Phosphatidylinositol 4,5-Bisphosphate-induced Ca$^{2+}$ Release from Skeletal Muscle Sarcoplasmic Reticulum Terminal Cisternal Membranes

Ca$^{2+}$ FLUX AND SINGLE CHANNEL STUDIES*

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We report here that the inositol 1,4,5-trisphosphate (IP$_3$) precursor, 1,α-phosphatidylinositol 4,5-bisphosphate (PIP$_2$) is a potent molecule (1 μM) which activates the ryanodine-sensitive Ca$^{2+}$ release channel from rabbit skeletal muscle terminal cisternae incorporated into a phospholipid bilayer. It also stimulates Ca$^{2+}$ release from these membrane vesicles. Therefore, it may play a modulating role in excitation-contraction coupling.

In the bilayer, PIP$_2$ added on the cytoplasmic side increased the mean channel opening probability 2–12-fold in the presence and absence of physiological Mg$^{2+}$ and ATP. From flux studies, PIP$_2$-induced Ca$^{2+}$ release, occurring through the ryanodine-sensitive Ca$^{2+}$ release channel, displayed saturation kinetics. The rate of Ca$^{2+}$ release induced by PIP$_2$ was approximately >50% slower than the rates induced by other agents (e.g. caffeine, Ca$^{2+}$, ATP).

PIP$_2$, and not IP$_3$, effectively elicited Ca$^{2+}$ release from terminal cisternae. On the contrary, IP$_3$, and not PIP$_2$, specifically mediated Ca$^{2+}$ release from dog brain cerebellar microsomes, where IP$_3$ receptors are known to be found. The PIP$_2$-induced Ca$^{2+}$ release from muscle membranes was not dependent on medium [Ca$^{2+}$] (from <10$^{-9}$ to ∼10$^{-4}$ M). However, IP$_3$ could activate the terminal cisternae Ca$^{2+}$ channel in the bilayer when there was low Ca$^{2+}$ (<10$^{-7}$ M). The data suggest that the ionic microenvironment around the Ca$^{2+}$ channel may be different for observing the two phosphoinositide actions.

Inositol 1,4,5-trisphosphate (IP$_3$)† has been established as a second messenger in many tissues in stimulus-response coupling (1). Since the release of Ca$^{2+}$ from sarcoplasmic reticulum (SR) triggers muscle contraction, and smooth muscle contraction responses to IP$_3$ (2), the possible role of IP$_3$ as a stimulatory factor in excitation-contraction (E-C) coupling has been examined. However, the role of IP$_3$ in skeletal muscle E-C coupling is not clear (3).

Other inositol derivatives in the biosynthetic pathway, tetra- and triphosphates, have also been suggested to play roles as second messengers in other cell types (4). Recently, the IP$_3$ precursor, PIP$_2$, has been demonstrated to elicit Ca$^{2+}$ release from nonmuscle tissue (5) and crude "heavy" SR membranes of skeletal muscle (6, 7). We proceed to investigate the efficacy of PIP$_2$ versus IP$_3$ on the skeletal muscle SR Ca$^{2+}$ release channel by incorporating purified functional terminal cisternal membranes into bilayers and to characterize the requirements and specificity for PIP$_2$-induced Ca$^{2+}$ release by flux studies performed in SR vesicles. We report here that PIP$_2$ can activate the ryanodine-sensitive SR Ca$^{2+}$ release channel in the presence of physiological Mg$^{2+}$ and ATP and that low concentrations of PIP$_2$ induce Ca$^{2+}$ release from terminal cisternae. The stimulatory actions on release from SR and on the channel appear to be unaffected by cytoplasmic [Ca$^{2+}$]. On the other hand, cytoplasmic IP$_3$ activates the same channel only in low [Ca$^{2+}$] (<10$^{-7}$ M).

MATERIALS AND METHODS

Reagents—A23187, ryanodine, the trithium salt of IP$_3$, and the triammonium salt of PIP$_2$ (solvability in water is 0.5 mg/ml or ∼9 mM) were purchased from CalBiochem (San Diego, CA). 45CaCl$_2$ was from Dupont-New England Nuclear. Phospholipids were from Avanti Polar Lipids, Inc. (Birmingham, AL). Natural phosphatidylicholine, phosphatidylethanolamine, and phosphatidylserine were from bovine brain. Other reagents were from Sigma including heparin from porcine intestinal mucosa (Mr ∼4,000-7,000). Thin layer chromatography of PIP$_2$ revealed only one spot, indicating no breakdown or contamination of IP$_3$.

