{2+} , Ba^{2+} , Sr^{2+} and Ca^{2+} in the absence of an agonist [13,38,46]; and (vii) are inhibited by 50–200 μM La^{3+} [41,47] and SK&F96365 [41,46], but are not

affected by 2-APB (TRP-3) [11]. Features of the diacylglycerol-activated bivalent cation channels in PC12 cells discovered in the present study include activation by diacylglycerol in a protein-kinase-C-independent manner, permeability to Sr^{2+} as well as to Ca^{2+} and Mn^{2+} , inhibition by extracellular Ca^{2+} , and inhibition by SK&F96365 and flufenamic acid, but not by 2-APB. Thus many of the properties of the diacylglycerol-activated bivalent cation channels in PC12 cells are exhibited by heterologously expressed TRP-3 and TRP-6 proteins. This comparison suggests that TRP-3 and/or TRP-6 may be the molecular correlate(s) of the endogenous diacylglycerol-activated bivalent cation channels observed in PC12 cells. Since it has been shown recently that heterologously expressed TRP-7 is activated by diacylglycerol [48], the possibility that TRP-7 is also a molecular correlate of the endogenous diacylglycerol-activated NSCC cannot be excluded.

The observation that the plateau of $[\text{Ca}^{2+}]_{\text{cyt}}$ reached during Ca^{2+} inflow through diacylglycerol-activated channels is greater than that reached after the activation of SOC channels suggests that the cellular functions of the diacylglycerol-activated NSCCs are different from those of SOC channels. Diacylglycerol-activated NSCCs might be involved in regulation mediated by neurotransmitters, NGF and other growth factors of a number of functions of PC12 cells. These include gene expression, neurite outgrowth and other steps in cell differentiation, the synthesis and storage of neurotransmitters, and membrane potential [49,50]. In this regard, it is of interest to note that it has recently been reported that a more distant TRP homologue, mucolipin, is mutated in the neurodegenerative disorder mucopolipidosis type IV [51], suggesting that there may also be diseases that originate from mutations in other TRP proteins, such as TRP-3 and TRP-6.

The technical assistance of Mike Calder, the advice of Mario Frosco, and the skilful assistance of Diana Tanevski (all of this department) in preparing the manuscript are gratefully acknowledged. This work was supported by a grant from the Australian Research Council.

REFERENCES

- Berridge, M. J. (1998) Neuronal calcium signaling. *Neuron* **21**, 13–26
- Bennett, D. L., Bootman, M. D., Berridge, M. J. and Cheek, T. R. (1998) Ca^{2+} entry into PC12 cells initiated by ryanodine receptors or inositol 1,4,5-trisphosphate receptors. *Biochem. J.* **329**, 349–357
- Taylor, S. C. and Peers, C. (1999) Store-operated Ca^{2+} influx and voltage-gated Ca^{2+} channels coupled to exocytosis in pheochromocytoma (PC12) cells. *J. Neurochem.* **73**, 874–880
- Barritt, G. J. (1999) Receptor-activated Ca^{2+} inflow in animal cells: a variety of pathways tailored to meet different intracellular Ca^{2+} signalling requirements. *Biochem. J.* **337**, 153–169
- Clementi, E., Scheer, H., Zacchetti, D., Fasolato, C., Pozzan, T. and Meldolesi, J. (1992) Receptor-activated Ca^{2+} influx. Two independently regulated mechanisms of influx stimulation coexist in neurosecretory PC12 cells. *J. Biol. Chem.* **267**, 2164–2172
- Partridge, L. D. and Valenzuela, C. F. (2000) Block of hippocampal CAN channels by flufenamate. *Brain Res.* **867**, 143–148
- Putney, Jr, J. W. (1999) “Kissin’ cousins”: Intimate plasma membrane–ER interactions underlie capacitative calcium entry. *Cell* **99**, 5–8
- Putney, Jr, J. W. (1999) TRP, inositol 1,4,5-trisphosphate receptors, and capacitative calcium entry. *Proc. Natl. Acad. Sci. U.S.A.* **96**, 14669–14671
- Hofmann, T., Schaefer, M., Schultz, G. and Gudermann, T. (2000) Transient receptor potential channels as molecular substrates of receptor-mediated cation entry. *J. Mol. Med.* **78**, 14–25
- Harteneck, C., Plant, T. D. and Schultz, G. (2000) From worm to man: three subfamilies of TRP channels. *Tr. Neurosci.* **23**, 159–166
- Ma, H.-T., Patterson, R. L., van Rossum, D. B., Birnbaumer, L., Mikoshiba, K. and Gill, D. L. (2000) Requirement of the inositol trisphosphate receptor for activation of store-operated Ca^{2+} channels. *Science* **287**, 1647–1651
- Li, H.-S., Xu, X. S. and Montell, C. (1999) Activation of a TRPC3-dependent cation current through the neurotrophin BDNF. *Neuron* **24**, 261–273

