Expression of *Drosophila trpl* cRNA in *Xenopus laevis* oocytes leads to the appearance of a Ca^{2+} channel activated by Ca^{2+} and calmodulin, and by guanosine 5′[γ-thio]triphosphate

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The effects of expression of the *Drosophila melanogaster* Trpl protein, which is thought to encode a putative Ca^{2+} channel [Phillips, Bull and Kelly (1992) Neuron **8**, 631–642], on divalent cation inflow in *Xenopus laevis* oocytes were investigated. The addition of extracellular Ca^{2+} ($[Ca^{2+}]_o$) to oocytes injected with *trpl* cRNA and to mock-injected controls, both loaded with the fluorescent Ca^{2+} indicator fluo-3, induced a rapid initial and a slower sustained rate of increase in fluorescence, which were designated the initial and sustained rates of Ca^{2+} inflow respectively. Compared with mock-injected oocytes, *trpl*-cRNA-injected oocytes exhibited a higher resting cytoplasmic free Ca^{2+} concentration ($[Ca^{2+}]_i$), and higher initial and sustained rates of Ca^{2+} inflow in the basal (no agonist) states. The basal rate of Ca^{2+} inflow in *trpl*-cRNA-injected oocytes increased with (1) an increase in the time elapsed between injection of *trpl* cRNA and the measurement of Ca^{2+} inflow, (2) an increase in the amount of *trpl* cRNA injected and (3) an increase in $[Ca^{2+}]_o$. Gd^{3+} inhibited the *trpl* cRNA-induced basal rate of Ca^{2+} inflow, with a concentration of approx. 5 μM Gd^{3+} giving half-maximal inhibition. Expression of *trpl* cRNA also caused an increase in the basal rate of Mn^{2+} inflow. The increases in resting $[Ca^{2+}]_i$ and in the basal rate of Ca^{2+} inflow induced by expression of *trpl* cRNA were inhibited by the calmodulin inhibitors W13, calmodazolium and peptide (281–309) of (Ca^{2+} and calmodulin)-dependent protein kinase II. A low concentration of exogenous calmodulin (introduced by microinjection) activated, and a higher concentration inhibited, the *trpl* cRNA-induced increase in basal rate of Ca^{2+} inflow. The action of the high concentration of exogenous calmodulin was reversed by W13 and calmodazolium. When rates of Ca^{2+} inflow in *trpl*-cRNA-injected oocytes were compared with those in mock-injected oocytes, the guanosine 5′-[β-thio]diphosphate-stimulated rate was greater, the onset of thapsigargin-stimulated initial rate somewhat delayed and the inositol 1,4,5-trisphosphate-stimulated initial rate markedly inhibited. It is concluded that (1) the divalent cation channel activity of the *Drosophila* Trpl protein can be detected in *Xenopus* oocytes; (2) in the environment of the *Xenopus* oocyte the Trpl channel admits some Mn^{2+} as well as Ca^{2+}, is activated by cytoplasmic free Ca^{2+} (through endogenous calmodulin) and by a trimeric GTP-binding regulatory protein, but does not appear to be activated by depletion of Ca^{2+} in the endoplasmic reticulum; and (3) expression of the Trpl protein inhibits the process by which the release of Ca^{2+} from intracellular stores activates endogenous store-activated Ca^{2+} channels.

INTRODUCTION

Most animal cells possess receptor-activated Ca^{2+} channels (RACCs) in the plasma membrane (reviewed in [1–5]). These channels are responsible for a closely controlled inflow of Ca^{2+} to the cytoplasmic space in response to an extracellular signal such as a hormone, neurotransmitter or growth factor. In non-excitable and in some excitable cells the inflow of Ca^{2+} through RACCs together with the action of inositol 1,4,5-trisphosphate [Ins(1,4,5)P_3], which releases Ca^{2+} from an Ins(1,4,5)P_3-sensitive store (most probably the smooth endoplasmic reticulum), is responsible for the increase in intracellular free Ca^{2+} concentration ($[Ca^{2+}]_i$) which acts as an intracellular messenger [1–5]. RACCs are also required for the replenishment of the Ins(1,4,5)P_3-sensitive Ca^{2+} stores after Ins(1,4,5)P_3-induced release of Ca^{2+} [1].

The structure and mechanism of activation of mammalian RACCs are not well understood at present [1,3–5]. So far there have been no reports of the purification of the channel protein(s) or isolation of the cDNA that encodes the channel protein(s). Recent evidence indicates that a number of cells possess at least two types of RACC: one type of channel that is highly selective for Ca^{2+} and a second non-specific cation channel [1,3,6]. The mechanism of activation of the Ca^{2+}-selective RACCs and some non-specific cation RACCs is proposed to involve the interaction of Ins(1,4,5)P_3 with Ins(1,4,5)P_3 receptors (Ca^{2+} channels) in the smooth endoplasmic reticulum and the release of Ca^{2+} from the endoplasmic reticulum. This, in turn, is believed to lead to opening of the plasma membrane RACCs. This is the store-operated, or 'capacitative', mechanism that was originally formulated by Putney [1,4,5,7]. The link between the smooth endoplasmic reticulum and the plasma membrane RACCs has not been elucidated, but it has been suggested that it might involve the Ins(1,4,5)P_3 receptor protein itself [8], a mobile intracellular messenger (e.g. the calcium influx factor) [9–12], a monomeric GTP-binding protein (G-protein) [13,14], and/or a trimeric G-protein [15–19]. One other type of RACC seems to be opened by the direct interaction of Ins(1,4,5)P_3 with the putative channel protein [1,3].

During the past five years two *Drosophila melanogaster* genes, *transient receptor potential*, *trp*, and *transient receptor potential like*, *trpl*, which encode putative plasma membrane Ca^{2+} channels

Abbreviations used: $[Ca^{2+}]_i$, concentration of intracellular free Ca^{2+}; $[Ca^{2+}]_o$, concentration of extracellular Ca^{2+}; $Ca^{2+}{}_o$, extracellular Ca^{2+}; 'caged' Ins(1,4,5)P_3, *myo*-inositol 4,5-diphosphate, $P^{4(5)}$-1-(2-nitrophenyl)ethyl ester; CamK(281–309), (Ca^{2+} and calmodulin)-dependent protein kinase peptide 281–309; CamK(281–290), (Ca^{2+} and calmodulin)-dependent protein kinase peptide 281–290; GDP[S], guanosine 5′-[β-thio]diphosphate; GTP[S], guanosine 5′-[γ-thio]triphosphate; Ins(1,4,5)P_3F, 3-dioxy-3 fluoroinositol 1,4,5-trisphosphate; MBS, modified Barth's solution; RACCs, receptor-activated Ca^{2+} channels.

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have been identified and sequenced [20–24]. These proteins, which seem to differ in their ion specificity and electrophysiological properties, are expressed in cells of the retina [20–24]. It has been suggested that the Trp and Trpl proteins represent *Drosophila* equivalents of mammalian Ins(1,4,5)P_3-activated RACCs [20–24]. The Trpl protein has two putative calmodulin-binding domains [22], a feature used in isolation of the *trpl* DNA by expression cloning [22]. Recently the Trp and Trpl proteins have been expressed in insect Sf9 cells and shown to lead to the appearance of new Ca^{2+} channels in these cells [25–28].

In view of the likely similarities between those types of mammalian RACCs that are non-selective cation channels and the *Drosophila* Trpl protein [3,4,22,24], it was considered that a study of the properties of the Trpl protein expressed in oocytes might provide a better understanding of this group of mammalian RACCs. Thus the aim of the present study was to express the *Drosophila* Trpl protein in *Xenopus* oocytes, to determine some of the characteristics of the cation channel formed by this polypeptide chain, and to study the mechanism of activation. In addition to an ability to express mammalian proteins, including ion channels [29,30], *Xenopus* oocytes allow the ready introduction by microinjection to the cytoplasmic space of membrane-impermeant compounds, such as Ins(1,4,5)P_3, which regulate cellular Ca^{2+} fluxes. However, *Xenopus* oocytes express at least two types of endogenous RACC [31–37]. Although these have been well characterized, it is necessary to distinguish divalent cation inflow through the expressed exogenous Trpl channel from that which occurs through these endogenous channels. The results of the present study have shown that, when expressed in *Xenopus* oocytes, the Trpl protein forms a divalent cation channel that admits Ca^{2+} and Mn^{2+}, seems to be constitutively activated by (Ca^{2+} and calmodulin), and can be further activated by a trimeric G-protein.

