Ca²⁺ release by inositol 1,4,5-trisphosphate is blocked by the K⁺-channel blockers apamin and tetrapentylammonium ion, and a monoclonal antibody to a 63 kDa membrane protein: reversal of blockade by K⁺ ionophores nigericin and valinomycin and purification of the 63 kDa antibody-binding protein

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Ins(1,4,5)P₃-induced Ca²⁺ release from platelet membrane vesicles was blocked by apamin, a selective inhibitor of low-conductance Ca²⁺-activated K⁺ channels, and by tetrapentylammonium ion, and was weakly inhibited by tetraethylammonium ion. Other K⁺-channel blockers, i.e. charybdotoxin, 4-aminopyridine and glibenclamide, were ineffective. A monoclonal antibody (mAb 213-21) obtained by immunizing mice with the InsP₃-sensitive membrane fraction from platelets also blocked Ca²⁺ release by InsP₃ from membrane vesicles obtained from platelets, cerebellum, aortic smooth muscle, HEL cells and sea-urchin eggs. ATP-dependent Ca²⁺ uptake and binding of [³H]InsP₃ to platelet membranes was unaffected by either K⁺-channel blockers or mAb 213-21. Blockade of Ca²⁺ release by apamin, tetrapentylammonium and mAb 213-21 was not affected by the Na⁺/H⁺ carrier monensin or the protonophore carbonyl cyanide *p*-

trifluoromethoxyphenylhydrazone (FCCP), but could be completely reversed by the K⁺/H⁺ ionophore nigericin and partially reversed by the K⁺ carrier valinomycin. The antibody-binding protein (ABP) solubilized from platelets, cerebellum, and smooth muscle chromatographed identically on gel filtration, anion-exchange and heparin-TSK h.p.l.c. ABP was purified to apparent homogeneity from platelets and aortic smooth muscle as a 63 kDa protein by immunoaffinity chromatography on mAb 213-21-agarose. These results suggest that optimal Ca²⁺ release by InsP₃ from platelet membrane vesicles may require the tandem function of a K⁺ channel. A counterflow of K⁺ ions could prevent the build-up of a membrane potential (inside negative) that would tend to oppose Ca²⁺ release. The 63 kDa protein may function to regulate K⁺ permeability that is coupled to the Ca²⁺ efflux via the InsP₃ receptor.

INTRODUCTION

Ins(1,4,5)P₃ serves as a second messenger for the mobilization of intracellular stores of Ca²⁺ in response to many types of cell-stimulating agents (Berridge and Irvine, 1989), and the InsP₃ receptor protein functions as a ligand-gated Ca²⁺ channel (Ferris et al., 1989). Among the many factors that may regulate Ca²⁺ release by InsP₃ are other ions in the cytoplasm. InsP₃ did not release Ca²⁺ from permeabilized liver cells (Joseph and Williamson, 1986) or liver rough-endoplasmic-reticulum membranes (Muallem et al., 1985) that were suspended in a sucrose medium devoid of univalent cations. Joseph and Williamson (1986) showed that Ca²⁺ release by InsP₃ was affected by the cation and anion composition of the medium, but, as it was insensitive to 4,4'-di-isothiocyanostilbene-2,2'-disulphonate (DIDS) and furosemide, they concluded that neither anion exchange nor cation/anion co-transport was involved. With permeant Cl⁻ as the anion, the cations K⁺, Na⁺, Rb⁺ and Cs⁺ were virtually equi-effective in supporting Ca²⁺ release by InsP₃, whereas K⁺ was the most effective cation with the relatively impermeant gluconate anion. K⁺ was also the most effective cation supporting Ca²⁺ release from brain microsomes by InsP₃ (Shah et al., 1987). The involvement of a K⁺ channel was further suggested by the finding

that InsP₃ induced ⁸⁶Rb⁺ influx into brain microsomes, and InsP₃-induced Ca²⁺ release was inhibited by tetraethylammonium chloride (TEA) in liver (Joseph and Williamson, 1986; Muallem et al., 1985), and by TEA and nonyltriethylammonium in brain microsomes (Shah and Pant, 1988). In the last study the response to InsP₃ was not blocked by L- or N-type Ca²⁺-channel inhibitors. Palade et al. (1989) also observed inhibition of InsP₃-induced Ca²⁺ release in cerebellar vesicles due to lack of K⁺ in the medium and by a wide variety of K⁺-channel blockers [e.g. TEA, tetrapentylammonium ion (TPA), quinine, Ba²⁺, 4-aminopyridine etc.]. However, they concluded that the inhibition exerted by the putative K⁺-channel blockers was due to their effects on the Ca²⁺ channel, because it was not reversed by the K⁺ carriers valinomycin or gramicidin D.

On the basis of the effects of the ionic composition of the medium on the release of Ca²⁺ by InsP₃, Joseph and Williamson (1986) concluded that the translocation of positive charge across the liver reticular membrane in the form of Ca²⁺ was electrogenic, and was balanced by ancillary movements of cations and anions. In the sarcoplasmic reticulum (SR) of various types of muscles, high-conductance voltage-sensitive univalent-cation channels, particularly selective for K⁺, have been proposed to function as an electrical shunt which compensates for the release of internal

Abbreviations used: HSA, human serum albumin; TEA, tetraethylammonium chloride; TPA, tetrapentylammonium chloride; ABP, antibody mAb 213-21-binding protein; NOG, *N*-octyl glucoside; HRP, horseradish peroxidase; FCCP, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone; SR, sarcoplasmic reticulum.

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Ca^{2+} that is associated with the contractile activation in skeletal, cardiac and smooth muscles (Fink and Stephenson, 1987; Abramchek and Best, 1989; Shen et al., 1990; Ide et al., 1991; Sokabe et al., 1991; Liu et al., 1991; Liu and Strauss, 1991; Uehara et al., 1991; Bezprozvanny et al., 1991). In contrast with the widely studied muscle systems, no K^+ channels, or other ion channels, present in the internal membrane systems that release Ca^{2+} in response to InsP_3 have been identified. Here we report that InsP_3 -induced Ca^{2+} release from platelet membrane vesicles is strongly inhibited by a specific subset of K^+ -channel blockers, namely the bee venom protein apamin and TPA, only weakly inhibited by TEA, and not inhibited at all by 4-aminopyridine, charybdotoxin or glybenclamide. Furthermore, we raised a monoclonal antibody (mAb 213-21) against the InsP_3 -sensitive human platelet membrane vesicle fraction which suppresses release of Ca^{2+} by InsP_3 from platelet membranes and from membrane vesicles of rat cerebellum, dog aortic smooth muscle, human erythroleukaemia (HEL) cells and sea-urchin eggs. Because the inhibitory effects of both mAb 213-21 and the K^+ -channel blockers were completely or partially reversed by addition of the K^+/H^+ ionophore nigericin or the K^+ carrier valinomycin, respectively, we suggest that both types of reagents are blocking a K^+ channel that acts in concert with the Ca^{2+} channel of the InsP_3 receptor. The monoclonal antibody was further used to immunopurify a 63 kDa membrane protein from human platelets and pig aorta. This protein, which is distinct from the InsP_3 receptor, may function in the regulation of K^+ permeability of the intracellular membranes that contain the InsP_3 receptor.

EXPERIMENTAL

Materials

InsP_3 was obtained from Amersham or Research Biochemicals (Natick, MA, U.S.A.). TPA was from Aldrich Chemical Co. and the other K^+ -channel blockers were from Sigma. Reagents for electrophoresis, and immunoaffinity kits (to couple monoclonal antibody to agarose beads), were purchased from Bio-Rad. $^{45}\text{CaCl}_2$ was obtained from New England Nuclear. $[^3\text{H}]\text{InsP}_3$ (17–60 Ci/mmol) and ^{35}S -SLR reagent (800 Ci/mmol; to label protein amino groups) were from Amersham. Cell-culture reagents were obtained from GIBCO, except for Controlled Process Serum Replacement (CPSR-3) and Hybrimax from Sigma, and Nutridoma from Boehringer Mannheim. Antibodies were obtained from the following sources: purified polyclonal human IgM (Cappel), mouse myeloma IgM (Sigma), horseradish peroxidase (HRP)-labelled goat anti-mouse IgM (μ -chain-specific) and goat anti-mouse IgG (γ -chain-specific) from Boehringer Mannheim. Antibodies to BSA and human serum albumin (HSA) were obtained from Calbiochem and Hyclone respectively. Anti-lipopolysaccharide antibody was from East Acres Biologicals. The cell lines used in these studies were originally obtained from the following sources: HL-60 (Dr. F. Rickles, University of Connecticut Health Center); GH3 pituitary and HEL cells (American Type Culture Collection); sea-urchin eggs (Dr. L. Jaffe, University of Connecticut Health Center); and Myeloma NSO non-producing variants of NS1 cells (Dr. M. Scharff, Albert Einstein Medical School).

