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Synthesis and Biological Activity of Phospholipase C-Resistant Analogues of Phosphatidylinositol 4,5-bisphosphate

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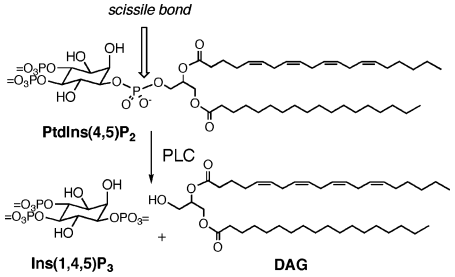
The membrane phospholipid phosphatidylinositol 4,5-bisphosphate (PtdIns(4,5)P_2) is an important regulator of cytoskeletal organization during a plethora of cellular functions, such as vesicle trafficking, endocytosis, phagocytosis, focal adhesion formation, and cell migration.[1] PtdIns(4,5)P_2 binds to and affects the function of many actin-binding and actin-remodeling proteins[2−4] and is a cofactor in enzyme activation.[5] In addition, PtdIns(4,5)P_2 regulates the activity of many ion channels and transporters.[6,7] PtdIns(4,5)P_2 is also the source of three second messengers: Ins(1,4,5)P_3, diacylglycerol (DAG),[8,9] and PtdIns(3,4,5)P_3.[10] In many cases, it is the decrease in PtdIns(4,5)P_2, resulting from hydrolysis by phospholipase C (PLC) (Scheme 1), and not the increase in Ins(1,4,5)P_3 and DAG that constitutes the physiologically relevant signal.[11,12] Hydrolysis of PtdIns(4,5)P_2 causes TRP channels to lose some activity.[13−19] Moreover, addition of PtdIns(4,5)P_2 restores sensitivity of TRPM4 and TRPM5 to activation by Ca^{2+} and restores the sensitivity of TRPM8 and TRPV1 to thermal and chemical stimuli.[15,16,18,19]

The availability of a metabolically stabilized analogue of PtdIns(4,5)P_2, that is, one that lacks the scissile P−O bond and thus could not be hydrolyzed by PLC activity, would have many applications in understanding the role of PtdIns(4,5)P_2 in cell physiology. α-Fluoroalkylphosphonates have emerged as important nonhydrolyzable mimics for phosphoesters in the synthesis of biologically active "unnatural products".[20−23] Herein we describe the first asymmetric total synthesis of isosteric and isoelectronic phosphonate analogues **1**−**5** of PtdIns(4,5)P_2 that cannot be hydrolyzed by PLC. The synthesis employs a Pd(0) coupling not previously exploited in phospholipid or phosphoinositide synthesis. Furthermore, we demonstrate that both saturated and unsaturated α-fluorophosphonate analogues can substitute for exogenous PtdIns(4,5)P_2 in restoring the sensitivity of the TRPM4 channel to Ca^{2+}.

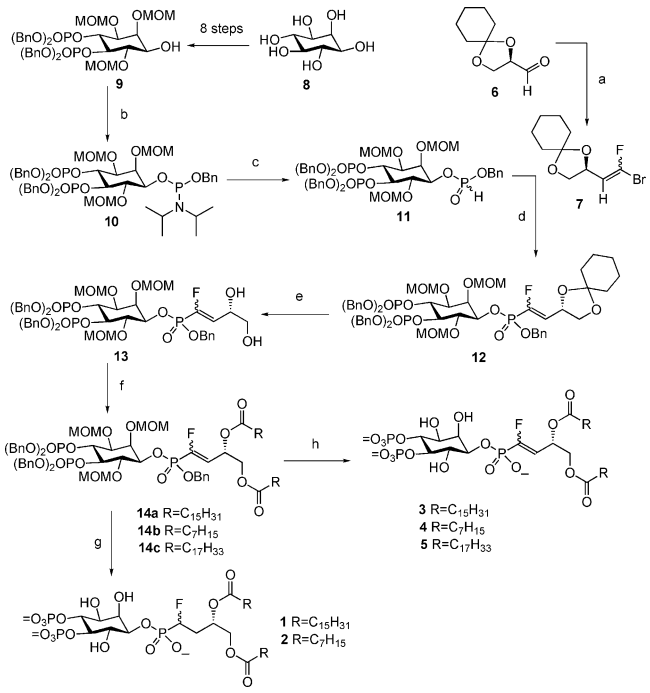
The synthetic sequence to the stabilized analogues **1**−**5** of PtdIns(4,5)P_2 is illustrated in Scheme 2. A variety of attempts to connect the intermediate **9**[24] with a fluoromethylenephosphonic acid synthon[21] failed. Eventually, we turned to the Pd(0)-catalyzed coupling of an *H*-phosphite with a vinyl bromide in order to form the desired C−P linkage. Thus, coupling the protected inositol **9** with dibenzyl *N*,*N*-diisopropylphosphoramidite gave the phosphoramidite intermediate **10**, which was converted to *H*-phosphonate **11** in 76% isolated yield in two steps.[25] The 1-bromo-1-fluoroolefin **7** (∼1:1 *E*/*Z*) was separately prepared via a Et_2Zn-promoted olefination reaction[26] of CBr_3F/PPh_3 with glyceraldehyde **6** in excellent yield.