EXPERIMENTAL

Materials

Drosophila trpl cDNA was kindly provided by Dr. Len Kelly, Department of Genetics, University of Melbourne, Parkville, Victoria. *Xenopus laevis* were obtained from Xenopus I (Ann Arbor, MI, U.S.A.); collagenase, guanosine 5′-[γ-thio]triphosphate (GTP[S]) and guanosine 5′-[β-thio]diphosphate (GDP[S]) from Boehringer Mannheim Australia (Castle Hill, N.S.W.); fluo-3 and fura 2 from Molecular Probes (Eugene, OR, U.S.A.); thapsigargin and pertussis toxin from Sigma-Aldrich (Castle Hill, N.S.W.); Ins(1,4,5)P_3, 3-dioxy-3-fluoro-inositol (1,4,5)-trisphosphate, *myo*-inositol 4,5-diphosphate,$P^{4(5)}$-1-(2-nitrophenyl)ethyl ester ('caged' Ins(1,4,5)P_3), W13, calmidazolium, calmodulin (high purity, bovine brain) and heparin from Calbiochem-Novabiochem Pty. Ltd. (Alexandria, N.S.W.). Peptides 281–309 [CamK(281–309)] and 281–290 [CamK(281–290)], which are part of the calmodulin binding domain of (Ca^{2+} and calmodulin)-dependent protein kinase II [38,39], were kindly provided by Dr. Bruce E. Kemp, St. Vincent's Institute of Medical Research, Melbourne, Victoria. All other chemicals were of the highest grade commercially available.

Preparation of *trpl* cRNA

Escherichia coli (ED 8799) were transfected with *trpl* cDNA in the pBluescript plasmid [22], and the plasmid DNA was isolated. After digestion with *Not*1, capped cRNA was prepared by transcription *in vitro* with the mMessage mMachine *in vitro* transcription kit, in accordance with the manufacturer's instructions (Ambion, Austin, TX, U.S.A.). Linearized cDNA (1 μg) was transcribed into cRNA by incubation with T7 RNA polymerase for 1 h at 37 °C. After removing template DNA by incubation for a further 15 min in the presence of DNAse I (2 units), and removing protein by phenol/chloroform extraction, the cRNA was recovered by precipitation with ammonium acetate. The yield of cRNA was estimated by determining the percentage of [^{32}P]UTP incorporated after precipitation with trichloroacetic acid.

Isolation and microinjection of oocytes

Ovary tissue was removed from mature egg-laying *X. laevis* under anaesthesia, and single oocytes at stages V and VI were isolated by microdissection after treatment of the oocytes with collagenase (2 mg/ml for 1 h at 17 °C) [30]. Oocytes were incubated in modified Barth's solution (MBS) [30] at 17 °C in a refrigerated incubator (Scientific Equipment Manufacturers, Adelaide, South Australia).

The procedures for the microinjection of cRNA into *Xenopus* oocytes and incubation of oocytes were as described previously [30]. *trpl* cRNA was dissolved in diethyl pyrocarbonate-treated sterile water and introduced into the cytoplasmic space of oocytes by microinjection (50 nl containing 50 ng of cRNA) with the use of a Nikon stereo microscope and a Nanolitre injector (World Precision Instruments, Sarasota, FL, U.S.A.). Oocytes treated in a similar manner but without the introduction of any fluid into the cytoplasmic space (mock-injected oocytes) were used as controls. After microinjection the oocytes were incubated at 17 °C in MBS for 3 days before the assay of divalent cation inflow. In a series of control experiments, Ca^{2+} fluxes in mock-injected oocytes were found to be similar to those in oocytes injected with 50 nl of diethyl pyrocarbonate-treated sterile water.

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

The fluorescence of oocytes loaded with fluo-3 (Ca^{2+}) or fura 2 (Mn^{2+}) [40] was measured with a Perkin-Elmer LS50B spectrofluorimeter fitted with a Well Plate Reader and Fibre Optic Accessory (Perkin-Elmer Pty. Ltd., Knoxfield, Victoria). Fluo-3 or fura 2 was loaded into oocytes (about 20 oocytes in one batch) by microinjection (36 nl/oocyte of 10 mM dye dissolved in 125 mM KCl) and the oocytes were incubated for 30 min {20 min [Ins(1,4,5)P_3F] or 15 min [Ins(1,4,5)P_3, GTP[S]]} in Ca^{2+}-free MBS before initiation of the measurement of fluorescence (t = 0 min in the figures). Ins(1,4,5)P_3, Ins(1,4,5)P_3F and GTP[S] were co-injected with fluo-3. After the microinjection of fluo-3 or fura 2 and the agent (or agents) under test, the oocytes were examined under a stereomicroscope. The few cells that exhibited damage (protrusion of cytoplasm from the injection site) were discarded.

For the measurement of fluorescence, each oocyte was transferred to a well in a multiwell plate (60-well Micromulti Test Tray, Disposable Products, Adelaide, South Australia) containing 30 μl of Ca^{2+}-free MBS and other additions as indicated. The oocytes were orientated with the pigmented animal pole downwards (furthest from the fibre-optic light guide) and the plane of the equator perpendicular to the light path. For measurements of Ca^{2+} inflow (fluo-3), excitation was at 490 nm and emission at 530 nm. For the measurement of Mn^{2+} inflow (fura 2), the excitation and emission wavelengths were 360 and 510 nM respectively. The fibre-optic light guide irradiates the bottom of a given well of the multiwell plate (including the whole oocyte) and detects the light emitted from this region (i.e. the fluorescence of the whole oocyte).

On the basis of a value of 1000 nl for the average volume of an individual oocyte [30] it was estimated that the dilution of a reagent microinjected into an oocyte (36 nl per oocyte) is approx.

25-fold. Thus the estimated intracellular concentration of fluo-3 and fura 2 is 0.4 mM. The estimated intracellular concentrations of other agents used are given in the legends of the Figures and in Tables 3 and 4.

Rates of Ca^{2+} inflow to individual oocytes were estimated by analysing plots of fluorescence as a function of time obtained after the addition of extracellular Ca^{2+} (Ca^{2+}_o) to oocytes that had been loaded with fluo-3 and incubated initially in MBS that contained no added Ca^{2+} (Ca^{2+}-free MBS). The principle underlying this method has been employed previously to measure Ca^{2+} inflow to many other cell types [15,41]. Experiments designed to test the effect of the absence of added Ca^{2+}_o from the MBS during the preincubation of oocytes on the rates of Ca^{2+} inflow showed that, in the absence of an agonist, similar rates of Ca^{2+}_o (13 mM)-induced increase in fluorescence were observed when oocytes were incubated in either the presence or absence of 0.74 mM Ca^{2+}_o in the MBS medium for 2 h before the injection of fluo-3 and for 20 min after the injection of fluo-3, then transferred to Ca^{2+}-free MBS for the measurement of fluorescence (results not shown). These results indicate that in the absence of an agonist preincubation of oocytes in the absence of added Ca^{2+}_o for 2 h does not lead to an increase in the observed rate of Ca^{2+}_o-induced increase in fluorescence (i.e. Ca^{2+} inflow) when compared with oocytes preincubated in the presence of added Ca^{2+}_o. When oocytes loaded with Ins(1,4,5)P_3F were preincubated in the presence of 0.74 mM Ca^{2+}_o for 20 min then transferred to Ca^{2+}-free MBS before beginning the measurement of fluorescence, the rate of Ins(1,4,5)P_3-stimulated Ca^{2+} inflow was 4.4 ± 0.7 (5) (mean $\pm$ S.E.M. with the number of experiments in parenthesis) fluorescence units/min (13 mM Ca^{2+}_o) compared with a rate of 9.0 ± 0.9 (3) fluorescence units/min observed for oocytes preincubated for 20 min in the absence of added Ca^{2+}_o. The results obtained in the presence of Ins(1,4,5)P_3F indicate that preincubation of oocytes in the absence of added Ca^{2+}_o (compare the results in the presence of added Ca^{2+}_o) does increase the initial rate of Ins(1,4,5)P_3F-stimulated Ca^{2+} inflow. This might be because preincubation in the presence of Ins(1,4,5)P_3 and absence of added Ca^{2+}_o increases the amount of Ca^{2+} transported out of oocytes [presumably by the plasma membrane ($Ca^{2+} + Mg^{2+}$)ATPase] and the emptying of intracellular Ca^{2+} stores when this is induced by the presence of Ins(1,4,5)P_3F in the preincubation period.

Verification of the source of Ca^{2+}_o-induced increase in fluorescence

In all experiments (see the Results section), except those in which GTP[S] was present, the resulting plot of fluorescence as a function of time consisted of an initial rapid increase in fluorescence (designated the initial rate of Ca^{2+} inflow) and a slower sustained increase in fluorescence (designated the sustained rate of Ca^{2+} inflow) {compare the sustained Ca^{2+}-activated chloride current observed by Parekh et al. [36] in *Xenopus* oocytes treated with Ins(1,4,5)P_3}. In a series of control experiments the fluorescence of fluo-3-loaded oocytes was also examined by confocal microscopy. In agreement with results reported by others for *Xenopus* oocytes [42], most of the fluorescence was found to arise from a region close to the oocyte plasma membrane (A. M. Auld, P. Kolesic and G. J. Barritt, unpublished work).