Preparation of InsP_3 -sensitive membranes

Membrane vesicles that release Ca^{2+} in response to InsP_3 were isolated from platelets, HEL and GH3 cells and sea-urchin eggs by centrifugation on Percoll gradients (O'Rourke et al., 1985,

1987; O'Rourke and Feinstein, 1990). InsP_3 -sensitive membrane vesicles from cerebellum were prepared as described by Supatapone et al. (1988). The media in all cases contained protease inhibitors: 10 μM leupeptin and pepstatin, and 1 mM phenylmethanesulphonyl fluoride. Aortic SR vesicles were isolated from homogenized dog and pig aortic smooth muscle by differential centrifugation as described by Watras and Benevolensky (1987), and 'heavy' SR vesicles from rabbit skeletal muscle were isolated as described by Kim et al. (1983).

InsP_3 binding to membranes and solubilized protein fractions

Binding of $[^3\text{H}]\text{InsP}_3$ to membranes was measured as described by O'Rourke and Feinstein (1990). $[^3\text{H}]\text{InsP}_3$ binding to solubilized protein fractions was assayed by using poly(ethylene glycol) as a protein precipitant (Chadwick et al., 1990): 75 μl of detergent-solubilized membranes at 1.5–2 mg/ml was diluted with 75 μl of 40 mM NaHCO_3 /1 mM EDTA, pH 8.3, and incubated with $[^3\text{H}]\text{InsP}_3$ for 10 min at 4 °C, followed by addition of 20 μl of rabbit IgG (50 mg/ml). Samples (75 μl) were removed for duplicate determinations and added to polycarbonate tubes containing 100 μl of ice-cold 30% poly(ethylene glycol) in dilution buffer. The samples were kept at 4 °C for 10 min, and then centrifuged in a Beckman TL100 centrifuge at 463 000 g for 10 min at 4 °C to pellet the precipitated proteins. The supernatant was aspirated and the pellet analysed for radioactivity.

Preparation and screening of mAb 213-21

Mice of Balb/C strain (Jackson Laboratories) were injected intraperitoneally with 25 μl of an emulsion of 100 μg of InsP_3 -sensitive platelet membrane protein (O'Rourke et al., 1985) in PBS and complete Freund's Adjuvant. Boosts of 50 μg of membranes were given at 2-week intervals. Serum antibody titres were measured by e.l.i.s.a. (see below) after each boost by using platelet InsP_3 -sensitive membrane vesicles adsorbed on microtitre-plate wells as previously described by O'Rourke et al. (1987). Immune sera inhibited InsP_3 -dependent Ca^{2+} release from platelet membrane vesicles, but preimmune serum was ineffective. Myeloma cells (NSO non-producing variants of NS1 cells) were fused with spleen cells (Harlow and Lane, 1988) from an immunized mouse and cultured in Dulbecco's Modified Eagle's (DME) medium supplemented with 10% CPSR-3 serum and 3-azaguanine to eliminate revertants. Supernatant media from hybridomas cultured in DME medium with 10% CPSR-3 were tested for ability to inhibit Ca^{2+} release by InsP_3 and were screened by e.l.i.s.a. for anti-(platelet membrane) antibody production. The same e.l.i.s.a. procedure was used to identify antibody-binding protein (ABP) during chromatographic purification steps. In the latter case, the detergent-solubilized membrane protein or fractions eluted from chromatography columns were adsorbed to microtitre plates and then probed with mAb 213-21 and HRP-labelled second antibody.

Production and purification of mAb 213-21

Clone 21 of hybridoma 213 produced the most potent antibody that inhibited Ca^{2+} release and was initially grown in serum-free medium supplemented with Nutridoma and then adapted to protein-free media (Hybrimax). The antibody, an IgM, was routinely purified from the supernatant by dialysis/ultrafiltration by using an Amicon CH2 concentrator equipped with a YM spiral filter cartridge having a 100 000-Da cut-off, or by concentration with Centricon 20 centrifugal ultrafilters. Concentrated supernatants were further purified by hydroxapatite h.p.l.c.

or an Immunopore IgM purification kit (Pierce Chemical Co.). Purified antibody was dialysed against Ca²⁺ transport buffer (see below) by using a Spectraphor 500000-Da cut-off membrane. Antibody purity was assessed as greater than 98% by SDS/PAGE using purified mouse and human IgM as standards. Radioactive mAb 213-21 was prepared by growing cells in medium containing [³H]lysine (97.5 Ci/mmol) (Galfre and Milstein, 1981).

E.I.s.a.

E.I.s.a.s were developed to detect monoclonal antibodies to platelet membranes and to detect the antigen(s) for these antibodies. Platelet InsP₃-sensitive membrane vesicles were employed to detect anti-membrane antibodies: 100 µl of platelet membrane protein (10 µg of protein/ml) in coating buffer containing 0.15 M Na₂CO₃ and 0.0349 M NaHCO₃, pH 9.6, were adsorbed to Falcon microtitre plates overnight at 4 °C. The uniformity of membrane protein adsorption to microtitre plates was verified in preliminary experiments by a BCA micro protein-assay method (Pierce); 200 µl of stock BCA reagent was added to each well and incubated for 1 h at 60 °C. Protein standards (BSA/HSA, Sigma) were incubated simultaneously on the same plate. The A₅₆₂ was measured with a Dynatech microplate reader. The microtitre plates with bound membrane protein were washed with PBS/Tween (140 mM NaCl, 1.5 mM K₂HPO₄, 2.7 mM KCl, 0.05% Tween 20, pH 7.4) and then incubated for 1 h with antibody-containing fluids, i.e. serum, hybridoma supernatants or purified antibody, diluted in PBS/Tween. After six washes with PBS/Tween, the plates were incubated for 1 h with affinity-purified goat second antibodies labelled with HRP to detect both heavy and light Ig chains. Peroxidase activity of the second antibody was assayed with 2,2'-azinobis-(3-ethylbenzthiazoline)-6-sulphonic acid in 0.1 M citrate, pH 4.5, as substrate. Colour development was stopped after 30 min with 5% SDS and the A₄₁₄ measured with a Dynatech microplate reader. Non-specific human IgGs and IgMs coated on wells were used as controls. The same procedure was used to identify mAb 213-21 antibody-binding protein during purification steps. In the latter case, the detergent-solubilized membrane protein or fractions eluted from chromatography columns were first adsorbed to microtitre plates and then probed with mAb 213-21 and HRP-labelled anti-IgM second antibody.

ATP-dependent Ca²⁺ uptake and InsP₃-induced Ca²⁺ release

K⁺-channel blockers, hybridoma culture media and mAb 213-21 were tested for possible effects on ATP-dependent ⁴⁵Ca²⁺ transport (uptake) and ⁴⁵Ca²⁺ release by InsP₃, as described by O'Rourke et al. (1985, 1987) and O'Rourke and Feinstein (1990). Freshly prepared InsP₃-sensitive membrane vesicles (0.1 mg of protein/ml) from platelets and other cell types were preloaded for 60 min with ⁴⁵Ca²⁺ at 23 °C in an uptake buffer, consisting of 100 mM KCl, 20 mM potassium phosphate, 5 mM MgCl₂, 1 mM ATP, 10 µM leupeptin and pepstatin, 0.4 mM CaCl₂, 0.5 µCi/ml ⁴⁵CaCl₂, 0.568 mM EGTA (free Ca²⁺ = 1.1 µM) and 20 mM Hepes, pH 7.1. ⁴⁵Ca²⁺ release was initiated by InsP₃, and the membrane pellets obtained after centrifugation were analysed as previously described. Ca²⁺ uptake and release in dog aortic SR vesicles, prepared as described by Watras and Benevolensky (1987), were measured by dual-wavelength spectrophotometry (720/790 nm) using the Ca²⁺ indicator antipyrilazo III. To test the effects of mAb 213-21, the SR vesicles were first preincubated for 30–60 min with antibody in 100 mM KCl/60 µM MgCl₂/20 mM Hepes, pH 6.8, 22–25 °C. Non-specific mouse IgM anti-

body was used as a control. The composition of the medium was then adjusted to measure Ca²⁺ uptake and release. The incubation medium contained 0.25 mg of SR protein/ml, 100 mM KCl, 0.5 mM MgCl₂, 5 mM phosphocreatine, 16 units/ml creatine kinase, 1.5 mM 2,3-diphosphoglycerate, 5 mM NaN₃, 0.4 mM antipyrilazo III and 20 mM Hepes, pH 6.8 (Watras and Benevolensky, 1987). Ca²⁺ uptake from the medium was initiated by addition of 1.5 mM Na₂ATP, and Ca²⁺ release was evoked 7 min later by addition of InsP₃. Total releasable intravesicular Ca²⁺ content was determined by addition of the Ca²⁺ ionophore A23187. All experiments were performed in triplicate unless otherwise stated.