Few examples exist of Pd(0)-catalyzed formation of P−CF bonds, and in our hands, only traces of coupled compound **12** with a majority of the P−O cleaved compound **9** were obtained under standard conditions using Et_3N or K_2CO_3 as base. It appeared that the rate of decomposition was faster than the rate of coupling for the more hindered *H*-phosphonate **11.** To overcome this problem, we selected propylene oxide as a weak Lewis base and an effective scavenger of HBr.[27] Using this modification, treatment of the *H*-phosphonate **11** with $Pd(OAc)_2$/dppf/propylene oxide in THF at 70 °C led to the formation of α-fluorovinylphosphonate **12** in 62%

Scheme 1. Phospholipase C Catalyzes Hydrolysis of PtdIns(4,5)P_2 to Two Second Messengers, Ins(1,4,5)P_3 and Diacylglycerol



Scheme 2. Synthesis of Phosphonates **1**−**5**[a]



[a] Conditions: (a) $CFBr_3$, PPh_3, Et_2Zn, THF, 76%; (b) $(BnO)_2P(NPr_2-i)_2$, *N*,*N*-diisopropylethylammonium •1*H*-tetrazole, CH_2Cl_2, rt; (c) H_2O, 1*H*-tetrazole, rt, 1 h, CH_2Cl_2, 76% for two steps; (d) $Pd(OAc)_2$, dppf, propylene oxide, THF, 70 °C, 62%; (e) 60% aqueous TFA, THF, 0 °C, 1 h, 86%; (f) EDCI, DMAP, fatty acid, CH_2Cl_2, rt; (g) H_2, Pd/C, MeOH, 6 h, EtSH; (h) TMBr/TMSI (5:1), rt, 1.5 h; MeOH, 1 h.

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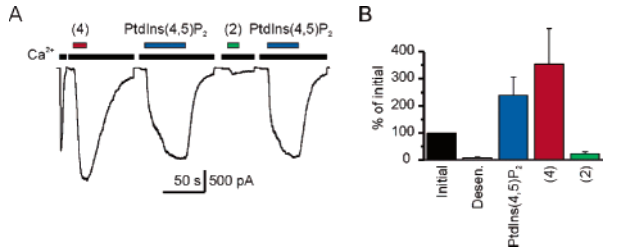

Figure 1. PtdIns(4,5)P_2 and analogues **2** and **4** restore TRPM4 currents following desensitization. (A) An excised inside-out patch from Chok1 cell expressing mouse TRPM4 (mTRPM4) shows activation and fast rundown of an inward current in the presence of 100 μM Ca^{2+} and recovery by dioctanoyl−PtdIns(4,5)P_2 and analogues **2** and **4** (V_m = 80 mV). (B) Initial magnitudes of the mTRPM4 currents, currents after rundown, and currents after recovery in response to 10 μM each of PtdIns(4,5)P_2, **2**, and **4** (averages, n = 8).