Membrane Preparations—Functional terminal cisterna (JTC) membrane vesicles were prepared from fast twitch skeletal muscles of rabbit hindlegs as previously described (8). Cerebellar microsomes (P2, a 17,000 × g pellet, and P3, a 100,000 × g pellet) were generously provided by Dr. P. Volpe (University of Texas Medical Branch, Galveston, TX). They were prepared from dog brains (9, 10). They contained ∼10 pmol of [3H]IP$_3$ binding site/mg protein, with K$_D$ of ∼20–30 nM, and their Ca$^{2+}$ stores could be released by IP$_3$.

Ca$^{2+}$ Uptake—Ca$^{2+}$ flux was measured by using radioisotope with a vacuum filtration method (11). The reaction mixture contained 1 mM MgCl$_2$, 80 mM KCl, 20 mM Tris-MOPS, pH 7, at room temperature (∼23°C), and various 45CaCl$_2$ (≥10,000 cpm/nmol) and protein concentrations as indicated in the figure legends. Na$_2$ATP (1 mM) was used to start the reaction. Aliquots (10-25 μg of protein) were filtered over time and the filters (type HA, 0.45 μm, Millipore Corp., Bedford, MA) were washed with 2 ml of a stop solution of 10 mM MgCl$_2$ in a basal buffer of 80 mM KCl, 1 mM Tris-EGTA, 20 mM Tris-MOPS, pH 7, at room temperature. When applicable, the basal buffer contained 10 μM IP$_3$ and no Mg$^{2+}$ (Fig. 6). The filters were subsequently processed for liquid scintillation counting.

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‡ The abbreviations used are: IP$_3$, d-my-o-inositol 1,4,5-trisphosphate; SR, sarcoplasmic reticulum; JTC, junctional terminal cisterna; PIP$_2$, 1,α-phosphatidylinositol 4,5-bisphosphate; MOPS, 3-(N-morpholino)propanesulfonic acid; EGTA, ethylene bis(oxetethylenedinitrilo)tetraacetic acid; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; CMS, cesium methanesulfonate.
**PI**P_{2}-induced Ca^{2+} Release from SR

Ca^{2+} Release—JTC vesicles (1 mg of protein/ml) were passively loaded with 1 mM 40CaCl{2} (=15,000 cpm/nmol), 80 mM KCl, and 20 mM Tris-MOPS, pH 7, for 15-20 h on ice. An aliquot was diluted 100-fold (v/v) into release medium containing basal buffer, at room temperature, and other agents as indicated in the figure legend. Aliquots of 10 μg of protein were then quickly filtered over time, and the filters were washed and processed for liquid scintillation counting as described above.

**Rapid Ca^{2+} Release**—For release studies of less than 1 s, JTC (50 μg of protein/ml) were actively loaded with 100 μM 40CaCl{2} (=10,000 cpm/nmol), 10 mM MgCl₂, 80 mM KCl, and 20 mM Tris-MOPS, pH 7, for 5 min at 25 °C with 2 mM acetyl phosphate. The entire volume was filtered over a 0.65-μm Millipore filter (type DA). The filter was then washed once with 2 ml of stop solution to remove the loading medium from the filter. The filter was then exposed to the medium containing basal buffer, at room temperature, and other agents as indicated in the figure legends. The time of exposure to release medium was controlled by the flow rate of a rapid filtration apparatus (Molecular Kinetics, Pullman, WA) (11). The filters were then processed for liquid scintillation counting without further washing.