In a separate series of experiments, conducted in a 35 mm Petri dish containing 3 ml of incubation medium in which the oocyte was immobilized with a wire grid, the fluorescence of a small region of the oocyte adjacent to the plasma membrane was measured with a Nikon microscope, photometer and pinhole diaphragm [15]. Results (not shown) similar to those obtained when fluorescence of the whole oocyte was measured (Figure 4a) for the Ca^{2+}_o-induced increase in fluorescence in mock-injected oocytes loaded with Ins(1,4,5)P_3F were obtained when the fluorescence of a small region of the oocyte (approx. 2 μm in diameter) adjacent to the plasma membrane on the vegetal side of the oocyte equator was measured as a function of time. These results indicate that (1) the rapid initial and slow sustained phases of Ca^{2+} inflow are also observed when the measurement of fluorescence is made close to the plasma membrane and (2) the use of a relatively small volume of medium in multiwell plates does not seem to have any detrimental effects on oocyte Ca^{2+} homoeostasis. Thus it is considered unlikely that the initial and sustained rates of Ca^{2+} inflow observed in all experiments except those involving GTP[S] are a consequence of the technique employed for the measurement of oocyte fluorescence (i.e. detection of light emitted from the whole oocyte by using a fibre-optic light guide) because similar kinetics were observed when the Ca^{2+}_o-induced increase in fluorescence of a region of the cytoplasmic space just underneath the plasma membrane was measured.

Calculation of rates of Ca^{2+} inflow

The initial rate of Ca^{2+} inflow was determined by calculating the slope of the plot of fluorescence as a function of time at 0, 1 and 3 min after the addition of Ca^{2+}_o. (The fluorescence at 0 min was obtained by extrapolation of the values obtained before Ca^{2+}_o addition.) The sustained rate of Ca^{2+} inflow was determined by calculating the slope of the plot of fluorescence as a function of time at 5, 10, 15, 20 and 25 min after addition of Ca^{2+}_o. For results obtained in the presence of GTP[S], it was not possible to define separate initial rapid and sustained slow phases of Ca^{2+} inflow, as discussed in the Results section. Thus, in the presence of GTP[S], the rate of Ca^{2+} inflow was determined from the values of fluorescence obtained at 0, 1, 3, 5 and 10 min after the addition of Ca^{2+}_o and was called an 'initial plus sustained' rate. All slopes were determined by linear regression.

Calculation of rates of Mn^{2+} inflow

Rates of Mn^{2+} inflow were estimated by measuring the rate of decrease in the fluorescence of intracellular fura 2 after the addition of Mn^{2+} to oocytes loaded with fura 2 and incubated in Ca^{2+}-free MBS, as described previously [43]. For each individual oocyte the rate of change in fluorescence was determined from the slope of the plot of fluorescence as a function of time (determined by linear regression) by using the values obtained from 1 to 25 min after the addition of Mn^{2+}.

Use of 'caged' Ins(1,4,5)P_3

'Caged' Ins(1,4,5)P_3 was co-injected with fluo-3. Oocytes were incubated in a 35 mm Petri dish fitted with a wire grid as described above. Photolysis was achieved by exposing the cell to the full spectrum of light from the mercury lamp for 1 s while the shutter of photometer was closed [16]. Control experiments (results not shown) showed that on photolysis there was no increase in fluorescence signal in oocytes loaded with 'caged' Ins(1,4,5)P_3 in the absence of fluo-3.

Presentation of results

Measurements were usually made with a group of about five oocytes in one experiment, and were repeated for the number of times indicated in the Figure legends and the Tables. The values

shown in plots of fluorescence as a function of time represent the means ± S.E.M. of the results obtained for a representative experiment. The bar graph and Tables show the means ± S.E.M. of the results obtained for all individual oocytes incubated under a given condition.

RESULTS

Basal (no agonist) Ca^{2+} inflow

X. laevis oocytes that had been injected with *trpl* cRNA (50 ng/oocyte) 3 days before being loaded with fluo-3 and incubated in the absence of added Ca^{2+}_o exhibited a greater basal fluorescence when compared with mock-injected oocytes (Figure 1). The addition of Ca^{2+}_o caused an initial rapid increase in fluorescence followed by a slower sustained increase (Figure 1). Both the initial and sustained rates of Ca^{2+} inflow were greater in *trpl*-cRNA-injected oocytes than in mock-injected oocytes (Figure 1).

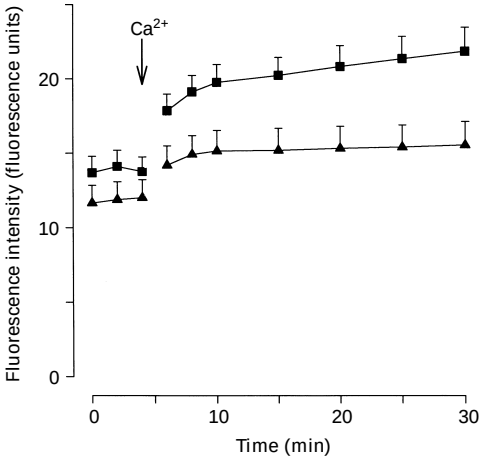

Figure 1 Effect of expression of *Drosophila trpl* cRNA on basal Ca^{2+} inflow (absence of agonists) in *Xenopus* oocytes

Fluorescence as a function of time for *trpl*-cRNA-injected (■) and mock-injected (▲) oocytes. Oocytes injected with fluo-3 were initially incubated for 30 min in the absence of added Ca^{2+}_o before beginning the measurement of fluorescence ($t = 0$ min). At the time indicated by the arrow 12.5 mM Ca^{2+} was added to the extracellular medium. The microinjection of oocytes with *trpl* cRNA (50 ng/oocyte, 3 days before measurement of fluorescence) and fluo-3, incubation of oocytes, and the measurement of fluorescence were conducted as described in the Experimental section. The results are the means ± S.E.M. for four to eight oocytes in one experiment and are representative of three similar experiments.

Table 2 Effect of amount of *trpl* cRNA injected on the *trpl*-cRNA-induced increase in the basal sustained rate of Ca^{2+} inflow

Oocytes were incubated for 3 days after the injection of *trpl* cRNA. The results are the means ± S.E.M. for the numbers of oocytes indicated in parenthesis. The microinjection of oocytes with *trpl* cRNA and fluo-3, incubation of oocytes, measurement of fluorescence and calculation of sustained rates of Ca^{2+}_o-induced increase in fluorescence were conducted as described in the Experimental section. The degrees of significance, determined with Student's *t*-test for unpaired samples, for comparison of *trpl*-cRNA-injected with mock-injected oocytes are $^{**}P \leqslant 0.01$, $^{***}P \leqslant 0.001$.

Amount of *trpl* cRNA injected (ng)	Rate of Ca^{2+}_o-induced increase in fluorescence (fluorescence units/min per oocyte)	
	Mock	*trpl*
10	0.019 ± 0.003 (18)	0.014 ± 0.009 (15)
30	0.026 ± 0.007 (12)	0.078 ± 0.01 (14)**
50	0.03 ± 0.005 (13)	0.12 ± 0.02 (12)***

A *trpl* cRNA-induced increase in the basal (no agonist present) initial and sustained rates of Ca^{2+} inflow was evident 2 days after injection of *trpl* cRNA (Table 1). The magnitude of the effect of *trpl* cRNA on the basal initial and sustained rates of Ca^{2+} inflow increased linearly with time over a period of 1–3 days after *trpl* cRNA injection and seemed to approach a plateau at 3 days (Table 1). A incubation period of 3 days with *trpl* cRNA was employed in all subsequent experiments. In *trpl*-cRNA-injected oocytes, the basal initial (results not shown) and sustained (Table 2) rates of Ca^{2+} inflow increased in a roughly linear manner as the amount of *trpl* cRNA injected was increased from 10 to 50 ng.

The degree of stimulation by *trpl* cRNA of the basal initial (results not shown) and sustained (Figure 2a) rates of Ca^{2+} inflow increased as the $[Ca^{2+}]_o$ was increased from 1 to 10 mM. The *trpl* cRNA-induced increases in the initial (results not shown) and sustained (Figure 2b) rates of Ca^{2+} inflow were completely inhibited by Gd^{3+}. Half-maximal inhibition was given by approx. 5 μM Gd^{3+} (Figure 2b).