Gel-filtration h.p.l.c. of ABP

The apparent molecular masses of ABP and the InsP₃ receptor were determined by gel-filtration h.p.l.c. The InsP₃-sensitive membrane fractions from platelets, pig aortic smooth muscle and rat cerebellum were solubilized in 50 mM *N*-octyl glucoside (NOG), 20 mM NaHCO₃/1 mM EDTA, pH 7.4. The solubilized membrane proteins were then concentrated and buffer-exchanged (by using Centricon 10) to the h.p.l.c. mobile phase. Samples were injected on to a calibrated DuPont G450 Zorbax column, by using mobile phases of either 'low salt' (25 mM NOG, 0.2 M sodium phosphate, pH 6.8) or 'high salt' (25 mM NOG, 20 mM NaHCO₃, 1 mM EDTA, 1.0 M NaCl, pH 7.4) at a flow rate of 0.5 ml/min. Eluted fractions were analysed for mAb 213-21 binding to e.i.s.a. and for radioligand binding of [³H]InsP₃ by using the solubilized-protein-binding assay. The G450 Zorbax columns were calibrated by separate injections of molecular-mass standards: IgM, 950 kDa; thyroglobulin, 670 kDa; phosphorylase *b*, 97 kDa; BSA, 66 kDa; ovalbumin, 43 kDa.

Immunoaffinity purification of ABP

Platelet membranes (20–30 mg of protein) were solubilized in 50 mM NOG/40 mM NaHCO₃/1 mM EDTA, pH 8.3, and centrifuged at 463 000 *g* for 20 min to remove insoluble material. Solubilized membrane proteins were loaded on to a heparin-TSK h.p.l.c. column and protein was eluted with a 0–1.0 M NaCl gradient in solubilization buffer with 25 mM NOG. Fractions were assayed for mAb 213-21 binding by e.i.s.a. E.I.s.a.-positive fractions were pooled and buffer-exchanged (by using Centricon-30) with 20 mM NaHCO₃/0.5 M NaCl/1 mM EDTA/25 mM NOG, pH 7.4. The e.i.s.a.-positive fractions were then applied to an immunoaffinity column prepared by coupling 5.0 mg of purified mAb 213-21 to agarose beads by using an Affi-Gel H2 immunoaffinity kit (BioRad). After incubation for 1 h, the column was washed sequentially with equilibration buffer containing 0.5 M NaCl (100 ml), 0.05 M NaCl (50 ml), 1.0 M NaCl (50 ml) and 0.5 M NaCl (100 ml). Antibody-bound protein was eluted from the column with 0.05 M diethylamine in 25 mM NOG, pH 11.5, and the pH of the eluate was immediately adjusted to 7.4 with 1.0 M HCl. The eluates were assayed by e.i.s.a. with mAb 213-21 and labelled with ³⁵S-SLR reagent (see below) and subjected to SDS/PAGE.

Protein measurements and protein labelling with ³⁵S-SLR

Protein in solution was assayed with the Bio-Rad Coomassie Blue Reagent by using BSA/HSA (Sigma) as the standard. Proteins purified by h.p.l.c. or immunoaffinity columns were labelled with ³⁵S-SLR labelling reagent: 100 µl of sample was incubated with 10 µCi of ³⁵S-SLR in 0.1 M borate buffer/25 mM NOG, pH 8.5, at 4 °C for 1 h. The reaction was quenched with

50 μ l of 0.2 M glycine in 0.1 M borate buffer, pH 8.5. The ^{35}S -labelled proteins were separated by SDS/PAGE on a 5–15% gradient gel. The gels were enhanced with Enlightening (New England Nuclear) for 30 min, then dried and autoradiographed for 24 h at -70°C with Kodak XAR2 film and an enhancing screen.

RESULTS

Release of Ca^{2+} by InsP_3 is blocked by certain K^+ -channel blockers

The release of Ca^{2+} by InsP_3 was studied in platelet membrane vesicles preloaded with $^{45}\text{Ca}^{2+}$ in the presence of ATP and phosphate. The medium required sufficient K^+ (or Rb^+) to support the active transport of Ca^{2+} . Uptake was negligible when Na^+ , Cs^+ or choline replaced K^+ . The various K^+ -channel blockers (i.e. 200 nM apamin, 100 nM charybdotoxin, 20 mM 4-aminopyridine, 10 μM glybenclamide, 20 mM TEA and 100 μM TPA) did not affect Ca^{2+} uptake. If the KCl in the medium was replaced with equiosmotic sucrose or other univalent-ion chloride salts after Ca^{2+} uptake was completed, there was a substantial leakage of Ca^{2+} out of the vesicles over time. This made it impossible to assess properly InsP_3 -induced Ca^{2+} release, so that

Figure 1 Inhibition of InsP_3 -induced Ca^{2+} release from platelet membrane vesicles by apamin and TPA

(a) Dose-response for inhibition of $^{45}\text{Ca}^{2+}$ release by apamin (●) and (insert) TPA (○). Ca^{2+} release was induced by 5 μM InsP_3 . Apamin and TPA were present throughout the initial 60 min Ca^{2+} -uptake period, but had no effect on amount of $^{45}\text{Ca}^{2+}$ accumulated. (b) Time course of $^{45}\text{Ca}^{2+}$ release; ▼, no additions; ○, InsP_3 alone; ●, InsP_3 + 20 nM apamin; ▽, InsP_3 + 200 nM apamin. InsP_3 (5 μM) was added at zero time.

Figure 2 Inhibition of InsP_3 -induced Ca^{2+} release from platelet membrane vesicles by mAb 213-21 and its correlation with antibody binding to membranes

Membrane vesicles were preloaded with $^{45}\text{Ca}^{2+}$ for 60 min in the presence of 0–50 nM mAb 213-21 (0–500 pmol/mg of protein). Ca^{2+} release was elicited by 5 μM InsP_3 . The IC_{50} for antibody was approx. 5 nM. Insert: ○, time course for inhibition of Ca^{2+} release by 15 nM mAb 213-21; ▼, time course for binding of mAb 213-21 to platelet membranes, measured by e.l.i.s.a.

all studies were conducted in medium containing 100 mM KCl. After preloading vesicles with $^{45}\text{Ca}^{2+}$, the release of Ca^{2+} was then elicited by InsP_3 in the presence or absence of the K^+ -channel inhibitors. Charybdotoxin, 4-aminopyridine and glybenclamide did not affect Ca^{2+} release (results not shown). Ca^{2+} release was also not affected by furosemide, an inhibitor of Na^+/Cl^- co-transport. The K^+ -channel blocker TEA was tested over a concentration range of 1–50 mM and was found to decrease the initial rate of Ca^{2+} release by a maximum of 40%. The total Ca^{2+} released by InsP_3 was decreased by only 20–24% (range; 3 experiments) by TEA. The IC_{50} for TEA was 2 mM, and its maximal inhibitory effect was attained at 5–10 mM. Apamin and TPA were much more effective, and each blocked InsP_3 -induced Ca^{2+} release by 80–100%; EC_{50} = 0.4 nM for apamin and 10 μM for TPA (Figure 1), comparable with their potencies in other systems (Banks et al., 1979; Hugues et al., 1982a,b; Palade et al., 1989).

Release of Ca^{2+} by InsP_3 is blocked by mAb 213-21

A monoclonal antibody (mAb 213-21) was raised by initially immunizing a mouse with human platelet InsP_3 -sensitive membrane vesicles (see the Experimental section). Purified antibody obtained from clone 213-21 bound with greatest specificity to the InsP_3 -sensitive platelet membrane fraction isolated by centrifugation on Percoll gradients (O'Rourke et al., 1985, 1987). The amount of ABP, estimated by specific binding of radioactive antibody and by e.l.i.s.a., was approximately equal to the content of platelet InsP_3 receptor, which was previously determined to be about 4 pmol/mg of vesicle protein (O'Rourke and Feinstein, 1990). The antibody inhibited InsP_3 -stimulated Ca^{2+} release from vesicles preloaded with $^{45}\text{Ca}^{2+}$. The inhibition was time- and dose-dependent (Figure 2a), with the maximum effect observed after 60 min at 23°C . The time course for inhibition of Ca^{2+} release was the same as the rate of binding of mAb 213-21 to platelet membranes as measured by e.l.i.s.a. (Figure 2b) or with [^3H]lysine-labelled antibody (results not shown). The IC_{50} of the most highly purified antibody obtained by hydroxyapatite h.p.l.c.