**Bilayer Technique**—The procedure developed by Hamilton et al. (1989) was used (12). A planar phospholipid bilayer of either a charged mixture phosphatidylcholine/phosphatidylethanolamine/phosphatidylserine = 5:5:2, 25 mg/ml, dissolved in decane) or a neutral mixture (phosphatidylcholine/phosphatidylethanolamine = 3:7, 50 mg/ml in decane) was “painted” with a glass rod across an aperture of ~300-μm diameter (septum depth ~200 μm) in a delrin cup. The cis chamber was the voltage control side connected to the negative input of a custom-made current/voltage converter, while the trans side was referred to the ground. The capacity transients were then monitored during the bilayer formation with a +50 mV/50 ms ramp. Ag/AgCl bridges were used to connect the chambers to Ag/AgCl electrodes immersed in 2-3 M KCl. Both chambers were initially filled with 50 mM methanesulfonate-CsOH (CMS) + 20 mM HEPES-CsOH, pH 7.4, with no added Ca^{2+}. The contaminating Ca^{2+} was <5 ± 14 μM, as measured by a Ca^{2+}-sensitive electrode (Radiometer, Copenhagen, Denmark). An asymmetric CMS gradient (250 mM cis, 50 mM trans) was established after the bilayer was formed. After the capacitance across the aperture reached 150-300 pF, incorporation of channels was facilitated by first dipping a glass rod into the JTC preparation and then painting across the aperture. The JTC protein was added to the cis chamber, thus by convention, it corresponds to the cytoplasmic side of the SR, while the trans side corresponds to the luminal side. To improve bilayer stability, we collapsed the Ca^{2+} gradient across the bilayer to a symmetric 250 mM cis/trans in some experiments. This procedure also prevented further fusion of channels into aggregates. Single channel data were collected either at steady state or during voltage clamp pulses (0.5-50 μV/50 ms). Capacity transients were eliminated by analog and digital subtraction with blank traces. Data acquisition software and hardware (pClamp, T1-1 interface, Axon Instruments, Burlingame, CA) were interfaced with a microcomputer with AD/DA converters (Scientific Solutions, Salem, OH). Channel activity was recorded either with a 16-bit VCR-based acquisition and storage system at a 20-KHz sampling rate or directly into the computer. A custom-made automated detection program for single channel events (provided by Drs. T. VanDongen and A. Brown, Baylor College of Medicine, Houston, TX) was used to analyze data. Unitary channel openings were detected by setting thresholds at half-amplitude on digitized data. Threshold values were obtained from amplitude distribution histograms of all the sampled points. Open and closed time intervals were obtained from idealized records. Dwell time distributions were logarithmically binned with equally spaced time intervals (6-8 bins/decade) and fitted to probability density functions by the method of maximum likelihood method (correlation coefficients >0.95) (13). In our figures, the channel openings are all shown as upward deflections.

**Identification of the Ca^{2+} Release Channel**—A convenient way to record channel activity of the Ca^{2+} release channel from native SR membranes was to use a CMS gradient because background CI channels and K’ channels were eliminated. This method and its rationale have been initially described by Fill and his colleagues (12, 14). The Ca^{2+} conducting channel displayed the pharmacology of the SR Ca^{2+} release channel. Some of its characteristics are shown in Fig. 1. These recordings were carried out in a 250 mM cis, 50 mM trans

**FIG. 1.** Pharmacology of single channel activities of JTC membranes. Ca^{2+} was the current carrier. The channel, opening upward, (arrowhead = base-line current), was recorded by pulsing ("Materials and Methods"). The holding potential was +20 mV. a and b, control activity in 250 mM/50 mM cesium methanesulfonate (cis/trans). The conductance of this channel was 407 pS. c and d, after 10 μM ryanodine (in 0.95% ethanol) was added to the cis chamber and stirred for 1 min. Note that the channel abruptly entered into a substrate with long openings and occasional closing. e, after 1.5 mM EGTA was added to the cis side (free Ca^{2+} ≈ 10^{-7} M), f, after 5 μM ruthenium red was added to the cis side. e and f, separate experiments from a-d.

Ca^{2+} gradient with contaminant Ca^{2+}. The control activity of the channel is displayed in the two upper records (a and b). Channel conductance of the largest level was 407 pS. Ryanodine (10 μM) affected the gating characteristics as described previously (14, 15) (Fig. 1, c and d). Reducing cytoplasmic Ca^{2+} by EGTA and ruthenium red blocked the channel activity (Fig. 1, e and f). The Ca^{2+} conducting channel was inhibited by 1 mM Mg and stimulated by ATP (1 mM) when both were added to the cytoplasmic side (not shown) (14). In summary, this pharmacological profile demonstrates beyond any doubt the identity of the Ca^{2+} release channel which was to be used in these studies. We have used nine separate JTC preparations, and at least five channels were studied from each preparation.

**Estimation of Free Ion Concentration**—Free and bound Ca^{2+} concentrations were determined following Fabiato (1988) (16). The value of contaminating Ca^{2+} of 2.3 μM in 10 μM PIP_{2} was used in some calculations (5). Accordingly, the maximal contaminating Ca^{2+} introduced by PIP_{2} in the bilayer experiments was ~0.1-0.2 μM, which cannot explain the stimulatory action we observed. The Ca^{2+} contamination by PIP_{2} is negligible in the experiments with 1 mM ATP. Furthermore, it is also negligible in passive Ca^{2+} loading experiments because we included EGTA to chelate residual Ca^{2+}. The small amount of Ca^{2+} introduced by 10 μM PIP_{2} in active Ca^{2+} loading experiments was also not likely to interfere with PIP_{2}-induced Ca^{2+} release because of Ca^{2+} reuptake by the Ca^{2+} pump (Fig. 4D).

Unless indicated otherwise, each experiment was repeated at least with three different membrane preparations. The temperature was ~22-25 °C. When applicable, the mean and standard deviations (S.D.) are presented.