Basal (no agonist) Mn^{2+} inflow

The addition of Mn^{2+} to mock-injected oocytes (loaded with fura 2 instead of fluo-3) incubated in the absence of added Ca^{2+}_o led

Table 1 Effect of incubation time on the *trpl*-cRNA-induced increase in the basal rate of Ca^{2+} inflow

Oocytes were injected with 50 ng of *trpl* cRNA. The results are the means ± S.E.M. for the numbers of oocytes indicated in parentheses. The microinjection of oocytes with *trpl* cRNA and fluo-3, incubation of oocytes, measurement of fluorescence and calculation of initial rates of Ca^{2+}_o-induced increase in fluorescence were conducted as described in the Experimental section. The degrees of significance, determined with Students *t*-test for unpaired samples, for comparison of *trpl*-cRNA-injected with mock-injected oocytes are $^{*}P < 0.05$, $^{**}P < 0.01$.

Time elapsed after cRNA injection (days)	Rate of Ca^{2+}_o-induced increase in fluorescence (fluorescence units/min per oocyte)			
	Initial		Sustained	
	Mock	*trpl*	Mock	*trpl*
1	0.19 ± 0.1 (10)	0.04 ± 0.1 (12)	0.03 ± 0.008 (10)	0.038 ± 0.009 (12)
2	0.23 ± 0.2 (13)	0.73 ± 0.4 (12)	0.025 ± 0.01 (13)	0.075 ± 0.02 (12)*
3	0.39 ± 0.003 (13)	1.13 ± 0.26 (15)**	0.02 ± 0.01 (13)	0.09 ± 0.03 (12)*

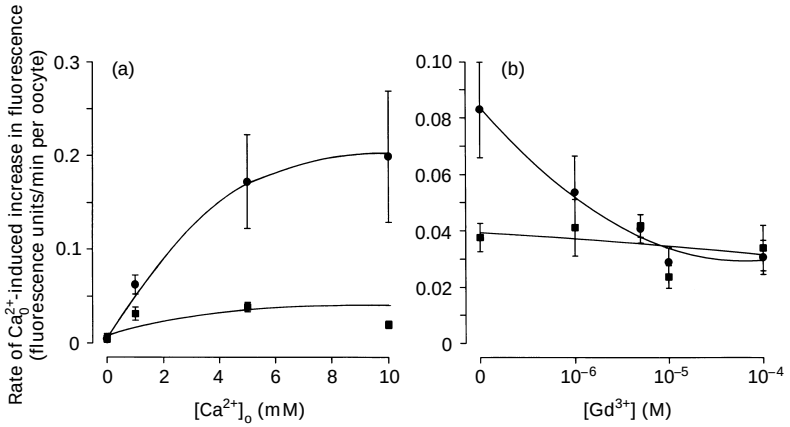

Figure 2 Effects of increasing $[Ca^{2+}]_o$ (a) and Gd^{3+} (b) on the basal sustained rate of Ca^{2+} inflow (absence of agonists) in *trpl*-cRNA-injected and mock-injected oocytes

(**a**) The basal sustained rate of Ca^{2+}_o-induced increase in fluorescence as a function of $[Ca^{2+}]_o$ for mock-injected (■) and *trpl*-cRNA-injected (●) oocytes. (**b**) The basal sustained rate of Ca^{2+}_o-induced increase in fluorescence (measured at 12.5 mM $[Ca^{2+}]_o$) as a function of the concentration of Gd^{3+} for mock-injected (■) and *trpl*-cRNA-injected (●) oocytes. Sustained rates of Ca^{2+}_o-induced increase in fluorescence were determined as described in the Experimental section. The values are the means ± S.E.M. for four to nine oocytes. For the batch of oocytes used in (**b**) the level of expression of *trpl* cRNA was lower than that observed in the experiment described in (**a**).

to a small decrease in fluorescence (Figure 3a). The rate of the Mn^{2+}-dependent decrease in fluorescence in *trpl*-cRNA-injected oocytes was greater than that in mock-injected oocytes (Figure 3a). The difference in the rate of Mn^{2+}-induced decrease in fluorescence between *trpl*-cRNA-injected and mock-injected oocytes was more clearly apparent when the means of the slopes obtained for individual oocytes in the presence and absence of Mn^{2+} were compared (Figure 3b). Although there was some variation in the absolute fluorescence from one oocyte to another (error bars in Figure 3a), there was considerably less variation in the slopes (error bars in Figure 3b). These results indicate that expression of *trpl* cRNA permits an increased inflow of Mn^{2+} as well as Ca^{2+} in the basal (no agonist) state.

Role of intracellular Ca^{2+} and calmodulin in *trpl* cRNA-induced basal Ca^{2+} inflow

The roles of Ca^{2+} and calmodulin in regulation of the *trpl* cRNA-induced basal rates of Ca^{2+} inflow were investigated with three inhibitors of calmodulin function: W13, calmidazolium and a site-specific synthetic peptide composed of residues 281–309 of the α-subunit of (Ca^{2+} and calmodulin)-dependent protein kinase II [CamK(281–309)] [38,39,44]. This peptide contains the calmodulin binding site of (Ca^{2+} and calmodulin)-dependent protein kinase II. W13 completely inhibited the *trpl* cRNA-induced increase in the basal initial (results not shown) and sustained (Table 3) rates of Ca^{2+} inflow whereas CamK(281–309) caused a 60 % inhibition of these parameters (Table 3). Little inhibition of *trpl* cRNA-induced Ca^{2+} inflow was observed when (Ca^{2+} and calmodulin) protein kinase II peptide 281–290 [CamK(281–290)], which does not inhibit calmodulin action [38,39], was injected into oocytes (results not shown). W13 and CamK(281–309) did not inhibit the basal initial (results not shown) and sustained (Table 3) rates of Ca^{2+} inflow in mock-injected oocytes. Calmidazolium (5 μM for 60 min) also completely inhibited the *trpl* cRNA-induced increases in basal, initial and sustained rates of Ca^{2+} inflow and had no effect on the rates in mock-injected oocytes (results not shown).

The injection of exogenous calmodulin (estimated intracellular concentration 1 μM) caused a small (50 %) increase in the initial and sustained rates of Ca^{2+} inflow of *trpl*-cRNA-injected but not mock-injected oocytes (results not shown). A higher concentration of exogenous calmodulin (3 μM) completely inhibited the basal initial (results not shown) and sustained (Table 3) rates of Ca^{2+} inflow in *trpl*-cRNA-injected oocytes, with no effect on mock-injected oocytes. The effect of 3 μM exogenous calmodulin on the initial (results not shown) and sustained (Table 3) rates of Ca^{2+} inflow in *trpl*-cRNA-injected oocytes was almost completely reversed by treatment of the oocytes with W13. Similar results were obtained with calmidazolium (results not shown). Because it was considered possible that insufficient W13 had been added to inhibit the actions of exogenous as well as endogenous calmodulin, the experiment was repeated with a higher concentration of W13 (240 μM). Results (not shown) similar to those shown in Table 3 (exogenous calmodulin plus W13) were obtained.

In another approach to investigating the mechanism(s) responsible for the constitutive activation of the Trpl protein in oocytes, the effects of pertussis toxin and GDP[S], inhibitors of trimeric G-proteins [45], were tested. Pretreatment of oocytes with pertussis toxin completely inhibited the *trpl* cRNA-induced increase in the basal initial (not shown) and sustained (Table 3) rates of Ca^{2+} inflow, but caused no inhibition of the basal rate of Ca^{2+} inflow in mock-injected oocytes (Table 3), and did not inhibit Ins(1,4,5)P_3F-stimulated Ca^{2+} inflow in mock-injected oocytes (results not shown). The microinjection of GDP[S] also completely inhibited the *trpl* cRNA-induced increase in basal Ca^{2+} inflow but did not affect basal Ca^{2+} inflow in mock-injected oocytes (Table 3).

Effects of Ins(1,4,5)P_3 and GTP[S] on *trpl* cRNA-induced Ca^{2+} inflow

The introduction of Ins(1,4,5)P_3 (intracellular concentration 7 μM) to mock-injected or *trpl*-cRNA-injected oocytes increased the initial and sustained rates of Ca^{2+} inflow when compared with the rates observed in mock-injected and *trpl*-cRNA-injected

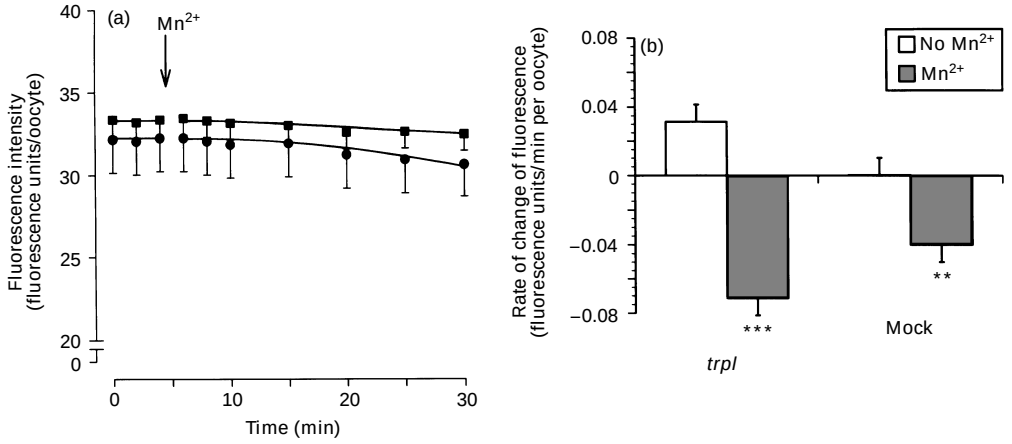

Figure 3 Stimulation of basal Mn^{2+} inflow (absence of agonists) by the expression of *trpl* cRNA