- 13 Hofmann, T., Obukhov, A. G., Schaefer, M., Harteneck, C., Gudermann, T. and Schultz, G. (1999) Direct activation of human TRPC6 and TRPC3 channels by diacylglycerol. *Nature (London)* **397**, 259–263
- 14 Lintschinger, B., Balzer-Geldsetzer, M., Baskaran, T., Graier, W. F., Romanin, C., Zhu, M. X. and Groschner, K. (2000) Coassembly of Trp1 and Trp3 proteins generates diacylglycerol- and Ca^{2+} -sensitive cation channels. *J. Biol. Chem.* **275**, 27799–27805
- 15 Brereton, H. M., Harland, M. L., Frosco, M., Petronijevic, T. and Barritt, G. J. (1997) Novel variants of voltage-operated calcium channels α_1 -subunit transcripts in a rat liver-derived cell line: deletion in the IVS4 voltage sensing region. *Cell Calcium* **22**, 39–52
- 16 Auld, A., Chen, J., Brereton, H. M., Wang, Y., Gregory, R. B. and Barritt, G. J. (2000) Store-operated Ca^{2+} inflow in Reuber hepatoma cells is inhibited by voltage-operated Ca^{2+} channel antagonists and, in contrast to freshly isolated hepatocytes, does not require a pertussis toxin-sensitive trimeric GTP-binding protein. *Biochim. Biophys. Acta* **1497**, 11–26
- 17 Greene, L. A. and Tischler, A. S. (1976) Establishment of a noradrenergic clonal line of rat adrenal pheochromocytoma cells which respond to nerve growth factor. *Proc. Natl. Acad. Sci. U.S.A.* **73**, 2424–2428
- 18 Di Virgilio, F., Fasolato, C. and Steinberg, T. H. (1988) Inhibitors of membrane transport system for organic anions block fura-2 excretion from PC12 and N2A cells. *Biochem. J.* **256**, 959–963
- 19 Thomas, A. P. and Delaville, E. (1991) The use of fluorescent indicators for measurements of cytosolic free calcium concentration in cell populations and single cells. In *Cellular Calcium: A Practical Approach* (McCormack, J. G. and Cobbold, P. H., eds.), pp. 1–54, Oxford University Press, Oxford
- 20 Petersen, C. C. H., Berridge, M. J., Borge, M. F. and Bennett, D. L. (1995) Putative capacitative calcium entry channels: expression of *Drosophila* trp and evidence for the existence of vertebrate homologues. *Biochem. J.* **311**, 41–44
- 21 Zhu, X., Jiang, M., Peyton, M., Boulay, G., Hurst, R., Stefani, E. and Birnbaumer, L. (1996) trp, a novel mammalian gene family essential for agonist-activated capacitative Ca^{2+} entry. *Cell* **85**, 661–671
- 22 Egea, J., Espinet, C. and Comella, J. X. (1999) Calcium influx activates extracellular-regulated kinase/mitogen-activated protein kinase pathway through a calmodulin-sensitive mechanism in PC12 cells. *J. Biol. Chem.* **274**, 75–85
- 23 Sutherland, C. A. and Amin, D. (1982) Relative activities of rat and dog platelet phospholipase A_2 and diglyceride lipase. Selective inhibition of diacylglyceride lipase by RHC80267. *J. Biol. Chem.* **257**, 14006–14010
- 24 Chaffoy de Courcelles, D., Roevens, P. and Belle, H. V. (1985) R59022, a diacylglycerol kinase inhibitor. Its effect on diacylglycerol and thrombin-induced C kinase activation in the intact platelet. *J. Biol. Chem.* **260**, 15762–15770
- 25 Tamaoki, T., Nomoto, H., Takahashi, I., Kato, Y., Morimoto, M. and Tomita, F. (1986) Staurosporine, a potent inhibitor of phospholipid/ Ca^{2+} dependent protein kinase. *Biochem. Biophys. Res. Commun.* **135**, 397–402