Figure 3 Inhibition of InsP₃-induced Ca²⁺ release from aortic smooth-muscle SR by mAb 213-21

Aortic SR vesicles (0.2–0.25 mg of protein/ml) were preincubated for 1 h (22 °C) with: (a) antibody solvent alone, (b) non-specific mouse IgM (225 nM), or (c) mAb 213-21 (225 nM). Ca²⁺ uptake reactions were initiated at zero time (60 min after mAb) by addition of ATP to the incubation media. Ca²⁺ in the medium was measured with antipyrilazo III (see the Experimental section). Ca²⁺ release was initiated at 7 min by adding 7.6 μM InsP₃ (single arrow). Residual Ca²⁺ was released by 4 μM A23187 (double arrow).

was about 5 nM (0.05 nmol/mg of membrane protein; $n = 6$) and complete inhibition was attained at 50 nM (0.5 nmol/mg of membrane protein) (Figure 2). The inhibition of Ca²⁺ release was not surmountable by a large excess of InsP₃, and the antibody did not affect ATP-dependent Ca²⁺ transport (uptake) and did not inhibit binding of [³H]InsP₃ to its receptor (results not shown). The specificity of mAb 213-21 was established in several ways: (i) non-specific purified mouse or human IgMs had no effect on Ca²⁺ release by InsP₃; (ii) the hybridoma culture medium carried through the same dialysis/ultrafiltration purification procedure for obtaining mAb 213-21 was also inactive; and (iii) mAb 213-21 solutions passed through an anti-IgM–agarose column lost all inhibitory activity. No evidence of proteolytic activity was found in mAb preparations (Bio-Rad protease assay kit), and a wide variety and combination of protease inhibitors did not affect the inhibitory action of mAb 213-21 on Ca²⁺ release.

InsP₃-sensitive Ca²⁺-accumulating membrane fractions from rat cerebellum, HEL cells and sea-urchin eggs were also found by e.l.i.s.a. to bind mAb 213-21. In each of these membrane systems the antibody effectively suppressed Ca²⁺ release by InsP₃ (not shown). Figure 3 illustrates a typical experiment ($n = 5$) in which the antibody inhibited InsP₃-induced Ca²⁺ release from dog aortic SR vesicles. The time course of mAb 213-21 binding to aortic SR, measured by e.l.i.s.a., was determined to be essentially complete in 1 h. Therefore, vesicles were incubated with buffer, mAb 213-21 or non-specific mouse IgM for 1 h before the initiation of Ca²⁺ uptake and release. mAb 213-21 had no significant effect on Ca²⁺ uptake (mean = 37 nmol of Ca²⁺/mg of protein) or the discharge of vesicular Ca²⁺ by ionophore A23187 (26.4 ± 1.5 nmol of Ca²⁺/mg of SR protein; $n = 13$). InsP₃ released Ca²⁺ from aortic SR with an EC₅₀ of 1 μM, and a maximum of 38% of the accumulated Ca²⁺ (13.3 nmol of Ca²⁺/mg of protein) was released by 7.6 μM InsP₃ (Figure 3a). In the experiment shown in Figure 3(c), in the same SR vesicles, mAb 213-21 inhibited InsP₃-induced Ca²⁺ release by 82%, and this effect was not surmountable by increasing InsP₃ to 30 μM. Non-specific mouse IgM at the same concentrations had no effect on either Ca²⁺ uptake or its release by InsP₃ (Figure 3b). The binding of [³H]InsP₃ (i.e. 10 nM [³H]InsP₃; 0.6 pmol/mg of protein) to aortic SR membranes was unaffected by mAb 213-21 (results not shown). Brief (30 s) incubation of SR vesicles with mAb 213-21 did not decrease the amount of Ca²⁺ released by InsP₃, eliminating the possibility that the antibody acted by rapidly binding InsP₃ in solution. Instead the antibody's effect was, as in the case with platelet membranes, clearly dependent on its rate of binding to the aortic SR membrane (results not

shown). The IC₅₀ for mAb 213-21 inhibition of Ca²⁺ release by InsP₃ was in the range 29–43 nM (0.12–0.17 nmol/mg of membrane protein; $n = 12$), so that, normalized per mg of membrane protein, the antibody was about one-third as potent on the smooth-muscle preparations as on the platelet membranes. In contrast with the marked effect of mAb 213-21 on InsP₃-gated Ca²⁺ release in smooth-muscle SR, Ca²⁺ release from rabbit skeletal-muscle SR vesicles by caffeine was not inhibited by mAb 213-21, and no binding of antibody to skeletal-muscle SR membranes was detected as measured by e.l.i.s.a. (results not shown).

Inhibition of InsP₃-induced Ca²⁺ release by K⁺-channel blockers and mAb 213-21 is reversed by nigericin and partially reversed by valinomycin

On the presumption that apamin, TPA and mAb 213-21 might all be inhibiting a K⁺ channel, we employed the K⁺ carrier valinomycin and the K⁺/H⁺ ionophore nigericin as potential exogenous K⁺ carriers in an attempt to circumvent the block. The Na⁺/H⁺ ionophore monensin and the protonophore carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone (FCCP) were used as controls for the possible involvement of H⁺. By themselves, none of the ionophores had any effect on ATP-dependent Ca²⁺ uptake or on passive Ca²⁺ efflux from the membrane vesicles, or on the normal response to InsP₃. To produce a block of Ca²⁺ release, vesicles were preloaded with ⁴⁵Ca²⁺ for 30 min in KCl medium (Na⁺-free), followed by addition of apamin or TPA for an additional 30 min of uptake. For experiments with mAb 213-21 the antibody was present during the entire 60 min uptake period. After Ca²⁺ uptake was completed, we added 2.6–5.0 μM nigericin, 5 μM monensin, 3.2 μM valinomycin or 10 μM FCCP to portions of the membrane vesicles. InsP₃ was added 60 s later, and the release of ⁴⁵Ca²⁺ was measured over a 1 min time period. Nigericin was able to reverse totally the block of InsP₃-induced Ca²⁺ release caused by mAb 213-21, apamin (Figures 4a and 4b) and TPA, but monensin and FCCP were without effect. Valinomycin also had little effect on the blockade of Ca²⁺ release when it was added just before InsP₃, but when it was preincubated with the membranes for 30 min before InsP₃ valinomycin partially reversed the block of InsP₃-induced Ca²⁺ release (Figure 4c).

Properties of the ABP

When platelet vesicles were treated with proteases (i.e. 10 μg/ml trypsin, papain and chymotrypsin), all antibody-binding activity

Figure 4 Nigericin reverses inhibition of Ca²⁺ release by apamin, mAb 213-21 and TPA in platelet membrane vesicles

(a) All vesicles were treated with 20 nM apamin for 60 min during the ⁴⁵Ca²⁺-uptake period: ●, 2.6 μM nigericin added alone at 0 s; ▽, 5 μM InsP₃ added at 60 s (no nigericin); ○, nigericin added at 0 s and InsP₃ at 60 s. (b) All vesicles were incubated with 50 nM mAb 213-21 during the 60 min Ca²⁺ uptake; ●, 2.6 μM nigericin added alone at 0 s; ▽, 5 μM InsP₃ added at 60 s (no nigericin); ○, nigericin added at 0 s and InsP₃ at 60 s. The release of ⁴⁵Ca²⁺ by InsP₃ in apamin/nigericin- or mAb 213-21/nigericin-treated vesicles was > 95% of the control response elicited by InsP₃ alone (results not shown). (c) ▲, Control response to 5 μM InsP₃ added at zero time. All other vesicles were incubated with 100 μM TPA for the last 30 min of ⁴⁵Ca²⁺ uptake: ▽, 5 μM InsP₃ added at zero time; ●, 3.2 μM valinomycin added during last 30 min of the 1 h ⁴⁵Ca²⁺-uptake period, and 5 μM InsP₃ added at zero time; ○, nigericin added 60 s before InsP₃, which was added at zero time. No Ca²⁺ release occurred during the initial 60 s incubation with nigericin in (a), (b) or (c) (results not shown).

was lost within 30 min (results not shown). Each of the proteases were inactivated by specific antagonists before measuring binding of mAb 213-21 by e.l.i.s.a. Oxidation of protein sugar moieties with 50 mM periodate (in 50 mM sodium acetate buffer, pH 5.8) for 1–5 h did not affect antibody binding of mAb 213-21 to platelet membranes, indicating that the antigenic determinants were not carbohydrate groups. As a positive control, the same periodate treatment of purified lipopolysaccharide completely abolished the binding of an anti-lipopolysaccharide antibody that is specific for lipopolysaccharide carbohydrate groups (Murayama et al., 1978). The binding of mAb 213-21 was also abolished when membranes were boiled or treated with 1% SDS,

and Western blotting after SDS/PAGE was unsuccessful, indicating that the antibody probably recognizes only conformation-dependent antigenic determinants present in the native ABP, a finding that is not unexpected, since undenatured membrane protein *in situ* was used as the immunogen. It was therefore necessary to develop an e.l.i.s.a. to detect ABP (see the Experimental section).