(**a**) Fluorescence as a function of time for mock-injected (■) and *trpl*-cRNA-injected (●) oocytes. Mn^{2+} (1.0 mM) was added at the time indicated by the arrow. Oocytes injected with fura 2 were initially incubated for 30 min in the absence of both added Ca^{2+}_o and Mn^{2+} before beginning the measurement of fluorescence ($t = 0$ min). The fluorescence of fura 2-loaded oocytes (360 nm excitation, 510 nm emission) was measured as described in the Experimental section. The results are the means ± S.E.M. for 27–32 oocytes. (**b**) Rates of change in fluorescence measured, over the period 1–25 min after the addition of Mn^{2+}, in the presence (solid bars) or absence (open bars) of 1.0 mM Mn^{2+} for *trpl*-cRNA-injected and mock-injected oocytes. Rates of change in fluorescence for individual oocytes were determined as described in the Experimental section. The results shown are the means ± S.E.M. for 27–32 individual oocytes. The degrees of significance, determined with Student's *t*-test for unpaired samples, for comparison of rates of change of fluorescence in the presence and absence of Mn^{2+} are $^{**}P \leqslant 0.01$, $^{***}P \leqslant 0.001$. The degree of significance for comparison of the rate of change of fluorescence in the presence of Mn^{2+} in *trpl*-cRNA-injected oocytes with the rate in mock-injected oocytes is $P \leqslant 0.05$.

Table 3 Effects of the calmodulin inhibitors W13 and CamK(281–309), purified calmodulin, pertussis toxin and GDP[S] on the basal sustained rate of Ca^{2+} inflow in mock-injected and *trpl*-cRNA-injected oocytes

W13 (120 μM) was added to the extracellular medium 60 min before beginning the measurement of fluorescence. CamK(281–309) (estimated intracellular concentration 1.2 μM) and calmodulin (Cam) (estimated intracellular concentration, 3 μM) were co-injected with fluo-3. Oocytes were pretreated with pertussis toxin (2 μg/ml) for 24 h at 17 °C. GDP[S] (estimated intracellular concentration 700 μM) was co-injected with fluo-3. Basal sustained rates of Ca^{2+}_o-induced increase in fluorescence were determined as described in the Experimental section and in the legend of Figure 1 by using 12.5 or 1.0 mM (GDP[S]) Ca^{2+}_o. The results are means ± S.E.M. for the numbers of oocytes indicated in parenthesis. The degrees of significance, determined with Student's *t*-test for unpaired samples, for a comparison of rates of Ca^{2+} inflow in *trpl*-cRNA-injected and mock-injected oocytes are $^{**}P \leqslant 0.01$, $^{***}P \leqslant 0.001$.

	Rate of Ca^{2+}_o-induced increase in fluorescence (fluorescence units/min per oocyte)	
Agent	Mock	*trpl*
None	0.05 ± 0.003 (40)	0.10 ± 0.01 (43)***
W13	0.05 ± 0.009 (33)	0.05 ± 0.01 (23)
CamK	0.05 ± 0.006 (22)	0.07 ± 0.01 (13)
Cam	0.05 ± 0.006 (32)	0.04 ± 0.008 (41)
Cam + W13	0.04 ± 0.006 (15)	0.10 ± 0.02 (17)**
Pertussis toxin	0.04 ± 0.002 (36)	0.038 ± 0.005 (33)
GDP[S]	0.03 ± 0.002 (43)	0.035 ± 0.004 (31)

oocytes incubated in the absence of exogenous Ins(1,4,5)P_3 [Figure 4a (compare Figure 1) and Table 4]. The effect of Ins(1,4,5)P_3 on the initial rate of Ca^{2+} inflow is consistent with that observed by others for untreated oocytes with different techniques [33,36]. Results similar to those shown in Figure 4a for mock-injected oocytes were obtained when the intracellular concentration of Ins(1,4,5)P_3 was reduced to 4 or 2 μM. However, no stimulation of Ca^{2+} inflow was observed at 0.3 μM Ins(1,4,5)P_3 (results not shown).

trpl-cRNA-injected oocytes loaded with Ins(1,4,5)P_3 exhibited lower initial rates of Ca^{2+} inflow and higher sustained rates of Ca^{2+} inflow than mock-injected oocytes (Figure 4a and Table 4). After subtraction of basal [no Ins(1,4,5)P_3] rates of Ca^{2+} inflow for mock-injected and *trpl*-cRNA-injected oocytes, it can be seen that the Ins(1,4,5)P_3-stimulated initial rate of Ca^{2+} inflow in *trpl*-cRNA-injected oocytes is substantially lower, and the sustained rate of Ca^{2+} inflow stimulated by 7 μM Ins(1,4,5)P_3 is substantially higher than the respective rates of Ca^{2+} inflow in mock-injected oocytes (Table 1). The Ins(1,4,5)P_3-stimulated Trpl-dependent sustained rate of Ca^{2+} inflow was completely abolished by the co-injection of heparin (0.9 mg/ml estimated intracellular concentration) with Ins(1,4,5)P_3 (results not shown).

Ins(1,4,5)P_3F (estimated intracellular concentration 7 μM) also stimulated the initial and sustained rates of Ca^{2+} inflow in *trpl*-cRNA-injected and mock-injected oocytes [Figure 4b (compare Figure 1) and Table 4]. However, the Ins(1,4,5)P_3F-stimulated initial and sustained rates of Ca^{2+} inflow in *trpl*-cRNA-injected oocytes were not significantly different from those in mock-injected oocytes (Table 1, 'Agent-stimulated' rate). There was also no significant difference observed between the Ins(1,4,5)P_3F-stimulated initial and sustained rates of Ca^{2+} inflow in *trpl*-cRNA-injected and mock-injected oocytes when a higher concentration (17 μM) of Ins(1,4,5)P_3F was used (Table 4).

Pretreatment of oocytes with thapsigargin also markedly stimulated both the initial and sustained rates of Ca^{2+} inflow in mock-injected and *trpl*-cRNA-injected oocytes compared with the rates in mock-injected and *trpl*-cRNA-injected oocytes incubated in the absence of thapsigargin [Figure 4c (compare Figure 1) and Table 4] (compare also the results obtained by others for the action of thapsigargin on untreated oocytes [35,37]). Oocytes injected with *trpl* cRNA exhibited higher initial and sustained rates of Ca^{2+} inflow in the presence of thapsigargin when

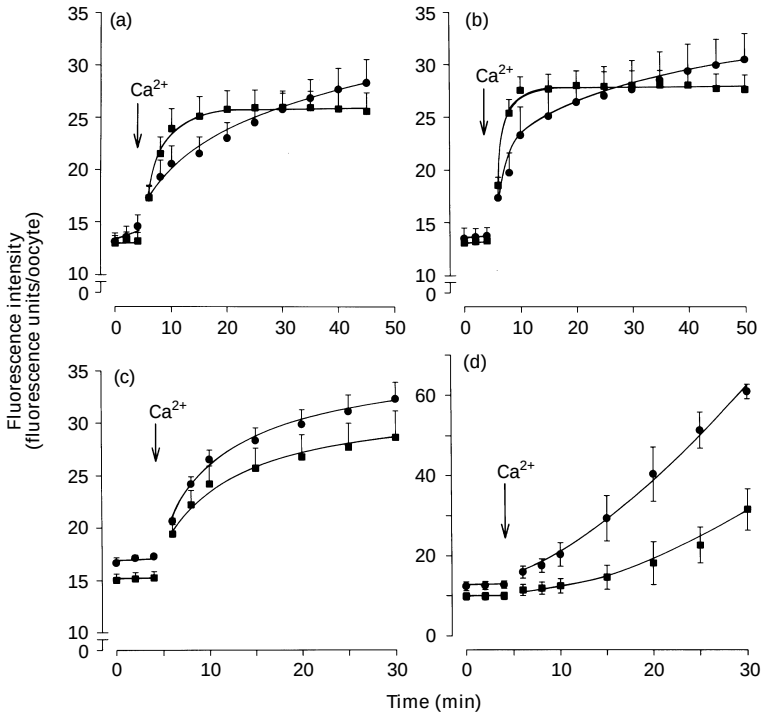

Figure 4 The stimulation by (a) Ins(1,4,5)P_3, (b) Ins(1,4,5)P_3F, (c) thapsigargin and (d) GTP[S] of $Ca^{2+}{}_o$-induced Ca^{2+} inflow in mock-injected (■) and *trpl*-cRNA-injected (●) oocytes