**RESULTS**

**Effects of PIP_{2} on the SR Ca^{2+} Release Channel**—The effect of low concentrations of PIP_{2} on steady state single channel activity of the ryanodine-sensitive Ca^{2+} release channel was examined. JTC membrane vesicles were incorporated into a phospholipid bilayer. When PIP_{2} was added to the cis chamber, in the presence of millimolar Mg^{2+} and ATP, an environment similar to physiological milieu, there was an increase in channel opening probability (P_{o}) (Fig. 2, A and B). In the absence of Mg^{2+} and ATP, the Ca^{2+} release channel was also stimulated by PIP_{2} (Fig. 2, C-E).

In Fig. 2A, recordings in control conditions were made in the presence of 2 mM Mg^{2+} and 1 mM ATP added to the cytoplasmic side. In the presence of Mg^{2+}, the channel activity was very infrequent and brief in nature, even after the addition of ATP. When 1 μM PIP_{2} was added to the same side (Fig. 2B), the channel was clearly activated: it opened more frequently, and with longer openings. Occasionally there was burst activity, but this did not occur with every channel. The amplitude of the channel was not altered by the addition of PIP_{2}. The bar graphs correspond to the P_{o} values over a
with time constants comitantly, PIPz reduced the mean closed lifetime values of components indicating that there were at least two open states events with longer lasting mean open lifetimes ($a_1$, $a_2$, and $a_3$). The mean open probability ($P_o$) increased from 0.06 to 0.19 by 1 $\mu$M PIPz. The activation by PIPz was evident immediately following the stirring (1 min), and it was clearly observed in 94% of all experiments (36 out of 38 channels from five different JTC preparations). The mean $P_o$ of the channels was enhanced between 2-5 min on a VCR tape, in a symmetric 250 mM cis/trans Ca$^{2+}$ medium (“Materials and Methods”). A-B, recorded in the presence of 2 mM MgCl$_2$ and 1 mM Na$_2$ATP in the cis chamber (pCa = 12). C-E, represented the channel where 0.5 $\mu$M PIPz increments were added sequentially to the cis chamber (pCa = 4.9). Channel opening is in the upward direction. The holding potentials were $-50$ mV (A-B) and $-40$ mV (C-E). The Ca$^{2+}$ conductances of the channels were 407 pS (A-B) and 396 pS (C-E). The mean opening probability ($P_o$) and the $P_o$ over a period of 45 s (a scale of 0 to 0.7 for all the y axes) are shown in the right panels of each corresponding channel recording. These channels were less active because there was no Ca$^{2+}$ gradient across the bilayer. In the absence of SR membranes, the bilayer was stable when 20 $\mu$M PIPz was added to both the cis and trans chambers.

**Fig. 2. Effect of PIPz on steady state single channel activities of JTC membranes.** Two separate Ca$^{2+}$ release channels from two different preparations are shown. Each channel was recorded for 2-5 min on a VCR tape, in a symmetric 250 mM cis/trans Ca$^{2+}$ medium (“Materials and Methods”). A-B, recorded in the presence of 2 mM MgCl$_2$ and 1 mM Na$_2$ATP in the cis chamber (pCa = 12). C-E, represented the channel where 0.5 $\mu$M PIPz increments were added sequentially to the cis chamber (pCa = 4.9). Channel opening is in the upward direction. The holding potentials were $-50$ mV (A-B) and $-40$ mV (C-E). The Ca$^{2+}$ conductances of the channels were 407 pS (A-B) and 396 pS (C-E). The mean opening probability ($P_o$) and the $P_o$ over a period of 45 s (a scale of 0 to 0.7 for all the y axes) are shown in the right panels of each corresponding channel recording. These channels were less active because there was no Ca$^{2+}$ gradient across the bilayer. In the absence of SR membranes, the bilayer was stable when 20 $\mu$M PIPz was added to both the cis and trans chambers.

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**Effects of PIPz on Dwell Time Distributions of Single Channels**—The actions of PIPz on dwell time distributions in the absence of Mg$^{2+}$ and ATP are shown in Fig. 3. PIPz (1 $\mu$M) increased the mean $P_o$ of one channel from 0.01 to 0.15. The duration and distribution of the open and closed events with the corresponding fitted curves are illustrated in the figure. The peaks of the histograms and the areas under these peaks correspond to the mean channel conductance ($G_m$) and the relative number of events/bin normalized to 1, respectively. Dwell time distributions of the open and closed states revealed multiple components indicating that there were at least two open states with time constants $\tau_1$ and $\tau_2$, and three closed states with time constants $\tau_1$, $\tau_2$, and $\tau_3$. PIPz slightly increased the values of the open lifetimes $\tau_1$ and $\tau_2$, and favored the proportion of events with longer lasting mean open lifetimes ($a_2$, $a_3$). Concomitantly, PIPz reduced the mean closed lifetime values of $\tau_2$ and $\tau_3$, and their proportions of closed states with longer lasting closures ($a_2$, $a_3$, and $a_4$, $a_5$). The closed state with the fast time constant ($a_1$, $\tau_1$) remained practically unmodified.