Gel-filtration h.p.l.c. of ABP

The apparent molecular mass of NOG-solubilized ABP from platelets, cerebellum and aortic smooth-muscle SR was estimated by gel filtration using a calibrated Dupont Zorbax GF-450 h.p.l.c. column (see the Experimental section). From each of these sources, protein reacting with mAb 213-21 (e.l.i.s.a.-positive) was eluted in two fractions by low salt, i.e. 0.2 M sodium phosphate buffer plus 25 mM NOG and 1 mM EDTA, pH 7.4. Figure 5 shows a typical chromatogram of platelet proteins. About 50% of the e.l.i.s.a.-positive protein was eluted earlier than IgM (> 950 kDa), while the remainder was eluted with a retention time close to that of a BSA (66 kDa) standard. The same distribution of e.l.i.s.a.-positive protein was obtained by gel filtration of the solubilized proteins from cerebellum and aortic smooth muscle. The [³H]InsP₃-binding activity was only present in the high-molecular-mass fraction from each of the tissue/cells analysed (results not shown). This is consistent with previous findings that solubilized native InsP₃ receptors from cerebellum and smooth muscle behave as entities with a molecular mass of about 1000 kDa, and appear to assemble as tetramers (Supattapone et al., 1988; Maeda et al., 1990; Chadwick et al., 1990). When solubilized membrane proteins from each of the sources were chromatographed in high-salt solutions (1.0 M NaCl + NOG/EDTA), virtually all (> 90%) the e.l.i.s.a.-positive material was eluted in the low-molecular-mass fraction close to the 66 kDa BSA standard and was completely separated from the [³H]InsP₃-binding fraction. The [³H]InsP₃-binding fractions did not react positively in the e.l.i.s.a. that detected ABP. When the low-molecular-mass fraction was pooled, concentrated and rechromatographed in low salt, the e.l.i.s.a.-positive protein again migrated as a single peak of about 60 kDa, and virtually no high-molecular-mass ABP was observed, indicating that the original presence of ABP in the high-molecular-mass fraction in low salt was not due to self-aggregation of ABP molecules. These findings suggest that in low salt the ABP may associate with some as yet unidentified protein in the same high-molecular-mass fraction that contains the InsP₃ receptor protein. High salt appears to disrupt these protein–protein associations. All the gel-filtration analyses, and the other h.p.l.c. procedures described below, were repeated 6–10 times.

Partial purification of ABP from platelet cell membranes by h.p.l.c.

ABP was partially purified from platelets by a three-step h.p.l.c. column-chromatography procedure employing a sequence of anion-exchange, heparin–TSK and gel-filtration h.p.l.c. A typical purification is shown in Figure 6. Platelet membrane vesicle protein (approx. 20 mg), solubilized in 0.1 M sodium phosphate containing 50 mM NOG, 1 mM EDTA, 10 μM leupeptin and pepstatin, pH 6.8, was loaded on to a Synchrom Synchropak AX300 anion-exchange h.p.l.c. column. Proteins were eluted with a linear sodium phosphate gradient from 0.01 to 0.35 M in 25 mM NOG, pH 6.8 (Figure 6a). The e.l.i.s.a.-positive fractions which were eluted with a peak at 0.25–0.28 M phosphate were pooled, and the buffer was exchanged to 40 mM NaHCO₃/

Figure 5 Gel-filtration h.p.l.c. of solubilized platelet membrane proteins: assay for mAb 213-21 binding protein by e.i.s.a.

(a) Gel filtration in low salt. Solubilized membrane protein (0.6 mg) was chromatographed on a GF-450 column in low salt: 25 mM NOG, 0.2 M sodium phosphate, 1 mM EDTA, pH 7.0. Open bars: e.i.s.a. absorbance of fractions. The molecular-mass standards were: ●, IgM (950 kDa); ▲, BSA (66 kDa). (b) Gel filtration in high salt. Running buffer was 25 mM NOG, 20 mM NaHCO₃, 1 mM EDTA, 1.0 M NaCl, pH 7.4. The molecular-mass standards were: ●, thyroglobulin (670 kDa); ■, phosphorylase *b* (92.5 kDa). The flow rate was 0.5 ml/min.

Figure 6 Partial purification of platelet ABP by h.p.l.c.

Platelet membranes (29 mg of protein) were solubilized in 25 mM NOG/0.1 M sodium phosphate, pH 6.8. (a) Anion-exchange h.p.l.c. with gradient elution from 0.01 to 0.35 M phosphate, pH 6.8. ○, E.i.s.a. results. [Phosphate] (●) was measured on-line with an h.p.l.c. conductivity flow-through cell. (b) Heparin-TSK h.p.l.c. of e.i.s.a.-positive protein (4.75 mg) eluted from anion-exchange column in (a). Protein was eluted with a 0–1.0 M NaCl gradient added to the NaHCO₃/NOG buffer. (c) The e.i.s.a.-positive fractions (0.155 mg of protein) eluted from heparin-TSK columns in (b) were concentrated and chromatographed by GF-450 gel-filtration h.p.l.c. with a running buffer of 1.0 M NaCl added to NaHCO₃/NOG. ●, BSA standard.

25 mM NOG/1 mM EDTA, pH 8.3, by using a Centricon device (10000 or 30000 Da cut-off). The protein (average = 4 mg) was then loaded on to a Supelco TSK gel heparin-5PW h.p.l.c. column and eluted with a gradient of 0–1.0 M NaCl added to the resuspension buffer. ABP was eluted with a peak at 0.75 M NaCl (Figure 6b). The [³H]InsP₃-binding activity present in the original membrane extract co-purified with ABP on the anion-

exchange column and partially overlapped the ABP-positive fractions eluted from the heparin h.p.l.c. column. The e.i.s.a.-positive fractions (average 155 µg of protein) from the heparin column were concentrated, and the buffer exchanged with high-salt solution (1.0 M NaCl, 1 mM EDTA, 10 mM Tris, 25 mM NOG, pH 7.1). The proteins were injected on to a Zorbax G-450 h.p.l.c. gel-filtration column equilibrated with the same high-salt

Figure 7 Immunaffinity purification of ABP on an agarose-mAb 213-21 column

(a) Solubilized platelet membrane protein (30 mg) was applied to a heparin-TSK column and eluted by a salt gradient (as in Figure 6a). The e.l.i.s.a.-positive eluate (5.22 mg of protein) contained two bands on SDS/PAGE, at 63 and 55 kDa. (b) Purification of heparin-TSK e.l.i.s.a.-positive eluate on an immunoaffinity column (mAb 213-21 coupled to agarose; see the Experimental section): e.l.i.s.a.-positive protein (3.96 μ g) was eluted by diethylamine, pH 11.5. The proteins in all experiments were labelled with 35 S-SLR reagent, separated by SDS/PAGE (5–15% gradient) and autoradiographed for 24 h. (c) E.l.i.s.a.-positive immunopurified fraction subjected to SDS/PAGE and stained with silver. Positions of molecular-mass markers (kDa) are indicated to the left of (a).

buffer. All of the [3 H]Ins P_3 -binding activity was eluted from the gel-filtration column in the high-molecular-mass fraction (≥ 1000 kDa), which was e.l.i.s.a.-negative and contained a major band of 254 kDa and no 63 kDa protein (results not shown). A single e.l.i.s.a.-positive fraction was obtained at a peak similar in apparent molecular mass to a BSA standard (Figure 6c). Two polypeptides of 63 kDa and 53 kDa were present in the e.l.i.s.a.-positive low-molecular-mass fraction (Figure 7a).