Ins(1,4,5)P_3, Ins(1,4,5)P_3F and GTP[S] were co-injected with fluo-3 into oocytes, as described in the Experimental section, 20 min [Ins(1,4,5)P_3] or 15 min (Ins(1,4,5)P_3, GTP[S]) before beginning the measurement of fluorescence ($t = 0$ min). The estimated intracellular concentrations of Ins(1,4,5)P_3, Ins(1,4,5)P_3F and GTP[S] were 7, 7 and 700 μM respectively. In (**c**), oocytes were incubated in Ca^{2+}-free MBS containing thapsigargin (5 μM) for 60 min, then washed and transferred to Ca^{2+}-free MBS before the microinjection of fluo-3. Fluorescence was measured as described in the Experimental section. At the time indicated by the arrow, $Ca^{2+}{}_o$ [12.5 mM in (**a**), (**b**) and (**c**) and 1.0 mM in (**d**)] was added. [Note that the time scale for (**c**) and (**d**) differs from that for (**a**) and (**b**), and the fluorescence intensity scale for (**d**) differs from those for (**a**), (**b**) and (**c**).] The results are the means ± S.E.M. for five oocytes in one experiment, and are representative of five or six separate experiments.

compared with mock-injected oocytes (Figure 4c and Table 4). However, when the values of Ca^{2+} inflow in the basal (no thapsigargin) state were subtracted, the thapsigargin-stimulated initial and sustained rates of Ca^{2+} inflow in mock-injected oocytes and in *trpl*-cRNA-injected oocytes were not significantly different (Table 4). For the experiments described in Table 4, some variation was observed in the initial rate of Ca^{2+} inflow in the absence of agent and in the degree of stimulation of the initial rate of Ca^{2+} inflow by Trpl for experiments conducted at 12.5 mM $Ca^{2+}{}_o$. This variation was due to variability in the values of the initial rate of Ca^{2+} inflow in different batches of oocytes, although in all cases a stimulation of the initial rate of Ca^{2+} inflow by expression of the Trpl protein was observed.

To further test whether Ins(1,4,5)P_3 can stimulate Ca^{2+} inflow in *trpl*-cRNA-injected oocytes over and above the ability of Ins(1,4,5)P_3 to release Ca^{2+} from intracellular stores and activate endogenous store-activated Ca^{2+} channels (i.e. after Ca^{2+} was released from the intracellular stores), the following experiment was performed. Oocytes were treated with thapsigargin for 2.5 h, loaded with fluo-3 and 'caged' Ins(1,4,5)P_3 and incubated in the absence of added $Ca^{2+}{}_o$. Subsequent addition of $Ca^{2+}{}_o$ caused an increase in fluorescence (Figure 5a). At the beginning of the sustained phase of Ca^{2+} inflow (plateau region) the 'caged' Ins(1,4,5)P_3 was photolysed. In mock-injected oocytes photolysis caused a very rapid decrease in fluorescence (most probably due to the photobleaching of some fluo-3). The subsequent plot of fluorescence as a function of time showed a small positive slope (Figure 5a). In *trpl*-cRNA-injected oocytes two differences were observed (Figure 5b). First, after the addition of $Ca^{2+}{}_o$ (before photolysis) the plots of fluorescence as a function of time often exhibited a lag. Secondly, after the photolysis of 'caged' Ins(1,4,5)P_3 a greater rate of increase in fluorescence was generally observed (Figure 5b). However, this increase was within the range observed for the effect of Trpl expression on basal Ca^{2+} inflow. In control experiments designed to verify that photolysis released free Ins(1,4,5)P_3, 'caged' Ins(1,4,5)P_3 in untreated oocytes incubated in the presence of $Ca^{2+}{}_o$ was photolysed. This caused a marked increase in fluorescence (Figure 5c) which was probably due to both the Ins(1,4,5)P_3-induced release of Ca^{2+} from intracellular stores and to enhanced plasma membrane Ca^{2+} inflow.

The ability of GTP[S], a slowly hydrolysable analogue of GTP that activates trimeric G-proteins [45], to activate the *trpl* cRNA-induced Ca^{2+} inflow process was also tested. The addition of $Ca^{2+}{}_o$ to mock-injected and *trpl*-cRNA-injected oocytes loaded with GTP[S] led to an increase in fluorescence (Figure 4d). However, in contrast with results obtained in the basal state (Figure 1), or in the presence of Ins(1,4,5)P_3, Ins(1,4,5)P_3F, or thapsigargin (Figures 4a to 4c), the plots of fluorescence as a function of time obtained in the presence of GTP[S] exhibited an increase in the rate of change in fluorescence with increasing time. Moroever, the pronounced initial increase in fluorescence observed on addition of $Ca^{2+}{}_o$ to oocytes loaded with Ins(1,4,5)P_3 or thapsigargin (Figures 4a to 4c) was absent in GTP[S]-loaded

Table 4 Effect of the expression of *trpl* cRNA on InsP_3-, InsP_3F-, thapsigargin- and GTP[S]-stimulated Ca^{2+} inflow in *Xenopus* oocytes

Initial and sustained rates of $Ca^{2+}{}_o$-induced increase in fluorescence were determined as described in the Experimental section. The extracellular concentration of thapsigargin was 5 μM. [The intracellular concentration was not determined (N.D.).] The parameter 'Agent-stimulated rate of $Ca^{2+}{}_o$-induced Ca^{2+} inflow' was calculated from the difference in the rates observed in the presence and absence of the agent under test. Variation in the initial rates of Ca^{2+} inflow in *trpl*-cRNA-injected oocytes is due to variation between different batches of oocytes. The results are the means ± S.E.M. for five or six experiments conducted with a total of 20–41 oocytes. Degrees of significance, determined with Student's *t*-test for unpaired samples, for a comparison of *trpl*-cRNA-injected with mock-injected oocytes are $^*P \leqslant 0.05$, $^{***}P \leqslant 0.001$.

Agent	Intracellular concn. of agent (μM)	$[Ca^{2+}]_0$ (mM)	Phase of Ca^{2+} inflow	Injection	Rate of $Ca^{2+}{}_o$-induced Ca^{2+} inflow (increase in fluorescence units/min per oocyte)		
					In the absence of agent	In the presence of agent	Agent-stimulated
InsP_3	7	12.5	Initial	*trpl*	0.81 ± 0.29	1.12 ± 0.17	0.30 ± 0.1***
				Mock	0.18 ± 0.06	1.37 ± 0.12	1.19 ± 0.1
InsP_3F	7	12.5	Initial	*trpl*	0.19 ± 0.07	1.49 ± 0.16	1.31 ± 0.1
				Mock	0.12 ± 0.03	1.69 ± 0.07	1.57 ± 0.07
InsP_3F	17	12.5	Initial	*trpl*	0.43 ± 0.09	1.20 ± 0.1	0.77 ± 0.1
				Mock	0.12 ± 0.02	1.01 ± 0.1	0.89 ± 0.1
Thapsigargin	N.D.	12.5	Initial	*trpl*	0.41 ± 0.07	0.86 ± 0.1	0.45 ± 0.1
				Mock	0.30 ± 0.05	0.74 ± 0.1	0.43 ± 0.1
GTP[S]	700	1.0	Initial + sustained	*trpl*	0.028 ± 0.003	1.17 ± 0.09***	1.15 ± 0.09***
				Mock	0.015 ± 0.003	0.47 ± 0.02	0.46 ± 0.02
InsP_3	7	12.5	Sustained	*trpl*	0.077 ± 0.03	0.218 ± 0.04***	0.141 ± 0.03*
				Mock	0.027 ± 0.01	0.072 ± 0.02	0.045 ± 0.02
InsP_3F	7	12.5	Sustained	*trpl*	0.182 ± 0.08	0.28 ± 0.04***	0.098 ± 0.04
				Mock	0.017 ± 0.01	0.11 ± 0.01	0.093 ± 0.01
InsP_3F	17	12.5	Sustained	*trpl*	0.09 ± 0.01	0.14 ± 0.02*	0.048 ± 0.02
				Mock	0.05 ± 0.01	0.07 ± 0.02	0.023 ± 0.02
Thapsigargin	N. D.	12.5	Sustained	*trpl*	0.05 ± 0.01	0.13 ± 0.02	0.08 ± 0.02
				Mock	0.02 ± 0.01	0.11 ± 0.02	0.09 ± 0.02