**Fig. 3. Effect of PIPz on the dwell time distribution of open and closed states of a JTC Ca$^{2+}$ channel.** Single channel activity was recorded as in Fig. 2. Dwell times were logaritmically binned (8 bin/decade, bars) and fitted to probability density functions (lines) (“Materials and Methods”). Due to the limit of the sampling frequency (5 kHz), it is not possible to accurately fit the fastest components ($\leq 0.1$ ms). We present here the open time (A) and closed time (B) histograms of one channel in the absence of Mg$^{2+}$/ATP, in the presence (long dash lines, hatched bars) and absence of 1 $\mu$M PIPz (solid lines, filled bars), respectively. The y axis is in arbitrary units. $a_1$, $a_2$, $a_3$ = amplitude factors, which are proportional to the areas under the peaks, which are time constants $\tau_1$, $\tau_2$, and $\tau_3 = $ time constants of components 1, 2, 3.

In summary, PIPz primarily reduced the proportion of long closures and favored long open states. No significant modifications were detected in the fast open and closed states. A similar action of PIPz was observed in six other different channels from five JTC preparations regardless of the presence of Mg$^{2+}$ and ATP.

**Effects of PIPz on $^{45}$Ca$^{2+}$ Flux Studies with Passive Ca$^{2+}$ Loading**—To gain insight on the properties of the PIPz-induced Ca$^{2+}$ release, we studied the action of PIPz on radio-labeled Ca$^{2+}$ fluxes under two different Ca$^{2+}$ loading conditions. We first determined the action of PIPz alone by measuring Ca$^{2+}$ fluxes from passively loaded JTC vesicles, i.e. in the absence of active Ca$^{2+}$ uptake and Ca$^{2+}$-induced Ca$^{2+}$ release. To this end, the PIPz effect was determined in the presence of 1 mM EGTA in the absence of ATP. Ca$^{2+}$ flux from the EGTA medium is therefore regarded as the basal efflux. A series of determinations were carried out to define the specificity and the action of PIPz. One $\mu$M PIPz rapidly depleted almost completely all the JTC Ca$^{2+}$ ($\approx 92\%$) (Fig. 4A). Previous reports in comparable conditions have shown less efficacy in releasing Ca$^{2+}$, even with 10 $\mu$M PIPz.

The action of PIPz is specific for the Ca$^{2+}$ release channel since PIPz did not change the rate of Ca$^{2+}$ release in the presence of 10 mM Mg$^{2+}$ or 20 $\mu$M ruthenium red. The rate of Ca$^{2+}$ release induced by PIPz is dependent on PIPz concentration. We find that the PIPz concentration for stimulating 50% of Ca$^{2+}$ release was $\approx 70$ nM ($7$ nmol PIPz/mg protein). This was derived under the condition of closed channel activity (in the presence of 10 mM Mg$^{2+}$, with 1 $\mu$M PIPz). Similarly, we determined a $K_m$ of $\approx 265$ $\mu$M ($27$ nmol/mg protein). In summary, low concentrations of PIPz effectively increased the rate of Ca$^{2+}$ release resulting from the activity of SR Ca$^{2+}$ channels. This finding coincides with our single channel results.

**Effects of PIPz on $^{45}$Ca$^{2+}$ Flux Studies with Active Ca$^{2+}$ Loading**—One important point to determine is whether PIPz is also effective in releasing Ca$^{2+}$ from SR vesicles that are actively transporting Ca$^{2+}$ in the presence of ATP and Mg$^{2+}$ as may be the case under physiological conditions. We there-
purposes, we show that Ca2+ released by the Caz+ ionophore, A23187, is virtually complete because being lipophilic, it rapidly dissipates the Ca2+ gradient across the SR membrane. This suggests that PIP2 operates in a manner other than a passive dissipator of Ca2+. In a similar way, 2 mM caffeine, an agent known to release SR Ca2+, released an equivalent amount of Ca2+ as 1 µM PIP2. For comparative purposes, we show that Ca2+ released by the Ca2+ ionophore, A23187, is virtually complete because being lipophilic, it rapidly dissipates the Ca2+ gradient across the SR membrane. This suggests that PIP2 operates in a manner other than a lipophilic effect.