Immunaffinity purification of ABP

Platelet and aortic ABP that were eluted from the heparin h.p.l.c. column were further purified to apparent homogeneity by immunoaffinity chromatography (see the Experimental section). The Ins P_3 receptor ([3 H]Ins P_3 -binding activity) did not bind to the column and was recovered entirely in the void volume. The ABP that was eluted from the column was tagged with 35 S-SLR protein-labelling reagent. The radioautographs and silver-stained gels of platelet ABP revealed a single band at 63 kDa (Figures 7b and 7c). This band did not react with anti-BSA or anti-HSA antibodies on Western blots. The amount of platelet ABP recovered from the immunoaffinity column was approx. 60 pmol, which was 30% (based on e.l.i.s.a.) of the estimated total amount of ABP in the original solubilized membrane extract. By the same procedure a single 63 kDa polypeptide was isolated from aortic smooth-muscle SR membranes.

DISCUSSION

As part of our ongoing studies on the platelet Ins P_3 receptor (O'Rourke et al., 1985, 1987; Feinstein and O'Rourke, 1987; O'Rourke and Feinstein, 1990), we have investigated the potential role of internal K $^+$ channels in the regulation of Ca $^{2+}$ release by Ins P_3 . An internal K $^+$ channel could conceivably function by

permitting an influx of K $^+$ to counterbalance an electrogenic outflow of Ca $^{2+}$ from the vesicles. This could prevent the build-up of an inside-negative membrane potential caused by outward Ca $^{2+}$ current, which would oppose Ca $^{2+}$ efflux from the vesicles. K $^+$ channels appear to provide such an electrical shunt in the SR of various types of muscle cells (see the Introduction). The possible involvement of similar K $^+$ channels in Ins P_3 -mediated Ca $^{2+}$ release has been inferred from studies of liver and brain internal membranes (Muallem et al., 1985; Joseph and Williamson, 1986; Shah et al., 1987; Shah and Pant, 1988). In our studies we employed a number of classical K $^+$ -channel blockers, and a monoclonal antibody which blocks Ca $^{2+}$ release from Ins P_3 -sensitive membrane vesicles of many cell types, to investigate the possible involvement of K $^+$ channels in responses to Ins P_3 . The K $^+$ -channel blockers that we used were TEA, TPA, charybdotoxin, apamin, 4-aminopyridine and glybenclamide. These reagents, and other selective toxins, have proved invaluable in establishing the types of K $^+$ channels involved in various cellular functions (Castle et al., 1989). TEA and other tetraalkylammonium compounds are well-known blockers of voltage-dependent K $^+$ channels, and TEA can also block some K $_{Ca}$ channels (Brayden and Nelson, 1992; Rane, 1991), but not others (Blatz and Magleby, 1986). There are at least three classes of K $_{Ca}$ channels (McManus, 1991), and certain subspecies are blocked most selectively by two highly specific toxins, i.e. charybdotoxin and apamin. ATP-activated K $^+$ channels, on the other hand, are sensitive to glybenclamide.

The release of Ca $^{2+}$ from platelet membrane vesicles by Ins P_3 was not affected by charybdotoxin, 4-aminopyridine or glybenclamide. It was weakly inhibited by TEA, but was strongly suppressed by the larger and more hydrophobic TPA ion and by the bee venom polypeptide apamin. This pharmacological profile fits that of certain low-conductance K $_{Ca}$ channels. The powerful effect of apamin on platelet membrane vesicles occurred over precisely the same concentration range at which it was effective against K $_{Ca}$ channels in other systems (Banks et al., 1979; Hugues et al., 1982a; Grissmer et al., 1992). This finding suggests that the internal platelet membrane system may contain an apamin-sensitive K $_{Ca}$ channel that influences Ca $^{2+}$ -release by Ins P_3 . Apamin, an octapeptide from bee venom (Habermann, 1972), whose structure-function features have been characterized (Labbe Jullie et al., 1991), is the most highly selective inhibitor of the class of low-conductance K $_{Ca}$ channels (Jenkinson, 1981; Romey and Ladzinski, 1984; Blatz and Magleby, 1986). It does not block the high-conductance (> 100 pS) charybdotoxin-sensitive K $_{Ca}$ channels (Miller et al., 1985) or other types of K $^+$ channels. Based on its pharmacological properties and intracellular location, the apamin-sensitive entity in platelet Ins P_3 -sensitive membranes is clearly distinct from the charybdotoxin-sensitive apamin-insensitive K $_{Ca}$ channel that was reported to be present in the surface membrane of intact platelets (Fine et al., 1989). Apamin receptors have not been fully characterized in any tissue. Previous studies using affinity-labelling techniques have identified putative apamin-binding proteins of 22, 27, 30, 33, 57, 59, 86 and 350 kDa (Schmid-Antomarchi et al., 1984; Seagar et al., 1987; Auguste et al., 1989; Leveque et al., 1990), but none of these apamin-binding proteins has yet been cloned and sequenced, so their structure and functions are not known. Interestingly, the binding of [125 I]-apamin to brain membranes was only partially inhibited by a high concentration of TEA (30 mM) and not inhibited at all by 4-aminopyridine (Seagar et al., 1987).

The weak inhibitory effect of TEA on Ca $^{2+}$ release by Ins P_3 is not unexpected when considered in context with the effect of apamin. Low-conductance K $_{Ca}$ channels that are blocked by apamin, but are insensitive to TEA, have been described in the

surface membrane of skeletal muscle (Blatz and Magleby, 1986) and N1E 115 mouse neuroblastoma cells (Moolenaar and Spector, 1979a,b; Hugues et al., 1982b). A low-conductance K_{Ca} channel highly sensitive to apamin, but not to charybdotoxin or 4-aminopyridine, was also found in human leukaemic T-cells (Grissmer et al., 1992). The weak inhibitory effect of TEA in our experiments on platelet membranes is, however, in contrast with the findings of Shah and Pant (1988), who observed an 80% decrease in InsP₃-induced Ca²⁺ release from brain microsomes by 1 mM TEA. Our results agree with those of Palade et al. (1989), in which total InsP₃-dependent Ca²⁺ release from dog brain microsomes was inhibited only 20% by 20 mM TEA. The greater effectiveness of TEA noted by Shah and Pant (1988) might be attributed to their use of only 0.5 μM InsP₃ as stimulus, as compared with 6.7–10 μM InsP₃ used by us and by Palade et al. (1989). Furthermore, unlike Shah and Pant (1988), we and Palade et al. (1989) preloaded membrane vesicles with Ca²⁺ in media that contained substantial concentrations of phosphate anion, which greatly enhances the total amount of Ca²⁺ uptake (O'Rourke et al., 1989). It is therefore possible that the effectiveness of TEA is lessened when the vesicles are more heavily loaded with Ca²⁺ and when the stimulus to the InsP₃ receptor is maximal. Palade et al. (1989) further reported stronger inhibitory effects of the more hydrophobic alkylammonium compounds, including TPA, as well as inhibition of Ca²⁺ release by Ba²⁺, quinine and 4-aminopyridine. The highly charged tetra-alkylammonium ions do not cross the vesicle membrane, and therefore, when applied to the medium, they act on an exterior side of the channel. The external TEA-receptor sites of voltage-dependent K⁺ channels in different cells range from highly sensitive (Hille, 1967) to totally insensitive (Wong and Binstock, 1980) to blockade by TEA. The protein structural requirements of voltage-dependent K⁺ channels that account for large differences in their sensitivity to TEA have been identified (Kavanaugh et al., 1991). The channels have the properties of a pore lined with aromatic residues (Heginbotham and MacKinnon, 1992). This could account for the higher affinity of a series of larger and more hydrophobic alkylammonium ions observed by Palade et al. (1989). Thus the C-5 and C-6 alkylammonium ions were the most potent, and TPA was 570 times more potent than TEA, comparable with our results obtained in platelet membrane vesicles.