- 26 Herbert, J. M., Augereau, J. M., Gleye, J. and Maffrand, J. P. (1990) Chelerythrine is a potent and specific inhibitor of protein kinase C. *Biochem. Biophys. Res. Commun.* **172**, 993–999
- 27 Parekh, A. B. and Penner, R. (1995) Depletion-activated calcium current is inhibited by protein kinase C in RBL-2H3 cells. *Proc. Natl. Acad. Sci. U.S.A.* **92**, 7907–7911
- 28 Shi, L. and Wang, C. (1999) Inhibitory effect of the kinase inhibitor chelerythrine on acetylcholine-induced current in PC12 cells. *Arch. Biochem. Biophys.* **368**, 40–44
- 29 Merritt, J. E., Armstrong, W. P., Benham, C. D., Hallam, T. J., Jacob, R., Jaxa-Chamiec, A., Leigh, B. K., McCarthy, S. A., Moores, K. E. and Rink, T. J. (1990) SK&F96365, a novel inhibitor of receptor-mediated calcium entry. *Biochem. J.* **271**, 515–522
- 30 Gregory, R. B., Rychkov, G. and Barritt, G. J. (2001) Evidence that 2-aminoethyl diphenylborate is a novel inhibitor of store-operated Ca^{2+} channels in liver cells, and acts through a mechanism which does not involve inositol triphosphate receptors. *Biochem. J.* **354**, 285–290
- 31 Braun, F.-J., Broad, L. M., Armstrong, D. L. and Putney, Jr, J. W. (2001) Stable activation of single Ca^{2+} release-activated Ca^{2+} channels in divalent cation-free solutions. *J. Biol. Chem.* **276**, 1063–1070
- 32 Gogelein, H., Dahlem, D., Englert, H. C. and Lang, H. J. (1990) Flufenamic acid, mefenamic acid and niflumic acid inhibit single non-selective cation channels in the rat of exocrine pancreas. *FEBS Lett.* **268**, 79–82
- 33 Garcia, M. C., Lopez, M. G., Garcia, A. G. and Sanchez-Crespo, M. (1992) Muscarinic acetylcholine receptor enhances phosphatidylcholine hydrolysis via phospholipase D in bovine chromaffin cells. *J. Neurochem.* **59**, 2244–2250
- 34 Horwitz, J. (1990) Carbachol and bradykinin increase the production of diacylglycerol from sources other than inositol-containing phospholipids in PC12 cells. *J. Neurochem.* **54**, 983–991
- 35 Altin, J. G. and Bradshaw, R. A. (1990) Production of 1,2-diacylglycerol in PC12 cells by nerve growth factor and basic fibroblast growth factor. *J. Neurochem.* **54**, 1666–1676
- 36 Garcia, R. L. and Schilling, W. P. (1997) Differential expression of mammalian TRP homologues across tissues and cell lines. *Biochem. Biophys. Res. Commun.* **239**, 279–283
- 37 Mizuno, N., Kitayama, S., Saishin, Y., Shimada, S., Morita, K., Mitsuhashi, C., Kurihara, H. and Dohi, T. (1999) Molecular cloning and characterization of rat trp homologues from brain. *Mol. Brain Res.* **64**, 41–51
- 38 Zhang, L. and Saffen, D. (2001) Muscarinic acetylcholine receptor regulation of TRP-6 Ca^{2+} channel isoforms: molecular structures and functional characterization. *J. Biol. Chem.* **276**, 13331–13339
- 39 Helliwell, R. M. and Large, W. A. (1997) α_1 -Adrenoceptor activation of a non-selective cation current in rabbit portal vein by 1,2-diacyl-*sn*-glycerol. *J. Physiol. (London)* **499**, 417–428