To quantify the effectiveness of PIP2-induced Ca2+ release, we varied the PIP2 concentration and the JTC protein concentration. We found that for a given PIP2 concentration between 0.5–10 µM, the extent of Ca2+ release became greater as the JTC protein concentration was diluted (Fig. 4C). The mechanism by which low PIP2 concentrations are more effective in releasing Ca2+ from diluted samples remains unclear. One may speculate that in concentrated JTC preparations, inositol phosphate-binding sites remained unoccupied and inactive. The fact that PIP2 could maximally release only 70% of the internal Ca2+ may relate to the activity of the Ca2+ pump in transporting the released Ca2+ back into the SR.

Effect of Ca2+ on PIP2-induced Ca2+ Release under Active Ca2+ Loading Conditions—Since cytoplasmic Ca2+ is an important factor for SR Ca2+ channel activation, we investigated the Ca2+ dependence of PIP2-induced Ca2+ release. Active Ca2+ uptake was initiated in the presence of Mg2+ and ATP, with Ca2+ mediums of pCa 5.7, 5, and 4.3. Ca2+ was optimal...
at pCa 5.7. The peak Ca\(^{2+}\) loaded at pCa 5 and 4.3 were decreased by \sim 36 and \sim 44\% relative to the value at pCa 5.7. But the proportion of Ca\(^{2+}\) released by PIP\(_2\) was neither affected by the changes in the Ca\(^{2+}\) load nor the medium [Ca\(^{2+}\)] (Fig. 4D). This suggests that PIP\(_2\)-induced Ca\(^{2+}\) release is not dependent on external Ca\(^{2+}\) from \sim 10^{-6} to \sim 10^{-4} M. Similarly, from Fig. 2, it is shown that PIP\(_3\) activated the SR Ca\(^{2+}\) channel at pCa \sim 5 and \sim 12. From Fig. 4A, PIP\(_3\) is shown to release Ca\(^{2+}\) at \sim 10^{-9} M medium free [Ca\(^{2+}\)], in the absence of Mg\(^{2+}\) and ATP. These results, when taken together with other data as described below, indicate that PIP\(_3\) elicits Ca\(^{2+}\) release from terminal cisternae regardless of [Ca\(^{2+}\)] and [Mg\(^{2+}\)] in the milieu.

**Rapid \(^{45}\)Ca\(^{2+}\) Flux Studies: Effects of PIP\(_3\), Caffeine, and Ca\(^{2+}\)-** The rate of rapid Ca\(^{2+}\) release as induced by PIP\(_3\) was compared to other agents known to induce Ca\(^{2+}\) from terminal cisternae (Fig. 5). In these experiments, although the JTC are actively loaded with Ca\(^{2+}\), the loading medium is removed by washing. There is no residual ATP. Residual [Ca\(^{2+}\)] is calculated to be \sim 10^{-6} M. The effective order of Ca\(^{2+}\) release was PIP\(_3\) < caffeine < Ca\(^{2+}\)/ATP alone. Release by the agents was more effective at more alkaline or physiologic pH. This coincides nicely with previous reports that the Ca\(^{2+}\) release channel activity and response to stimulatory agents are more sensitive at alkaline pH (17, 18).

We have, therefore, demonstrated that PIP\(_3\) is slower than other well known Ca\(^{2+}\) releasing agents in eliciting Ca\(^{2+}\) release. It is about 45\% slower than caffeine at alkaline pH. Micromolar Ca\(^{2+}\) is \sim 71\% faster than caffeine at pH 7.5.

**Specificity of Inositol Polyphosphates—** Since IP\(_3\) is formed from PIP\(_3\), we therefore carried out the following two sets of experiments to examine the ability of IP\(_3\) to induce Ca\(^{2+}\) release. First, we investigated the specificity of the inositol polyphosphates in inducing Ca\(^{2+}\) release by comparing the Ca\(^{2+}\) release properties of canine brain cerebellum microsomes and JTC membranes. Cerebellar microsomes contain Ca\(^{2+}\) stores which can be released by IP\(_3\), and this IP\(_3\)-induced release can be inhibited by heparin (10). Our results demonstrate a striking difference between IP\(_3\) and PIP\(_3\) actions in the two types of tissues (Fig. 6). As anticipated for cerebellum membranes, IP\(_3\) and not PIP\(_3\) elicited Ca\(^{2+}\) release, about 26 \pm 1\% (n = 3). For JTC membranes, although IP\(_3\) consistently caused a small, transient release of 7 \pm 4\% (n = 5), PIP\(_3\) effectively induced a larger extent of release of 37 \pm 11\% (n = 4).

The experiments indicate that (a) PIP\(_3\) does not act chaotropic because it does not induce Ca\(^{2+}\) release from brain cerebellum, (b) PIP\(_3\) is a very potent inositol polyphosphate for activating the SR Ca\(^{2+}\) channel, much more effective than IP\(_3\), and (c) we confirm previous reports that IP\(_3\) effects are difficult to demonstrate in Ca\(^{2+}\) flux studies (19) and that a miniscule amount of Ca\(^{2+}\) can be released by IP\(_3\) from SR (20).