The inhibitory effects of apamin and TPA on Ca²⁺ release by InsP₃ implicates, but does not prove, the involvement of a K⁺ channel. As a critical test of this hypothesis we employed the electroneutral K⁺/H⁺ exchanger carrier nigericin, the Na⁺/H⁺ carrier monensin, and the electrogenic K⁺ carrier valinomycin, reasoning that the creation of an alternative route for K⁺ within the membrane would overcome the blockade of the natural K⁺ channel by TPA, apamin and mAb 213-21. Nigericin, at the concentrations employed, did not by itself release Ca²⁺ or affect the normal response to InsP₃, yet in each case it totally or substantially reversed the inhibition of InsP₃-induced Ca²⁺ release caused by the K⁺-channel blockers and the antibody. In contrast, monensin, whose structure is very similar to that of nigericin, but which is much more selective for Na⁺ than for K⁺ (Pressman and Haynes, 1969), as well as the protonophore FCCP, did not reverse the blockade of Ca²⁺ release. The reversal of the effects of the K⁺-channel blockers by nigericin implies that they were not acting directly on the Ca²⁺ channel of the InsP₃ receptor. Palade et al. (1989) came to the opposite conclusion about the K⁺-channel blockers which they employed, because the addition of the electrogenic K⁺ ionophore valinomycin (or gramicidin) did not overcome the blockade by TEA. We also tested valinomycin and found it to be unable to reverse the blockade of Ca²⁺ release if added 60 s before InsP₃. However, when preincubated with the

membranes for 30 min valinomycin partially reversed the blockade of Ca²⁺ release. The reason for the slow onset of action of valinomycin is not clear, but some property of the membranes may impede the insertion of positively charged valinomycin-K⁺ complexes into the lipid bilayer. Thus a more prolonged period of incubation with valinomycin might be necessary for it to exert an effect in our system. Because nigericin, in contrast with valinomycin or gramicidin, can also transport H⁺, we also tested the protonophore FCCP, but found it to be without effect. Similarly, the Na⁺/H⁺ ionophore monensin was also ineffective, which is additional evidence that the effectiveness of nigericin does not result from its ability to collapse a transmembrane proton gradient.

The possibility, raised by Palade et al. (1989), that the K⁺-channel blockers act directly on the InsP₃ receptor's Ca²⁺ channel must be further considered. There are no experiments on the purified InsP₃ receptor in lipid bilayers that would decide this issue. L-type Ca²⁺ channel blockers did not block InsP₃-induced Ca²⁺ release from cerebellar membrane vesicles (Shah and Pant, 1988), indicating that the receptor's Ca²⁺ channel is distinct from the voltage-sensitive L-type Ca²⁺ channel. On the other hand, Bkaily et al. (1985) observed that 0.05–0.1 mM apamin produced an irreversible block of spontaneous and isoprenaline-induced slow action potentials in chick-embryo cultured ventricle-cell aggregates and intact chick embryonic heart, which suggested that the peptide had an extraordinarily potent effect on L-type Ca²⁺ channels. Indeed, the effective concentrations of apamin in this case were below those normally effective against low-conductance K_{Ca} channels. In contrasting findings, Baro and Escande (1989) reported that L-type Ca²⁺ channels of guinea-pig atrial myocytes were not affected by apamin until extraordinarily high concentrations of at least 1 μM were attained, i.e. 100 times the effective concentrations in our experiments. Nevertheless, if we consider the possibility that apamin might block the InsP₃ receptor's Ca²⁺ channel, how then could we account for the rapid reversal of its effects by nigericin? Since nigericin is not a Ca²⁺ ionophore, and transport of protons does not account for its action, its only known property that provides an explanation for its ability to reverse the effects of apamin, TPA and mAb 213-21 is its ability to transport K⁺. This leads to the conclusion that apamin's action against InsP₃ is based on its ability to block certain K⁺ channels.

It is also possible that the K⁺ channel in question is the InsP₃ receptor itself, and that the blockers are affecting the channel's K⁺ conductance. The InsP₃ receptor protein from smooth muscle, when reconstituted into lipid bilayers, had an estimated Ca²⁺/K⁺ permeability ratio of 2:1 (Mayrleitner et al., 1991). However, there are no measurements of the magnitude of the K⁺ current that is possible under the condition of massive Ca²⁺ efflux from the lumen of the membrane vesicles. Furthermore, if a K⁺ current is necessary to balance the outward Ca²⁺ current through the receptor's channel, the K⁺ must move in an opposite direction to the Ca²⁺, and there is no evidence that the InsP₃ receptor has this countercurrent ion-transport capability. Despite these arguments, we cannot totally rule out the receptor as a site of action for the K⁺-channel blockers TPA and apamin until direct experiments are performed on purified receptor protein. With respect to the action of mAb 213-21, which acts like apamin and TPA, the evidence is more conclusive and direct. This antibody does not interact directly with the IP₃ receptor, and its target, the ABP, is not a fragment of the receptor (see below).

The mAb 213-21, which was raised against the InsP₃-sensitive membrane fraction of platelets, also blocked InsP₃-induced Ca²⁺ release from membrane vesicles obtained from platelets, and from membranes isolated by similar methods from cerebellum,

sea-urchin eggs, HEL cells and aortic smooth-muscle SR. The ability of mAb 213-21 to hinder response to InsP_3 in membrane vesicles obtained from a wide variety of cell types indicates the presence of a conserved common target, ABP. ABP was indeed detected by e.l.i.s.a. with mAb 213-21 in InsP_3 -sensitive membrane fractions from all the sources listed above, but not in skeletal-muscle SR. The specificity of the antibody's action on Ca^{2+} release was established by several lines of evidence: (i) mAb 213-21 did not affect ATP-dependent Ca^{2+} uptake by membrane vesicles and did not affect the release of Ca^{2+} caused by the Ca^{2+} ionophore A23187; (ii) supermaximal concentrations of InsP_3 did not overcome a partial blockade by the antibody; (iii) mAb 213-21 did not affect [^3H] InsP_3 binding to its receptor at concentrations that blocked Ca^{2+} release; (iv) the inhibitory effects of mAb 213-21 on Ca^{2+} release were not duplicated by several non-specific mouse and human IgMs; (v) all biological activity of mAb 213-21 solutions was lost upon passage through an agarose-anti-IgM column; (vi) mAb 213-21 did not bind to skeletal-muscle SR membrane proteins, and did not inhibit the caffeine-stimulated Ca^{2+} channel of skeletal SR membranes. Furthermore, the inhibitory action of mAb 213-21 on InsP_3 -induced Ca^{2+} release was reversed by nigericin. These results indicate that the antibody acts similarly to apamin and TPA.

The availability of mAb 213-21 provided the means to purify the ABP. ABP from platelets, cerebellum and aortic smooth muscle chromatographed virtually identically in several h.p.l.c. systems: (i) gel filtration, (ii) anion exchange and (iii) heparin-TSK. Furthermore, in contrast with the InsP_3 receptor, the ABP did not bind to concanavalin A-agarose (result not shown). The apparent molecular masses of ABP from all tissues/cells were identical when run on calibrated gel-filtration h.p.l.c. columns. At low salt concentrations ABP was eluted in each case in two fractions, one at an apparent molecular mass of ≥ 1000 kDa and another at approx. 66 kDa. We attribute the presence of about 50 % of the total ABP in the high-molecular-mass fraction to its association with another protein oligomer, or complex of proteins, in that fraction. High salt (1.0 M NaCl) dissociated the e.l.i.s.a.-positive ABP from the high-molecular-mass fraction so that it then chromatographed exclusively in the 66 kDa peak fraction, and was completely separated from the InsP_3 -binding activity. When ABP eluted with high salt was rechromatographed at low salt, there was no ABP in the high-molecular-mass fraction. This is convincing evidence that the presence of some ABP in the high-molecular-mass fraction after low-salt gel filtration was due to an association with some other entity and not to self-aggregation. Consistent with the results of gel-filtration h.p.l.c., we conclude that the ABP is a polypeptide of 63 kDa, for the following reasons. After a sequence of h.p.l.c. steps on an anion exchanger, heparin-TSK and gel filtration, only two polypeptides, of 63 and 53 kDa, were found by SDS/PAGE in the final e.l.i.s.a.-positive eluate. Furthermore, the strongly e.l.i.s.a.-positive eluate obtained by immunoaffinity chromatography on agarose-mAb 213-21 contained a single polypeptide of 63 kDa. By the same immunoaffinity method we also isolated a 63 kDa protein from aortic smooth muscle.

The 63 kDa ABP is distinct from the InsP_3 receptor on several grounds: (i) as determined by e.l.i.s.a., mAb 213-21 did not bind to the chromatographic fractions that were devoid of ABP but contained substantially purified platelet IP_3 receptor; (ii) the antibody did not bind to the platelet InsP_3 -receptor protein that was purified to homogeneity as a 260 kDa protein; and (iii) sequence analysis of cloned cDNA isolated from a $\lambda\text{gt}11$ HEL-cell expression library revealed no similarity of ABP to the published InsP_3 -receptor sequences (J. LaPlante, F. O'Rourke and M. B. Feinstein, unpublished work). Further work is

necessary to determine if the ABP is indeed a K^+ channel, or functions in some other way to regulate the Ca^{2+} channel of the InsP_3 receptor. Further elucidation of its molecular structure, which is in progress, and its expression in a functional form is essential to elucidate the precise role of the ABP in InsP_3 -mediated Ca^{2+} release.