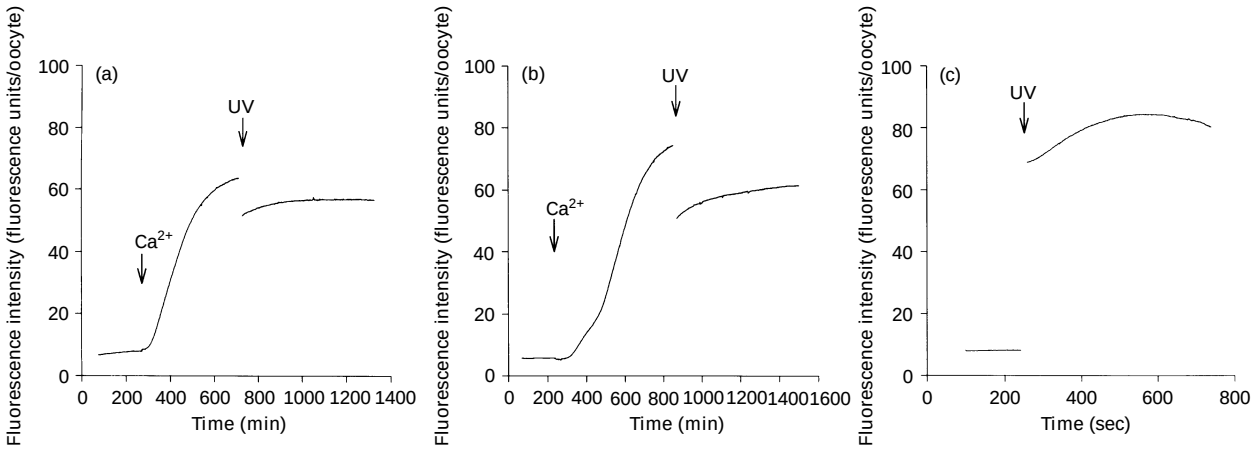

Figure 5 Effect of release of Ins(1,4,5)P_3 [after photolysis of 'caged' Ins(1,4,5)P_3] on the fluorescence of intracellular fluo-3 in (a) mock-injected oocytes pretreated with thapsigargin, (b) *trpl*-cRNA-injected oocytes pretreated with thapsigargin and (c) untreated oocytes

'Caged' Ins(1,4,5)P_3 was co-injected with fluo-3, the oocytes were preincubated in Ca^{2+}-free MBS, and fluorescence was measured as a function of time by using a fluorescence microscope, as described in the Experimental section. At the times indicated by the arrows 12.5 mM $Ca^{2+}{}_o$ was added (**a**,**b**) and the 'caged' Ins(1,4,5)P_3 was photolysed (UV) for 1 s, as described in the Experimental section. Pretreatment with 5 μM thapsigargin for 2.5 h was conducted as described in the legend to Figure 4. For each of (**a**), (**b**) and (**c**) the trace shown is a representative of four similar traces. Abbreviation: sec, seconds.

oocytes (Figure 4d). However, it is not possible to determine from the present results whether the initial (rapid) phase of Ca^{2+} inflow has been completely abolished (leaving only the sustained phase) or whether the initial phase is present but is markedly reduced.

The observed rate (initial plus sustained) of Ca^{2+} inflow in *trpl*-cRNA-injected oocytes loaded with GTP[S] was substantially greater than that in mock-injected oocytes loaded with GTP[S] (Figure 4d and Table 4). The basal (no agonist) rates of Ca^{2+} inflow were subtracted from those obtained in the presence of GTP[S] to obtain the GTP[S]-stimulated rates of Ca^{2+} inflow. Comparison of the values obtained indicates that the GTP[S]-stimulated rate of Ca^{2+} inflow in *trpl*-cRNA-injected oocytes is more than twice than that in mock-injected oocytes (Table 4).

DISCUSSION

Evidence that the Trpl protein encodes a divalent cation channel

The idea that the Trpl protein encodes a divalent cation channel is consistent with the observation that expression of this protein in *Xenopus* oocytes leads to an increase in the initial rates of Ca^{2+} and Mn^{2+} inflow in the basal state, and to an increase in the GTP[S]-stimulated rate of Ca^{2+} inflow. The observation that the apparent concentration of Gd^{3+} that gives a half-maximal inhibition of the *trpl* cRNA-induced initial rate of Ca^{2+} inflow in oocytes (5 μM) is about 10 times greater than that which inhibits mammalian L-type voltage-operated Ca^{2+} channels [46] and some RACCs [25,27,47], which are considered to be quite selective for Ca^{2+}, and the ability of the putative Trpl channel to admit Mn^{2+} suggests that Trpl encodes a non-specific cation channel [25,27]. It is possible that expression of the Trpl protein in oocytes leads to activation of endogenous channels in the oocyte plasma membrane rather than the creation of new channels. Although the former possibility cannot be completely excluded it is considered unlikely.

The conclusion reached here that the Trpl protein is a non-selective cation channel located in the plasma membrane confirms the conclusions derived from the expression of Trpl in insect Sf9 cells [25,27,28]. Thus the present results show that Trpl divalent cation channel activity can also be expressed and detected in the environment of the *Xenopus* oocyte. The concentration of Gd^{3+} that inhibits basal Ca^{2+} inflow in *trpl*-cRNA-injected oocytes (the present study) is lower than that required to inhibit *trpl* cRNA-stimulated Ca^{2+} inflow in Sf9 cells [25]. This difference might be due to differences in the cell incubation media employed for the two cell types (this could alter the concentration of extracellular free Gd^{3+}) or to differences in the ability of Gd^{3+} to distribute in the cytoplasmic space and bind to other proteins in Sf9 cells and oocytes.

Effects of the Trpl protein on the sustained rate of Ca^{2+} inflow

In *trpl*-cRNA-injected oocytes incubated in the presence of Ins(1,4,5)P_3, Ins(1,4,5)P_3F or thapsigargin, a substantial slow and sustained rate of Ca^{2+} inflow was observed after the initial rapid phase of Ca^{2+} inflow. It is noteworthy that this sustained rate of Ca^{2+} inflow continued during the period of measurement of fluorescence so that a new steady state (no further change in fluorescence) was not reached during this time (compare [48]). In mock-injected oocytes incubated in the absence of other agents, the slow sustained rate of Ca^{2+} inflow was very low or negligible. The present results do not permit definition of the mechanism(s) giving rise to the slow sustained rate of increase in fluorescence, which might reflect the complex interaction of a number of processes. These might include the slow recruitment of additional fluo-3 as Ca^{2+} diffuses further into the interior of the oocyte coupled with increased Ca^{2+} inflow through Trpl channels in the plasma membrane, inhibition by the Trpl protein of the plasma membrane and/or endoplasmic reticulum ($Ca^{2+} + Mg^{2+}$)ATPase, and/or a time-dependent relaxation of feedback inhibition of the Trpl Ca^{2+} channel (compare the inhibition by protein kinase C of endogenous receptor-activated Ca^{2+} channels in *Xenopus* oocytes [37]). The kinetics of the slow sustained phase of $Ca^{2+}{}_o$-induced increase in fluorescence observed in the present experiments are similar to those of the sustained secondary Ca^{2+}-activated Cl^- currents observed by Parekh et al. [36] in *Xenopus* oocytes (expressing exogenous 5-hydroxytryptamine receptors) treated with 5-hydroxytryptamine or Ins(1,4,5)P_3. It is noteworthy that these workers suggested that the sustained secondary Ca^{2+}-activated Cl^- current is likely to be due to a sustained rate of Ins(1,4,5)P_3F-stimulated Ca^{2+} inflow [36].

Roles of Ca^{2+} and calmodulin in activation of the Trpl channel in the basal state

The conclusion that Ca^{2+} and calmodulin activate the putative Trpl Ca^{2+} channel is consistent with the observations that (1) W13, calmidazolium and CamK(281–309), which are inhibitors of calmodulin action [38,39,44], block the *trpl* cRNA-induced increase in the initial rate of basal Ca^{2+} inflow, and (2) the low concentration of exogenous calmodulin (1 μM) activated the basal initial rate of Ca^{2+} inflow in *trpl*-cRNA-injected oocytes. Thus the *trpl* cRNA-induced increases in (1) the basal value of $[Ca^{2+}]_i$, and (2) the basal initial rate of Ca^{2+} inflow are likely to be due to activation of the putative Trpl Ca^{2+} channel by Ca^{2+} and endogenous calmodulin. The observation that a higher concentration (3 μM) of exogenous calmodulin inhibited Ca^{2+} inflow in *trpl*-cRNA-injected oocytes, and the observation that this inhibitory effect was blocked by W13 or calmidazolium, might be explained by the binding of exogenous calmodulin to a second putative calmodulin binding site that is predicted to be present on the C-terminus of the Trpl protein [22]. If this second calmodulin site has a lower affinity for calmodulin than the first, and if, in oocytes, site one is normally complexed to endogenous calmodulin, an inhibition of Trpl-mediated Ca^{2+} inflow might be expected when exogenous calmodulin is introduced. Such an inhibition of the Trpl channel by (Ca^{2+} and calmodulin) and a time-dependent loss of calmodulin from the cell have been proposed to explain the observation that, in insect Sf9 cells expressing the Trpl protein, whole-cell divalent cation currents, which are a measure of Trpl channel activity, increase with time [49].