**Interaction of IP\(_3\) with the SR Ca\(^{2+}\) Channel: Single Channel Studies—** Since it is difficult to ascertain any substantial IP\(_3\) effect on Ca\(^{2+}\) release, we proceeded to test the effect of IP\(_3\) on the single channel activity of JTC membranes. At resting [Ca\(^{2+}\)] levels (pCa 7.2), the activity of the channel is low (Fig. 7A), as is illustrated by the P\(_0\) over 50 s and also the mean P\(_0\) (Fig. 7C). IP\(_3\) (10 \mu M) activated the channel, increasing the mean P\(_0\) \sim 4-fold (Fig. 7, B and D). The P\(_0\) over time was also enhanced. We did not find any activation of the channel by 10 \mu M IP\(_3\) in the presence of our usual CMS medium containing Ca\(^{2+}\). Our finding that IP\(_3\) activates the ryano-
dine-sensitive Ca\(^{2+}\) channel in native SR membranes is in agreement with previous reports (19–21). But the conditions for observing the activation are variable, particularly with respect to the free [Ca\(^{2+}\)] in the medium.

**Conclusions**—From the data above, we conclude that PIP\(_2\) specifically stimulates the ryanodine-sensitive SR Ca\(^{2+}\) channel activity. The activation is dependent on PIP\(_2\) concentration but is relatively independent of [Ca\(^{2+}\)]. On the other hand, IP\(_3\) also activates the channel, but the efficacy is dependent on the [Ca\(^{2+}\)] in the milieu. It is not effective in the range of Ca\(^{2+}\) where PIP\(_2\) exhibits potent stimulation.

**DISCUSSION**

We have demonstrated that low concentrations of PIP\(_2\) consistently stimulate Ca\(^{2+}\) release by activating the SR Ca\(^{2+}\) release channel in the terminal cisternae of skeletal muscle (Fig. 2). Dwell time analyses of single channels reveal multiple open (~2) and closed (~3) states of the channel. PIP\(_2\) activates the channel by increasing the opening frequency, accompanied by longer openings and occasional bursting activity. These effects are achieved by the following PIP\(_2\) actions: (a) promotes the proportion of channel openings to longer open times, (b) decreases the longer lasting closed lifetimes, and (c) reduces the proportion of closed states with long closures (Fig. 3).

In conjunction with our single channel data, we find that PIP\(_2\)-induced Ca\(^{2+}\) release is concentration dependent, and is mediated via the SR Ca\(^{2+}\) channel, which is ryanodine-sensitive, Mg\(^{2+}\)-, and ruthenium red-inhibited. We have tested the PIP\(_2\)-induced Ca\(^{2+}\) release process in two experimental conditions, passive and active Ca\(^{2+}\) loading, because we wish to achieve optimal loading prior to release so that the extent of release induced by inositol phosphate reagents is significant (Fig. 4, A and B). Despite the difference in PIP\(_2\) sensitivity in the two loading methods, resulting from the fact that there is ongoing Ca\(^{2+}\) uptake that opposes the releasing action of PIP\(_2\) in the presence of an active Ca\(^{2+}\) loading medium, we find that low concentrations of PIP\(_2\) release Ca\(^{2+}\) under both experimental conditions. Furthermore, PIP\(_2\) effects are not likely chaotropic effects because (a) it does not induce Ca\(^{2+}\) release from brain microsomes, (b) the dose-response curve for PIP\(_2\) appears saturable (Fig. 4C), (c) the lipid bilayer is stable despite the addition of high [PIP\(_3\)], and (d) PIP\(_2\) is amphipathic, and is soluble in aqueous medium up to millimolar concentrations.

An important consideration is whether PIP\(_2\) has a functional and regulatory role during the Ca\(^{2+}\) release process under physiological conditions. The fact that PIP\(_2\) can release Ca\(^{2+}\) and activate the channel under physiological ATP and Mg\(^{2+}\) concentrations strongly suggests that this might be the case. Another consideration is to estimate the PIP\(_2\) concentration in the membrane. We have calculated the range of PIP\(_2\) concentration in the transverse tubular membrane to be 1–3 mM, using the reported values of 4–8 nmol/g muscle in resting cells (22). This is derived by assuming that TT occupies 0.3% by cell volume (23), and 1 g of muscle occupies a volume of 1 ml. If we further assume a mol/mol production of IP\(_3\) from PIP\(_2\) and that PIP\(_2\) is localized at the triadic gap, then the range of PIP\(_2\) is 6–10 μM, based on the values of 120 pmol IP\(_3\)/mg TT protein (38 μM) (23) and 8 nmol/mg phospholipid (=10 μM) (3, 23). Although we have made several assumptions, the micromolar values of PIP\(_2\) are in the same range as those used in our stimulation experiments, and these values make possible a physiological role of PIP\(_2\) in E-C coupling. However, we do not know whether PIP\(_2\) is released toward the SR membranes upon muscle stimulation, or whether it is an integral constituent of the SR membrane, where it may have an important regulatory role as a set point for the activation of the Ca\(^{2+}\) release channel. It is also not yet clear whether PIP\(_2\) is normally present in the mammalian SR membrane (Ref. 3 versus 24).