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REFERENCES

- Abramchek, C. W. and Best, P. M. (1989) *J. Gen. Physiol.* **93**, 1–21
- Auguste, P., Hugues, M. and Lazdunski, M. (1989) *FEBS Lett.* **248**, 150–154
- Banks, B. E. E., Brown, C., Burgess, G. M., Burnstock, G., Claret, M., Cocks, T. M. and Jenkinson, D. H. (1979) *Nature (London)* **282**, 415–417
- Baro, I. and Escande, D. (1989) *Eur. J. Physiol.* **415**, 63–71
- Berridge, M. J. and Irvine, R. F. (1989) *Nature (London)* **341**, 197–205
- Bezprozvanny, I. B., Benevolensky, D. S. and Naumov, A. P. (1991) *Biochim. Biophys. Acta* **1064**, 75–80
- Bkaily, G., Sperelakis, N., Renaud, J.-F. and Payet, M. D. (1985) *Am. J. Physiol.* **248**, H961–H965
- Blatz, A. L. and Magleby, K. L. (1986) *Nature (London)* **323**, 718–720
- Brayden, J. E. and Nelson, M. T. (1992) *Science* **256**, 532–535
- Castle, N. A., Haylett, D. G. and Jenkinson, D. H. (1989) *Trends Neurosci.* **12**, 59–65
- Chadwick, C. C., Saito, A. and Fleischer, S. (1990) *Proc. Natl. Acad. Sci. U.S.A.* **87**, 2132–2136
- Feinstein, M. B. and O'Rourke, F. (1987) in *Current Communications in Molecular Biology: Inositol Lipids in Cellular Signalling* (Michell, R. H. and Putney, J. W., Jr., eds.), pp. 141–143. Cold Spring Harbor Laboratory, Cold Spring Harbor
- Ferris, C. D., Haganir, R. L., Supattapone, S. and Snyder, S. H. (1989) *Nature (London)* **342**, 87–89
- Fine, B. P., Hansen, K. A., Salcedo, J. R. and Aviv, A. (1989) *Proc. Soc. Exp. Biol. Med.* **192**, 109–113
- Fink, R. H. and Stephenson, D. G. (1987) *Pflügers Arch.* **409**, 374–380
- Galfre, G. and Milstein, C. (1981) *Methods Enzymol.* **73**, 3–32
- Grissmer, S., Lewis, R. S. and Calahan, M. D. (1992) *J. Gen. Physiol.* **99**, 63–84
- Habermann, E. (1972) *Science* **177**, 314–322
- Harlow, E. and Lane, D. (1988) *Antibodies: A Laboratory Manual*, pp. 196–238, Cold Spring Harbor Laboratory, Cold Spring Harbor
- Heginbotham, L. and MacKinnon, R. (1992) *Neuron* **8**, 483–491
- Hille, B. (1967) *J. Gen. Physiol.* **50**, 1287–1302
- Hugues, M., Schmid, H., Romey, G., Duval, D., Frelin, C., and Lazdunski, M. (1982a) *EMBO J.* **1**, 1039–1042
- Hugues, M., Romey, G., Duval, D., Vincent, J. P. and Lazdunski, M. (1982b) *Proc. Natl. Acad. Sci. U.S.A.* **79**, 1308–1312
- Ide, T., Morita, T., Kawasaki, T., Taguchi, T. and Kasai, M. (1991) *Biochim. Biophys. Acta* **1067**, 213–220
- Jenkinson, D. H. (1981) *Trends Physiol. Sci.* **2**, 318–320
- Joseph, S. K. and Williamson, J. R. (1986) *J. Biol. Chem.* **261**, 14658–14664
- Kavanaugh, M. P., Varnum, M. D., Osborne, P. B., Christie, M. J., Busch, A. E., Adelman, J. P. and North, R. A. (1991) *J. Biol. Chem.* **266**, 7583–7587
- Kim, D., Ohmishi, S. and Ikemoto, N. (1983) *J. Biol. Chem.* **258**, 9662–9668
- Labbe Jullie, C., Grainer, C., Albericio, F., Defendini, M. L., Ceard, B., Rochat, J. and Van Rietschoten, J. (1991) *Eur. J. Biochem.* **196**, 639–645
- Leveque, C., Marqueze, B., Couraud, F. and Seager, M. (1990) *FEBS Lett.* **275**, 185–189
- Liu, Q. Y. and Strauss, H. C. (1991) *Biophys. J.* **60**, 198–203
- Liu, Q. Y., Lai, F. A., Shen, W. K., Meissner, G. and Strauss, H. C. (1991) *FEBS Lett.* **291**, 13–16
- Maeda, N., Niinobe, M. and Mikoshiba, K. (1990) *EMBO J.* **9**, 61–67
- Mayrleitner, M., Chadwick, C. C., Timmerman, A. P., Fleischer, S. and Schlinder, H. (1991) *Cell Calcium* **12**, 505–514
- McManus, O. B. (1991) *J. Bioenerg. Biomembr.* **23**, 537–560
- Miller, C., Moczydlowski, E., Latorre, R. and Phillips, M. (1985) *Nature (London)* **313**, 316–318
- Moolenaar, W. H. and Spector, I. (1979a) *J. Physiol. (London)* **292**, 297–306
- Moolenaar, W. H. and Spector, I. (1979b) *J. Physiol. (London)* **292**, 307–323
- Muallem, S., Schoeffield, M., Pandolf, S. and Sachs, G. (1985) *Proc. Natl. Acad. Sci. U.S.A.* **82**, 4433–4437
- Murayama, A., Shimada, K. and Yamamoto, T. (1978) *Immunochimistry* **15**, 523–528
- O'Rourke, F. and Feinstein, M. B. (1990) *Biochem. J.* **267**, 295–302
- O'Rourke, F., Halenda, S. P., Zavoico, G. B. and Feinstein, M. B. (1985) *J. Biol. Chem.* **260**, 956–962

- O'Rourke, F., Zavoico, G. B., Smith, L. H. and Feinstein, M. B. (1987) *FEBS Lett.* **214**, 176–180
- O'Rourke, F., Zavoico, G. B. and Feinstein, M. B. (1989) *Biochem. J.* **257**, 715–721
- Palade, P., Dettbarn, C., Volpe, P., Alderson, B. and Otero, A. S. (1989) *Mol. Pharmacol.* **36**, 664–672
- Pressman, B. and Haynes, D. H. (1969) in *The Molecular Basis of Membrane Function* (Tosteson, D. C., ed.), pp. 221–246, Prentice-Hall, Eaglewood Cliffs, NJ
- Rane, S. G. (1991) *Am. J. Physiol.* **260**, C104–C112
- Romey, G. and Ladzunski, M. (1984) *Biochem. Biophys. Res. Commun.* **118**, 669–674
- Schmid-Antomarchi, H., Hugues, M., Norman, R., Ellory, C., Borsotto, M. and Lazdunski, M. (1984) *Eur. J. Biochem.* **142**, 1–6
- Seagar, M. J., Marqueze, B. and Couraud, F. (1987) *J. Neurosci.* **7**, 565–570
- Shah, J. and Pant, H. C. (1988) *Biochem. J.* **250**, 617–620
- Shah, J., Cohen, R. S. and Pant, H. C. (1987) *Brain Res.* **419**, 1–6
- Shen, W. K., Hill, J. A. Jr., Rasmussen, R. and Strauss, H. C. (1990) *Prog. Clin. Biol. Res.* **334**, 205–230
- Sokabe, M., Kasai, M., Nomura, K. and Naruse, K. (1991) *Comp. Biochem. Physiol.* **98**, 23–30
- Supattapone, S., Worley, P. F., Baraban, J. M. and Snyder, S. H. (1988) *J. Biol. Chem.* **263**, 1530–1534
- Uehara, A., Yasukohchi, M., Ogata, S. and Imanaga, I. (1991) *Pflugers Arch.* **417**, 651–653
- Watras, J. and Benevolensky, D. (1987) *Biochim. Biophys. Acta* **931**, 354–363
- Wong, B. S. and Binstock, L. (1980) *Biophys. J.* **32**, 1037–1042