The possibility that the effects of the high exogenous calmodulin concentration are due to buffering by the calmodulin protein of the increase in $[Ca^{2+}]_i$ is considered unlikely because the capacity of the native cytoplasmic space to buffer increases in $[Ca^{2+}]_i$ is very high [50]. However, the possibility that a high intracellular concentration of exogenous calmodulin could activate other processes such as the plasma-membrane ($Ca^{2+} + Mg^{2+}$)ATPase [51] (which would lower the $Ca^{2+}{}_o$-induced increase in fluorescence) cannot be eliminated.

The observations that pertussis toxin, which ADP-ribosylates certain trimeric G-proteins [45], and GDP[S], which inhibits the actions of trimeric G-proteins [45], each block the *trpl* cRNA-induced increase in basal Ca^{2+} inflow indicate that a pertussis toxin-sensitive trimeric G-protein is required for maintenance of the basal activity of the Trpl Ca^{2+} channel. As it has been shown that in untreated oocytes PtdIns(4,5)P_2-specific phospholipase C can be activated by a pertussis toxin-sensitive trimeric G-protein [52], one possibility is that the requirement for a trimeric G-protein in the activation of the putative Trpl Ca^{2+} channel reflects the formation, in the basal state, of some Ins(1,4,5)P_3, which, in turn, increases $[Ca^{2+}]_i$ sufficiently to cause activation of the Trpl channel.

Effect of depletion of intracellular Ca^{2+} stores on Trpl channel activity

The observation that the thapsigargin-stimulated initial rate of Ca^{2+} inflow in *trpl*-cRNA-injected oocytes was not significantly different from the corresponding thapsigargin-stimulated initial rate of Ca^{2+} inflow in mock-injected oocytes provides evidence that the putative Trpl Ca^{2+} channel is not activated by store depletion in the environment of the oocyte. This confirms the conclusion reached from the results obtained by others when the

Trpl protein was expressed in Sf9 cells [25,27,28]. The observation that the Ins(1,4,5)P_3-stimulated initial rate of Ca^{2+} inflow in *trpl*-cRNA-injected oocytes was substantially less than that in mock-injected oocytes also suggests that store depletion does not activate the Trpl channel.

The inhibition by *trpl* cRNA expression of the Ins(1,4,5)P_3-stimulated initial rate of Ca^{2+} inflow and the delayed onset of thapsigargin-stimulated initial rate of Ca^{2+} inflow observed in *trpl*-cRNA-injected oocytes in some experiments indicate that the presence of the Trpl protein in oocytes inhibits endogenous store-activated Ca^{2+} inflow. This suggests that there is some interaction between the Trpl protein and (1) the action of Ins(1,4,5)P_3 and the Ins(1,4,5)P_3 receptor in releasing Ca^{2+} from intracellular stores and/or (2) the process by which store depletion activates endogenous store-activated Ca^{2+} channels. One possible explanation for the inhibition by Trpl of the Ins(1,4,5)P_3-stimulated initial rate of Ca^{2+} inflow is that increased $[Ca^{2+}]_i$ caused by the presence of the constitutively active Trpl channel inhibits the Ins(1,4,5)P_3-induced release of Ca^{2+} from the endoplasmic reticulum, as suggested by Fournier et al. [50] for the expression of voltage-operated Ca^{2+} channels in *Xenopus* oocytes. However, it is also of interest to note that one explanation proposed by Dong et al. [49] for the observation that Ins(1,4,5)P_3 activates the Trpl protein when this is expressed in insect Sf9 cells is that the effect is mediated by an interaction between the Ins(1,4,5)P_3 receptor (and possibly other proteins) and the Trpl protein.

Effects of Ins(1,4,5)P_3 on Trpl channel activity

The observations that (1) the Ins(1,4,5)P_3- and Ins(1,4,5)P_3F-stimulated initial rates of Ca^{2+} inflow in *trpl*-cRNA-injected oocytes were less than or equal to the corresponding rates in mock-injected oocytes and (2) the release of Ins(1,4,5)P_3 from 'caged' Ins(1,4,5)P_3 during the sustained phase of Ca^{2+} inflow in thapsigargin-treated *trpl*-cRNA-injected oocytes did not further stimulate Ca^{2+} inflow suggest that in *Xenopus* oocytes the Trpl channel is not activated (either directly or indirectly) by Ins(1,4,5)P_3. This contrast with the results obtained by Dong et al. [49] for the expression of Trpl in insect Sf9 cells where evidence for an activation of Trpl by Ins(1,4,5)P_3 (through some mechanism other than the depletion of intracellular Ca^{2+} stores) was obtained.

However, it is possible that the activation of Trpl by Ins(1,4,5)P_3 in *Xenopus* oocytes may have been masked by (1) the apparent inhibition by Trpl of the Ins(1,4,5)P_3-stimulated initial rate of Ca^{2+} inflow, (2) the fact that in the experiments that employed 'caged' Ins(1,4,5)P_3 the photolysis of 'caged' Ins(1,4,5)P_3 was conducted under conditions where $[Ca^{2+}]_i$ was high so that any further increase in $[Ca^{2+}]_i$ due to the direct activation of Trpl by Ins(1,4,5)P_3 might have been small, (3) the relatively large flux of Ca^{2+} through endogenous store-activated Ca^{2+} channels present in oocytes, (4) complexities (discussed above) in identifying and measuring rates of Ca^{2+} inflow to *Xenopus* oocytes by using fluorescent dyes such as fluo-3, and (5) the location in the oocyte of the Trpl protein in a different environment (e.g. associated with different membrane proteins) from that in insect Sf9 cells. Furthermore, although the mechanism that generates the sustained phase of Ca^{2+} inflow is not known (as discussed above) it remains a possible interpretation of the observations that (1) the Ins(1,4,5)P_3-stimulated sustained rate of Ca^{2+} inflow in *trpl*-cRNA-injected oocytes is substantially greater than the corresponding rate in mock-injected oocytes and (2) this difference is not observed in the presence of heparin is that the Trpl protein is indeed activated by Ins(1,4,5)P_3 in oocytes.

Activation of the Trpl channel by GTP[S]

The observation that the GTP[S]-stimulated rate of Ca^{2+} inflow in *trpl*-cRNA-injected oocytes was greater than the corresponding rate in mock-injected oocytes indicates that the putative Trpl Ca^{2+} channel can be activated directly or indirectly by GTP[S]. However, as mentioned in the Results section, the interpretation of the data obtained in the presence of GTP[S] is complicated by the fact that the shapes of the plots of fluorescence as a function of time are quite different from those obtained with other agents. This may be due to the large number of intracellular sites at which GTP[S] can interact (these include all trimeric and monomeric G-proteins present in the oocyte) and the consequences of the activation of these G-proteins [53], including the activation of protein kinase C [37,53].

Because GTP[S] is known to activate trimeric G-proteins [45] it is likely that the activation of Trpl by GTP[S] is initiated by the activation of one or more trimeric G-proteins, which could exert an effect either through the activation of phosphoinositide-specific phospholipase Cβ and the subsequent formation Ins(1,4,5)P_3, or by a process independent of Ins(1,4,5)P_3. Because the kinetic properties of GTP[S]-stimulated Ca^{2+} inflow differ greatly from those of Ins(1,4,5)P_3-induced Ca^{2+} inflow it is considered unlikely that GTP[S] activates Trpl by increasing $[Ca^{2+}]_i$. The observation that the Trpl protein expressed in *Xenopus* oocytes is activated by GTP[S] is in agreement with the results of studies with insect Sf9 cells that have shown that Trpl expressed in this cell type can be activated by agonists that bind to trimeric G-protein-coupled receptors that activate phospholipase Cβ [25,27] or by intracellular GTP[S] [28].

Relationship of the *Drosophila* Trpl channel to mammalian RACCs

Among the various types of RACC present in mammalian cells are store-activated Ca^{2+}-selective channels and a variety of non-selective cation channels, some of which are activated by store depletion and others by increases in $[Ca^{2+}]_i$ or Ins(1,4,5)P_3 concentration [1–5,54]. The present results, which provide further evidence that the *Drosophila* Trpl protein is a non-specific cation channel and show that, in the environment of the *Xenopus* oocyte, this channel is activated by (Ca^{2+} and calmodulin), indicate that the *Drosophila* Trpl channel has similarities with those mammalian non-selective cation RACCs that are activated, at least in part, by an increase in $[Ca^{2+}]_i$ [55]. Further studies of the regulation of the *Drosophila* Trpl channel in *Xenopus* oocytes should contribute to knowledge of the structure and mechanism of activation of these non-selective cation RACCs.

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