One interesting property of the PIP\(_2\) effect is that the PIP\(_2\) stimulation of Ca\(^{2+}\) release and activation of the channel are unaffected by a wide range of medium [Ca\(^{2+}\)], from <10\(^{-9}\) to ~10\(^{-4}\) M (Figs. 2, 4 A, and D, and 5). The presence or absence of physiological Mg\(^{2+}\) concentrations (~1–2 mM) did not appear to interfere with PIP\(_2\)-induced release. This lack of Ca\(^{2+}\) dependence in a microenvironment that undergoes large changes in Ca\(^{2+}\) concentration upon muscle stimulation imply that the PIP\(_2\) stimulation of the Ca\(^{2+}\) release channel should be always present regardless of the local Ca\(^{2+}\) concentration.

The release of Ca\(^{2+}\) from terminal cisternae of PIP\(_2\), however, is relatively slower than that induced by other known stimulating agents of the SR Ca\(^{2+}\) release channel, e.g. caffeine, Ca\(^{2+}\), and ATP (Fig. 5). The data suggest that PIP\(_2\) can modulate Ca\(^{2+}\) release but is not likely to be a candidate as the trigger for rapid Ca\(^{2+}\) release in E-C coupling. But if we consider the possibility that the rate of Ca\(^{2+}\) release induced by caffeine in skinned fibers is relatively rapid, ~0.8–14 μM/ ms (25), and that from our vesicle studies in Fig. 5, the PIP\(_2\)-induced Ca\(^{2+}\) release is only ~50% less than that induced by caffeine, then PIP\(_2\) is an adequate trigger for Ca\(^{2+}\) release. This question needs to be further investigated in more physiological preparations of skeletal muscle since it is difficult to extrapolate from vesicular Ca\(^{2+}\) release studies the rate of Ca\(^{2+}\) release in an intact cell.

The PIP\(_2\) product, IP\(_3\), has first been shown to elicit muscle contraction in frog muscle (26). Since then, IP\(_3\) has also been shown to elicit contraction from mechanically skinned mammalian skeletal muscle fibers (27). But the suggestion that IP\(_3\) is a chemical coupling factor in skeletal muscle E-C has been controversial due to discrepancies between whole muscle and SR vesicle studies (Ref. 3). When the reports are positive, the IP\(_3\)-elicited response is slow, as in the case of skinned muscle.
fibers (27), or small, as in the case of triadic vesicles (20) and from our data (Fig. 6), or requiring high [IP_3], as in the case of JTC vesicles (28) and triadic vesicles (20). It is possible that an IP_3-sensitive factor is lost during the preparation of membrane vesicles. But it is difficult to explain the fact that IP_3 activates the purified ryanodine-sensitive SR Ca^{2+} release channel itself (19). It has been suggested that IP_3-sensitive SR Ca^{2+} channels appear to occur in only 50% of a population of native SR vesicles and that IP_3 activation of these channels is at submicromolar [Ca^{2+}]. Our data on the IP_3 stimulation of the SR Ca^{2+} channel activity (Fig. 7) support his latter observation. His results may also explain the finding that only low affinity IP_3-binding sites have been reported (29).

Moreover, it is possible that Ca^{2+} inhibits the IP_3 receptor, thus negating positive IP_3 responses when it is present. In fact, this has been implicated in IP_3-mediated Ca^{2+} release from permeabilized cells of peripheral tissue (30).

It is possible that PIP_2, being an amphipathic molecule, readily penetrates the phospholipid membrane and interacts with a putative inositol polyphosphate-binding site, effecting Ca^{2+} release from the SR Ca^{2+} release channel. This suggests that PIP_2 may be pharmacologically more important and potent than IP_3. Our data and others (6, 7) support this hypothesis. Furthermore, our observations that PIP_2 induce Ca^{2+} release from SR but not from brain cerebellum suggest that there is a specific site in SR and that the Ca^{2+} release processes in these two tissues are regulated differently.

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