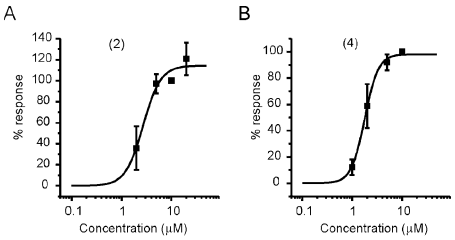

Figure 2. Dose-response for recovery of TRPM4 currents by **2** and **4**. After TRPM4 desensitization, recovery was assessed. Data were normalized to the response to 10 μM of each analogue in the same patch. (A) Averaged data (n = 5) for recovery of TRPM4 currents by **2** (EC_{50} = 2.7 ± 0.6 μM and n_H = 2.5 ± 1.2). (B) Averaged data (n = 6) for **4** (EC_{50} = 1.8 ± 0.1 μM and n_H = 3.2 ± 0.5).

yield. Acetal **12** was selectively deprotected by treatment with 60% aqueous trifluoroacetic acid in tetrahydrofuran at 0 °C to give diol **13**. Next, acylation of **13** with either octanoic acid, palmitic acid, or oleic acid provided the fully protected phosphonates **14a**, **14b**, and **14c** in 80, 73, and 82% yields, respectively. Hydrogenolysis of **14a** and **14b** removed the benzyl groups, and then reaction with ethanethiol removed the MOM groups to give the α-fluoromethylenephosphonate analogues **1** and **2**.[28] The α-fluorovinylphosphonates **3**−**5**[28] were obtained by deprotection of benzyl and MOM groups simultaneously with TMSBr/TMSI (5:1).

Recently, the hydrolysis of the water-soluble dioctanoyl PtdIns(4,5)P_2 was found to be important in the desensitization of TRPM4 channel (activated by cytoplasmic Ca^{2+}). Exogenous PtdIns(4,5)P_2 could restore the sensitivity of TRPM4 channels to Ca^{2+}, demonstrating that PtdIns(4,5)P_2 was a general regulator for the gating of TRPM4 ion channels.[15] The ability of the two dioctanoyl−PtdIns(4,5)P_2 analogues **2** and **4** to restore TRPM4 currents following rundown is shown in Figure 1. Both analogues restored TRPM4 sensitivity following desensitization, but the α-fluorovinylphosphonate **4** was more potent. Indeed, the unsaturated phosphonate **4** was even more effective than the hydrolyzable dioctanoyl−PtdIns(4,5)P_2 at restoring TRPM4 sensitivity. This provides further evidence that the regulation of TRPM4 by dioctanoyl−PtdIns(4,5)P_2 and the ability of dioctanoyl−PtdIns(4,5)P_2 to restore TRPM4 currents following rundown is not due to effects of products of PLC hydrolysis.[15]

To determine sensitivity of TRPM4 currents to **2** and **4**, we measured the effects of varying concentrations of both compounds on the recovery of TPRM4 currents in excised inside-out patches evoked in response to 100 μM Ca^{2+} (Figure 2). Maximal recovery of TRPM4 currents was observed upon reaching 10 μM for both **2** and **4**, and half-activation was observed at ∼2 μM for both compounds, which is similar to the concentration of PtdIns(4,5)P_2 that promoted half-activation of TRPM4 (6 μM).[15] The difference between the effectiveness of **2** and **4** in restoring TRPM4 currents (Figure 1) appears to result from differential abilities to promote activation of the TRPM4 channel. Taken together, these data suggest that the α-fluorovinylphosphonate **4** is a biologically active, long-lived mimic of PtdIns(4,5)P_2.

In conclusion, we developed an efficient synthesis of two nonhydrolyzable PtdIns(4,5)P_2 analogues, and we showed that α-fluorovinylphosphonate **4** optimally restored the sensitivity of TRPM4 currents. These results suggest that metabolically stabilized analogues of PtdIns(4,5)P_2 will have a wide variety of applications in separating the role of the phosphoinositide per se from activities that result when Ins(1,4,5)P_3, DAG, Ca^{2+}, or other downstream signals are generated from the hydrolysis of PtdIns(4,5)P_2 by PLC.

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Supporting Information Available: Experimental details for synthesis and characterization of new compounds, and protocols for TRPM4 channel activity measurement. This material is available free of charge via the Internet at http://pubs.acs.org.

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(28) Note on stereochemistry. Both compounds **1** and **2** are inseparable mixtures of diastereomers at the C−F stereocenter, and the chiral phosphorus atom is racemic. Similarly, compounds **3**, **4**, **5**, and **12**−**14** are inseparable *E*/*Z* mixtures.

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