- 40 Estacion, M., Sinkins, W. G. and Schilling, W. P. (2001) Regulation of *Drosophila* transient receptor potential-like (TrpL) channels by phospholipase-C-dependent mechanisms. *J. Physiol. (Cambridge, U.K.)* **530**, 1–19
- 41 Osipenko, O. N., Barrie, A. P., Allen, J. M. and Gurney, A. M. (2000) Pituitary adenylyl cyclase-activating peptide activates multiple intracellular signaling pathways to regulate ion channels in PC12 cells. *J. Biol. Chem.* **275**, 16626–16631
- 42 Boulay, G., Zhu, X., Peyton, M., Jiang, M., Hurst, R., Stefani, E. and Birnbaumer, L. (1997) Cloning and expression of a novel mammalian homolog of *Drosophila* Transient Receptor Potential (Trp) involved in calcium entry secondary to activation of receptors coupled by the G_q class of G protein. *J. Biol. Chem.* **272**, 29672–29680
- 43 Zitt, C., Obukhov, A. G., Strübing, C., Zobel, A., Kalkbrenner, F., Lückhoff, A. and Schultz, G. (1997) Expression of TRPC-3 in Chinese hamster ovary cells results in calcium-activated cation currents not related to store depletion. *J. Cell Biol.* **138**, 1333–1341
- 44 Mizuno, N., Kitayama, S., Saishin, Y., Shimada, S., Morita, K., Mitsuhashi, C., Kurihara, H. and Dohi, T. (1998) Molecular cloning and characterization of rat trp homologues from brain. *Mol. Brain Res.* **64**, 41–51
- 45 Preuss, K. D., Nöller, J. K., Krause, E., Göbel, A. and Schulz, I. (1997) Expression and characterization of a Trp1 homolog from rat. *Biochem. Biophys. Res. Commun.* **240**, 167–172
- 46 Zhu, X., Jiang, M. and Birnbaumer, L. (1998) Receptor-activated Ca^{2+} influx via human TRP-3 stably expressed in human embryonic kidney (HEK) 293 cells. *J. Biol. Chem.* **273**, 133–142
- 47 Kamouchi, M., Philipp, S., Flockerzi, V., Wissenbach, U., Mamin, A., Raeymaekers, L., Eggermont, J., Droogmans, G. and Nilius, B. (1999) Properties of heterologously expressed hTRP3 channels in bovine pulmonary artery endothelial cells. *J. Physiol. (London)* **518**, 345–358
- 48 Okada, T., Inoue, R., Yamazaki, K., Maeda, A., Kurosaki, T., Yamakuni, T., Tanaka, I., Shimizu, S., Ikenaka, K., Imoto, K. and Mori, Y. (1999) Molecular and functional characterization of a novel mouse transient receptor potential protein homologue TRP7. *J. Biol. Chem.* **274**, 27359–27370
- 49 Buess, M., Engler, O., Hirsch, H. H. and Moroni, C. (1999) Search for oncogenic regulators in an autocrine tumor model using differential display PCR: identification of novel candidate genes including the calcium channel mtrp6. *Oncogene* **18**, 1487–1494
- 50 Magoski, N. S., Knox, R. J. and Kaczmarek, L. K. (2000) Activation of a Ca^{2+} -permeable cation channel produces a prolonged attenuation of intracellular Ca^{2+} release in *Aplysia* bag cell neurones. *J. Physiol. (Cambridge, U.K.)* **522**, 271–283
- 51 Sun, M., Goldin, E., Stahl, S., Falardeau, J. L., Kennedy, J. C., Acierio, Jr, J. S., Bove, C., Kaneski, C. R., Nagle, J., Bromley, M. C., Colman, M. et al. (2000) Mucopolidosis type IV is caused by mutations in a gene encoding a novel transient receptor potential channel. *Hum. Mol. Genet.* **9**, 2471–2478

Received 1 March 2001/11 May 2001; accepted 19 July 2001